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IN-VITRO GENE DISRUPTION USING TN7-BASED TRANSPOSITION: EVALUATION OF ITS UTILITY AND EFFICIENCY IN GENERATING A BRUCELLA VIRB1 KNOCKOUT

A Thesis

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 Master of Science

in

The Department of Biological Sciences

by

Jeffrey W. Mercante
B.A., Southern Methodist University, 1998
August 2002
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In addition, the author expresses gratitude to Andy McLemore for his assistance and thanks to all those in Dr. Battista’s and Dr. Elzer’s laboratories. And not least, sincere appreciation goes to Miss Alexandra Dubón for her never-ending patience.
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ABSTRACT

A Tn7-based transposition system carrying a kanamycin resistance gene has been used to disrupt a known virulence gene of Brucella melitensis 16M and Brucella abortus 2308 in-vitro. The suicide plasmid pBAvirB1::kan-29 was created, in part, by cloning the B. abortus virB1 gene into a vector that is not stably maintained in Brucella spp. The cloned virB1 gene was then mutagenized by in-vitro transposition of a Tn7-based transposon. This construct was used in-vivo to insert a disrupted virB1 gene in place of the wild type virB1 found in B. abortus 2308 and B. melitensis 16M by homologous recombination. The resulting virB1 mutant bruccellae exhibited resistance to kanamycin, decreased survival in murine macrophages and attenuated virulence in a BALB/c mouse model. This method is proposed to be a simple, efficient means of generating gene knockouts in Brucella spp., and may be useful in targeting virulence factors for the creation of live, attenuated vaccines. The genus Brucella consists of bacteria that are facultative intracellular pathogens. Individuals in this genus of Gram-negative coccobacilli are responsible for causing late term spontaneous abortion in cattle and goats as well as the human disease brucellosis (undulant fever). The success of Brucella is dependent on their ability to invade, survive and multiply within phagocytes. The virB operon, consisting of eleven genes in most Brucella species, is thought to contribute to a type IV secretion system responsible for maintaining infection in a mammalian host. Further results of this study suggest that the Brucella virB1 gene product, like its Agrobacterium counterpart, may not be essential for virulence; disruption of the Brucella virB1 gene results in a one to two log decrease in intracellular survival at forty-eight hours in an in-vitro macrophage model and by four weeks in an in-vivo mouse model.
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

During the late nineteenth century a German physician named Robert Koch set out to prove the germ theory of disease, which had been stated by Girolamo Fracastoro of Verona over three hundred years earlier. From his studies with anthrax, Koch formulated a set of postulates that are still used today for the identification of etiological agents (Atlas 1997). With the connection between microorganisms and disease established there came an understanding of vaccination, the discovery of antimicrobial agents and the study of virology and immunology. As a result of these and other technological advances, the average human life expectancy rose dramatically and once common ailments such as measles, diphtheria, pneumonia and tuberculosis became treatable and sometimes curable. One such treatable disease of humans and a wide variety of mammals is brucellosis, which is caused by bacteria of the genus *Brucella*.

While stationed on the island of Malta, the British army physician, David Bruce, was the first to successfully isolate and culture *Micrococcus melitensis* in 1897, which was later renamed *Brucella melitensis* in his honor (Spink 1956; Dalrymple-Champneys 1960). As many British sailors discovered, *Brucella* infection may lead to the debilitating, chronic disease known as brucellosis. If it is not treated with antibiotics, brucellosis can result in lymphoid organ inflammation together with bone and joint problems, and in rare cases death (Spink 1956; Dalrymple-Champneys 1960; Thimm 1982; FAO/WHO 1986; Jawetz, Melnick et al. 1989; Madkour 1989; Paul 1993; Young 1995; Comerci, Martinez-Lorenzo et al. 2001; DelVecchio, Kapatral et al. 2002).

*Brucella* is also responsible for late-term abortion in several domestic and wild mammals, including swine, goats, cattle, sheep, bison and elk (Spink 1956; Dalrymple-Champneys 1960; WHO/EMC 1998). Although the incidence of *Brucella* infection has
been greatly reduced or eliminated in many countries, brucellosis is still the world’s most widespread zoonoses and is prevalent in many underdeveloped nations (Ruben, Band et al. 1991). Of additional importance is the designation of *Brucella* as a biosafety level III disease organism and its use as an agent of biological warfare. According to the Centers for Disease Control and Prevention (CDC) in Atlanta, which considers *Brucella* a select agent with Class B status as a biological threat (same category as the agents which cause Q fever and encephalitis), brucellae are listed among the top ten most important biological warfare agents for public health preparedness (Frederick, Takafuji et al. 1997; Rotz, Khan et al. 2002). Outlined in a report by the WHO in 1970 (Health Aspects of Chemical and Biological Weapons), it was calculated that upon biological attack, brucellae have the potential to incapacitate as many people as an equivalent dose of *Bacillus anthracis* or *Coxiella burnetii* (causative agent of Q fever) (as reviewed by Christopher, Cieslak et al. 1997; Madigan, Martinko et al. 2000). But while vaccines have been developed to protect against other biological diseases such as tularemia, Q fever and anthrax, a *Brucella* vaccine has been illusive, being unsuitable for human use (Huxsoll et al. 1989 as cited in Franz, Parrot et al. 1997).

1.1 General Characteristics of *Brucella* spp.

Brucellae are small, non-motile, Gram-negative coccobacilli about 0.6µm - 1.5µm in length and 0.5µm - 0.7µm in width, found in the \( \alpha_2 \) subdivision of the phylum *Proteobacteria* (Thimm 1982; FAO/WHO 1986; Moreno, Stackebrandt et al. 1990). The majority of species in this subdivision are associated with eukaryotes as endosymbionts or pathogens (Fig. 1) (Ugalde 1999; Sieira, Comerci et al. 2000). Individuals in the genus *Brucella* are intracellular pathogens of mammals including humans (Dalrymple-
Champneys 1960). All species of *Brucella* are non-spore forming and aerobic, except for *B. abortus* and *B. ovis*, which are carboxyphilic, requiring an increased CO\(_2\) tension of 5% for optimal growth (Thimm 1982; FAO/WHO 1986; Thoen, Enright et al. 1993).

Brucellae are currently categorized into at least six different species encompassing 18 biovars, with several undesignated strains that infect marine mammals (Bricker 2000; Moreno and Moriyon 2002). There is evidence, however, to suggest that the genus is monospecific with *B. melitensis* considered the type species (Verger, Grimont et al. 1985; Michaux-Charachon, Bourg et al. 1997). Each species is specialized to an existence within a particular mammal host: *B. abortus* in cattle, bison and elk; *B.*
Brucella melitensis in goats and sheep; B. suis in pigs; B. canis in dogs; B. ovis in sheep and B. neotomae has been found in the desert wood rat (W. Wundt 1956 and FAO/WHO 1971 as cited in Thimm 1982; FAO/WHO 1986; Madkour 1989). None of the Brucella spp. have plasmids and all species contain 2 chromosomes with a G + C content between 57-59%, except for B. suis biovar 3, which has a single combined chromosome (Michaux, Paillisson et al. 1993; Corbel 1997; Michaux-Charachon et al. 1997; Jumas-Bilak, Michaux-Charachon et al. 1998a; O'Callaghan, Cazevieille et al. 1999; DelVecchio et al. 2002).

Brucellae are ubiquitous, and can be found in almost every country and environment where there are mammals, from the Mediterranean basin to Japan and North America through parts of Mexico, Central and South America to Australia (Thimm 1982; Young 1995). According to Madkour (1989), only 17 countries have been declared Brucella-free as of 1989. Brucellosis is a disease problem in many underdeveloped areas such as Africa, China and the Middle East as well as Latin America (Ruben et al. 1991; WHO/EMC 1998); however, the incidence of Brucella infection in both man and animal has been greatly reduced or eliminated in most developed nations (Corbel 1997).

In those affected areas, Brucella is of economic importance because it readily causes late term abortion, decreased milk production and infertility in infected animals. Brucella abortus infection may cause late term abortion and sterility in cattle, (Bang’s disease) (Huddleson 1947a; Spink 1956; Cloeckaert, Verger et al. 1996b) while B. suis infects swine with resulting abortion in pregnant females and orchitis, epididymitis, and sterility in males (Manthei 1948 as cited in Spink 1956; Dalrymple-Champneys 1960).

Brucella ovis is responsible for abortion and sterility in sheep (Buddle 1956; Cloeckaert
et al. 1996b) while *B. melitensis* is a causative agent of abortion in both goats and sheep (Dalrymple-Champneys 1960).

### 1.2 *Brucella* spp. Infection and Dissemination

Human transmission and *Brucella* infection may occur by inhalation of infectious aerosols, passage across mucous membranes, through a percutaneous route such as cracks or cuts in the skin or even through unbroken skin (Kaufmann, Fox et al. 1980; Thimm 1982; Smith and Ficht 1990; Ruben et al. 1991). The route of *Brucella* dissemination throughout the body is dependent on dedicated phagocytic cells such as macrophages and neutrophils (Mims 1987; Enright 1990). Once the bacterium is engulfed by a phagocyte it resists bactericidal mechanisms and begins to multiply while it is transported through the lymphatic system and localized in lymph nodes (Mims 1987; Jawetz et al. 1989). *Brucellae* multiply within lymph nodes causing hemorrhage at 2 to 3 weeks post-infection and then invade the blood stream leading to eventual bacteremia and dissemination throughout the body (Enright 1990; Thoen et al. 1993).

Studies reveal that in ruminants and humans *brucellae* localize to the reticulo-endothelial system, including the liver, spleen, kidneys, mammary glands and bone marrow and to nontraditional phagocytic cells such as those of the brain, endocardium, bones and joints (Spink 1956; Dalrymple-Champneys 1960; Smith and Ficht 1990). *Brucellae* may colonize both the male and pregnant female reproductive tracts, especially the chorionic trophoblasts of the placenta (Smith 1919; Spink 1956; Dalrymple-Champneys 1960; Meador and Deyoe 1989).

### 1.3 *Brucella* spp. Reproductive Tropism

In the ruminant, *brucellae* have a tropism for reproductive organs in both males and females locating to the testis, uterus and especially placental tissues (Smith 1919;
Placental destruction and necrosis resulting in ulcerated chorionic membranes releases large numbers of *Brucella* into the uterine lumen that are transferred to chorionic villi and fetal tissues (Anderson, Meador et al. 1986a, b; Enright 1990). *Brucella* induced abortion is thought to be caused by placentitis followed by vasculitis, decreased placental function (Anderson et al. 1986b; Enright 1990), and direct transmission to the fetus (Payne 1959; Thoen et al. 1993). But as noted by Enright (1990), even with severe placentitis, the endometrium is left largely untouched. A combination of factors, in addition to decreased placental function, appears to contribute to abortion. Possibilities include fetal stress due to steroid production and the presence of a weak *Brucella* endotoxin. *Brucella* infection of both sheep and cows results in increased cortisol production with a subsequent decrease in progesterone and increase in estrogen secretion by the placenta (Enright 1990). This sudden change in hormonal levels results in increased endometrial PGF2α production leading to premature delivery or possibly abortion (Liggins 1981 as cited in Enright 1990; Elzer, Phillips et al. 1996).

Additionally, the ruminant fetus’ immune system is less able to control *Brucella* infection compared to the adult (Enright 1990). Large granulomatous lesions, composed of macrophages, epithelioid cells, and other polymorphs, form in infected fetal tissues such as the lung, kidney, liver and lymphoid tissues. Fetal infection is characterized by elevated cortisol production, presumably due to fetal stress, together with an increase in the neutrophil population and a decrease in the number of lymphocytes and eosinophils. The presence of a *Brucella* endotoxin, consisting of lipopolysaccharide (LPS), is also thought to contribute to abortion, possibly due to vasculitis and placentomal lesions.
Anderson et al. 1986b; Huddelson 1953 and Molello 1963 as reviewed by Samartino and Enright 1993). This notion is plausible since LPS and lipid A from other Gram-negative bacteria have abortive properties when present in living microorganisms or administered in purified form (Enright 1990). Presently, however, the process of abortion due to Brucella infection and colonization is still not completely understood.

Why brucellae have a tropism for reproductive organs is still debated. An initially accepted theory held that because brucellae preferentially metabolize the sugar erythritol, they locate to areas which contain this carbohydrate, such as the placenta (Pearce 1962 as reviewed by Tripathi, Bhatnagar et al. 1977; Samartino and Enright 1993). Brucella spp. share this ability to metabolize erythritol with only one other pathogenic bacterium, Serratia marcescens (Slotnick and Dougherty 1972). Several studies have supported this theory, such as those of Tripathi et al. (1977), and Keppie et al. (1965) (as reviewed by Tripathi et al. 1977). Recently Sangari et al. (2000) and DelVecchio et al. (2002) have identified the genes responsible for the erythritol catabolic pathway in Brucella. It was proposed that the absence of erythritol in the placentas of humans, rats, mice, rabbits and guinea-pigs could explain the rare occurrence of Brucella in the reproductive systems of these animals (Keppie 1964 as cited in Samartino and Enright 1993). However, it has been shown that Brucella can infect the placenta of all these species including humans (Kniazeff, Elberg et al. 1964; Bosseray 1980; Madkour 1989; Samartino and Enright 1993). Additionally, Brucella erythritol catabolizing gene (ery) knockout strains proved to be just as virulent as wild type Brucella (Elzer 2002a). Thus, it appears that the ability to catabolize erythritol may not be the determining factor that allows Brucella to colonize the placenta and cause abortion, but it may confer an advantage.
1.4 *Brucella* Infection and Man

*Brucella* is also an important zoonotic pathogen, ranking among the world’s top five most common bacterial zoonoses (Sola-Landa, Pizzaro-Cerda et al. 1998). Members of the genus known to infect man include *B. melitensis*, *B. abortus*, *B. suis* and *B. canis* (Spink 1956; Thimm 1982; Ramacciotti 1984; Madkour 1989; Elzer 2002a). Of the six known species of *Brucella*, *B. melitensis* is considered the most infectious and virulent to man, followed by *B. suis* and then *B. abortus* (Table 1) (Spink 1956; Madkour 1989; Smith and Ficht 1990).

Table 1. Relative *Brucella* infectious dose for man of the four most virulent species (Young 1995; Elzer 2002a).

<table>
<thead>
<tr>
<th>Virulence to Humans</th>
<th>Species</th>
<th>Relative infectious dose</th>
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<tr>
<td>Most Virulent</td>
<td><em>B. melitensis</em></td>
<td>~1-10 organisms</td>
</tr>
<tr>
<td></td>
<td><em>B. suis</em></td>
<td>~1000</td>
</tr>
<tr>
<td></td>
<td><em>B. abortus</em></td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>Least Virulent</td>
<td><em>B. canis</em></td>
<td>~1,000,000</td>
</tr>
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</table>

The most common causes of human infection include laboratory acquired infection, ingestion of unpasteurized milk, contaminated cheese, and contact with diseased animals, especially placental tissues (Dalrymple-Champneys 1960; Young 1975; Fox and Kaufmann 1977; Thimm 1982; 1991; Ruben et al. 1991; Corbel 1997; Hong, Tsolis et al. 2000).

*Brucella* infection in man causes a systemic, febrile disease known as brucellosis. It has been referred to as undulant fever, Malta fever (*B. melitensis*), Mediterranean
remittent fever, Gibraltar fever, Cyprus fever and neapolitan fever, among others (Spink 1956; Thimm 1982; Jawetz et al. 1989; Madkour 1989). Acute, human brucellosis is characterized by a cyclic fever, shaking chills, profuse, drenching night and evening sweats, weakness, weight loss, nervousness, joint pain, insomnia, headache and respiratory trouble in the short term (Spink 1956; FAO/WHO 1986; Jawetz et al. 1989; Madkour 1989; DelVecchio et al. 2002). Other clinical short term signs include lymphadenopathy, splenomegaly, hepatomegaly, skin rash, depression and mental fatigue, with CNS invasion occurring in < 2% of cases (Spink 1956; Madkour 1989; Young 1995). Chronic, long term complications include weakness, sacroiliitis, orchitis, epididymo-orchitis, spondylitis, osteomyelitis, bursitis, bone and joint infection, dementia, neurological disorders and relapse with recurrent bacteremia (Spink 1956; Dalrymple-Champneys 1960; Thimm 1982; FAO/WHO 1986; Jawetz et al. 1989; Young 1995; Comerci et al. 2001). Severe cases of brucellosis can demonstrate as bronchitis, meningitis and in < 2% of all cases, endocarditis, which is responsible for the majority of Brucella-related deaths (Bouza 1987 as cited in Hart, Morgan et al. 1951; Dalrymple-Champneys 1960; Al-Harthi 1989; Young 1995; Comerci et al. 2001).

Full recovery from brucellosis is possible with antibiotic therapy. Rifampicin (600-900mg/day) and doxycycline (200mg/day) given for 6 weeks is the most effective treatment for eliminating Brucella infection (FAO/WHO 1986). The incubation period between infection and outward clinical signs is thought to average 1-3 weeks, however it may be as long as several months (Madkour 1989). According to an FAO/WHO report (1986), natural recovery from brucellosis of average severity takes 1-3 months with weakness and fatigue lasting much longer. As a result of this ability to incapacitate its
host, some species of *Brucella* have been investigated as biological warfare agents by several governments including the United States (Christopher et al. 1997; Franz et al. 1997). Beginning in 1942 and under the direction of the War Reserve Service, several potential pathogenic and nonpathogenic biological agents, including *Brucella suis*, were tested as candidates for weaponization (Christopher et al. 1997). By 1954 *B. suis* was loaded into biological cluster bombs for use by the U.S. Airforce (Franz et al. 1997).

1.5 *Brucella* Intracellular Colonization

1.5.1 *Brucella* General Intracellular Characteristics

Members of the genus *Brucella* do not evade phagocytosis by professional phagocytes. According to Spink (1956), phagocytosis of brucellae by macrophages and polymorphonuclear (PMN) cells may be necessary for protection and dissemination of the bacterium throughout the host. Brucellae are non-motile and readily ingested by macrophages where they multiply, eventually establishing infection within both professional and non-professional phagocytic cells (Smith and Ficht 1990). Other virulent facultative intracellular pathogens with this ability to colonize phagocytic cells include *Mycobacterium, Legionella, Shigella, Listeria, Salmonella, Yersinia, Nocardia* and possibly *Neisseria* (Kiderlen, Kaufmann et al. 1984; Frenchick, Markham et al. 1985; Mims 1987; Salyers and Whitt 1994; Russell 2000; Cianciotto 2001). Exactly how brucellae withstand the bactericidal environment of such cells as macrophages and neutrophils is not entirely understood; however, there appears to be more than one mechanism involved. Studies conducted by Detilleux, Deyoe et al. (1990a, b, 1991) show that brucellae do not induce their own uptake by non-phagocytic cells. After phagocytosis, brucellae are found in coated pits, suggesting they are phagocytized by non-professional phagocytes, such as Vero cells, through a receptor-mediated process.
that has not yet been elucidated. One particular study has confirmed these observations with non-professional phagocytes and provided new evidence that *Brucella* uptake into macrophages is also receptor mediated. Using transposon mutagenesis, Sola-Landa et al. (1998) have uncovered a possible two component sensory and regulatory (BvrR-BvrS) system employed by *B. abortus*, which may be responsible for invasion, intracellular survival and replication within Hela cells and macrophages. The BvrR-BvrS system of *Brucella* is closely related to similar sensory/regulatory systems in *Rhizobium meliloti* (Chvl-ExoS) and *Agrobacterium tumefaciens* (Chvl-ChvG) (Sola-Landa et al. 1998). It appears that the *Brucella* BvrR-BvrS system is responsible for outer membrane properties that control bacterial susceptibility to polycations and surfactants, host cell penetration and invasion, and intracellular survival and phagosomal trafficking (Sola-Landa et al. 1998).

### 1.5.2 *Brucella* Within Professional Phagocytes

Within professional phagocytes, such as macrophages, brucellae appear to survive and multiply within the phagosome and phago-lysosome, similar to pathogens like *Leishmania* spp. and *Coxiella burnetti* (Harmon, Adams et al. 1988; Rabinovitch 1996 as cited in Sinai and Joiner 1997; Porte, Liautard et al. 1999). Recent work by Rittig, Alvarez-Martinez et al. (2001) suggests that when brucellae (*B. suis* and *B. melitensis*) are phagocytized by human monocytes, they are sequestered in phagosomes with either tightly (TP) or loosely (SP) apposed walls. Brucellae found in the TP phagosomes readily survive acidification whereas brucellae found in SP phagosomes are killed in the acidic environment resulting from phagosome-lysosome fusion.

Once phagosome-lysosome fusion occurs, brucellae appear to be resistant to degradation at a pH of 3.2-4.0 and begin multiplying within the acidified compartment
(Kulakov, Guigue-Talet et al. 1997; Porte et al. 1999; Arenas, Staskevich et al. 2000; Endley, McMurray et al. 2001). This resistance to lysosomal degradation is also seen in other bacteria such as *Mycobacterium lepraemurium*, which is protected by a hydrophobic mycoside capsule, and *Salmonella typhimurium* (Mims 1987). Evidence suggests that acidification of the *Brucella* containing phagosome promotes the synthesis of bacterial stress proteins including GroEL and DnaK which are essential for replication of the bacteria within macrophages (Lin and Ficht 1995; Kohler, Teyssier et al. 1996; Rafie-Kolpin, Essenber et al. 1996; Porte et al. 1999; Arenas et al. 2000; Rittig, Alvarez-Martinez et al. 2001; Boschirol, Ouahrani-Bettache et al. 2002). Brucellae share this unique environment for transcriptional activation with at least four other pathogens such as *Leishmania*, *Coxiella*, *Legionella pneumophila* (Sturgill-Koszychi and Swanson 2000), and *Salmonella typhimurium*. Upon phagosome acidification *Salmonella* activates pag gene expression, which are virulence and survival genes under the direction of the *phoP/phoQ* sensory/regulatory system that are regulated by stressful conditions such as carbon limitation and low pH typically found within the phagosome of a macrophage (Aranda, Swanson et al. 1992; Salyers and Whitt 1994; Rathman, Sjaastad et al. 1996; Joiner 1997; Russell 2000; Cianciotto 2001; Rittig et al. 2001; Boschirol et al. 2002).

1.5.2.1 Brucellacidal Activity of PMNs and Macrophages

Virulent strains of *Brucella* can readily invade and multiply within both macrophages and PMN cells (Young 1995). Macrophages, however, are considered the primary phagocytic host (Mims 1987; Madkour 1989). Why brucellae are rarely found in PMN cells such as neutrophils probably has to do with both their increased bactericidal activity and limited life span (Madkour 1989; Thoen et al. 1993). PMN cells are known
to produce a larger quantity of antimicrobial oxygen species, thus making them more
effective than monocytes or macrophages at killing certain fungi and bacteria (Mims
1987). In addition, activation may be required for full macrophage antimicrobial activity,
as evidenced by the parasites *Leishmania, Listeria* and *Toxoplasma gondii*, which can
survive phagocytosis by non-activated macrophages (Mims 1987). However, compared
to *Listeria*, *Brucella* is more resistant to killing by activated macrophages (FAO/WHO
1986). The limited life span of a PMN, usually two to three days in man, compared to
several weeks or months for macrophages, may also be a limiting factor, not allowing for
*Brucella* replication and persistence (Mims 1987; Atlas 1997). The basic bactericidal
mechanisms of both macrophages and PMN cells will be discussed in section 1.6.

### 1.5.3 *Brucella* Within Non-Professional Phagocytes

Within non-professional phagocytes, like Vero cells, Hela cells and trophoblasts,*
*Brucella* takes a different course altogether, and is not solely located in phagosomes or
phago-lysosomes. As with professional phagocytic cells, brucellae may be found within
phagosomes of non-dedicated phagocytic cells (Detilleux, Deyoe et al. 1990a; Pizzaro-
Cerda, Moreno et al. 1998). According to Detilleux et al. (1990a), the fate of these
brucellae after phagosome-lysosome fusion appears to be death and degradation. An
important discovery made by Anderson et al. (1986a, b) in two studies revealed that
within the placenta of goats, *B. abortus* is endocytosed by erythrophagocytic trophoblasts
and subsequently localize and multiply within the rough endoplasmic reticulum (RER) of
chorioallantoic trophoblasts. This localization of brucellae to cisternae of the RER or ER
(endoplasmic reticulum)-like structures has also been well established in other non-
phagocytic cells including Vero cells (Detilleux, Deyoe et al. 1990b; Detilleux et al.
1990a; Detilleux, Deyoe et al. 1991), Hela cells (Pizzaro-Cerda et al. 1998; Delrue,
Martinez-Lorenzo et al. 2001), as well as in further experiments using trophoblasts (Anderson et al. 1986a, b; Samartino and Enright 1993). The only other bacterium that finds a similar, although not identical, ER-like intracellular niche is \textit{Legionella pneumophila} (Katz and Hashemi 1982; Horwitz 1983). The purpose for RER colonization by \textit{Brucella} has only been speculative, but possibilities include use of trophoblastic RER produced proteins for bacterial metabolism or as glycoprotein components of the bacterial membrane (Anderson et al. 1986b).

1.6 \textit{Brucella} Intracellular Survival

1.6.1 Intracellular Environmental Conditions

Once phagocytized by macrophages or PMN cells, brucellae must endure an onslaught of bactericidal elements. Antimicrobial mechanisms available to phagocytes can be separated into those that are oxygen-dependent, non-oxygen dependent or nitrogen dependent (Mims 1987; Atlas 1997). Once phagocytized, brucellae encounter an initial oxidative burst which generates reactive oxygen intermediates such as superoxide anions, hydrogen peroxide and hydroxyl radicals (Mims 1987). In addition, PMN cells and circulating monocytes catalyze the myeloperoxidase-mediated halogenation of hydrogen peroxide, leading to the production of hypohalides (OCL⁻) and singlet oxygen (Elsbach and Weiss 1983; Mims 1987; Baldwin, Jiang et al. 1993; Paul 1993). These oxygen and halogenated radicals damage DNA, proteins and bacterial membrane lipids (Mims 1987; Paul 1993). Nitrogen-dependent antimicrobial activity includes the production of reactive nitrogen intermediates like nitrate, nitrite and nitric oxide (Atlas 1997).

Brucellae must also contend with oxygen independent bactericidal mechanisms used by professional phagocytes that result from phagosome-lysosome fusion and acidification of the phagosome to a pH of 3.5 (Mims 1987; Porte et al. 1999).
Phagocytes release hydrolytic enzymes, such as lysozyme and acid hydrolases, into the phagosome as well as antimicrobial agents like defensins and cationic proteins which are found in PMN cells and some macrophages (Mims 1987; Paul 1993; Porte et al. 1999). Professional phagocytes also use nutritional deprivation against ingested microbes. PMN cells secrete a B12-binding protein, while both macrophages and PMN cells discharge lactoferrin into the phago-lysosome, which competes with intracellular bacteria for free iron (Mims 1987; Atlas 1997).

1.6.2 Intracellular Survival Mechanisms

1.6.2.1 Brucella Manipulation of Phagosomal Maturation

After phagocytosis, *Brucella* spp., like *Mycobacterium*, *Legionella*, *Chlamydia*, *Toxoplasma*, *Salmonella* and *Listeria*, alter the process of phagosome maturation and phagosome acidification (Aranda et al. 1992; Alvarez-Dominguez, Roberts et al. 1997; Sinai and Joiner 1997; Arenas et al. 2000). Upon phagocytosis by macrophages, *B. abortus* may alter phagosomal maturation and intracellular transport in at least half of their vacuoles, significantly delaying interaction with lysosomes (Arenas et al. 2000; Endley et al. 2001; Rittig et al. 2001). Recently, Naroeni, Jouy et al. (2001) have shown with *B. suis* that delayed phagosomal maturation may be due to a phagosomal membrane modification that prevents or delays lysosomal recognition. This modification is believed to be due to an active process by which brucellae alter the phagosomal membrane since killed brucellae do not cause delayed phagosomal maturation. Frenchick et al. (1985) suggest this modification is not associated with the *Brucella* LPS.

1.6.2.2 Brucella Inhibition of Phagocyte Degranulation

Instead of directly challenging the host’s natural bactericidal machinery, brucellae may use stealth to establish an intracellular replication niche. Studies conducted by Riley
and Robertson (1984a, b) suggest that virulent, smooth strains of \textit{B. abortus} fail to stimulate human or bovine PMN cell degranulation at levels comparable to extracellular parasites such as \textit{Staphylococcus epidermidis}. Intracellular degranulation by PMN cells normally involves the fusion of microbe-containing phagosomes with lysosomes, releasing bactericidal agents such as reactive oxygen species, proteases and defensins (Riley and Robertson 1984b, a). Furthermore, the level of degranulation caused by glutaraldehyde-killed \textit{B. abortus} was comparable to viable \textit{B. abortus}, suggesting that a surface molecule of \textit{Brucella} either allows the bacteria to enter the macrophage undetected or inhibits normal degranulation by some unknown mechanism (Riley and Robertson 1984b, a). Other researchers have obtained similar results showing inhibited degranulation while also using bovine PMN cells (Canning, Roth et al. 1986).

A major departure from the theory of a surface macromolecule as the inhibitor of degranulation is the identification of two molecules, produced by live \textit{B. abortus}, which alone suppress PMN cell degranulation (Canning, Roth et al. 1985; Canning et al. 1986). Adenine and 5’-guanosine monophosphate (GMP) were found to inhibit up to 80% and 65% respectively, of the release of peroxidase-positive granules from bovine PMN cells (Canning et al. 1986). Similar results have been obtained in experiments with another pathogen, \textit{Haemophilus somnus} (Chiang, Kaeberle et al. 1986), where the secretion of GMP and adenine also inhibit PMN function (Canning et al. 1986).

\textbf{1.6.2.3 \textit{Brucella} Inhibition of Macrophage Activation}

Macrophage activation is essential for immune control of microbial infections (Mims 1987). Activated macrophages have increased lysosomal enzyme content and bactericidal mechanisms, which are necessary for controlling intracellular pathogens (Cossart, Boquet et al. 2000). Thus, activated as opposed to non-activated macrophages
have an enhanced ability to resist infection and/or manage *Brucella*, mycobacteria, *Listeria monocytogenes*, protozoans such as *Toxoplasma gondii* and *Leishmania*, as well as some viral agents (Pomales-Lebron and Stinebring 1957; Fitzgeorge, Solotorovsky et al. 1967; Ralston and Elberg 1968; Mims 1987; Unane 2000). Activation may occur through a T-cell dependent or independent manner. The T-cell independent pathway relies on a cytokine cascade that begins when the macrophage encounters a pathogen, triggering the release of interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α). IL-12 and/or TNF-α stimulate natural killer (NK) cells to secrete interferon gamma (IFN-γ) that, in turn, activates other macrophages (Paul 1993; Unane 2000). The T-cell dependent pathway involves the digestion and presentation of bacterial proteins on the surface of macrophages and other antigen presenting cells (APCs), which are attached to major histocompatibility complexes (MHCs). Bacterial antigens bound to these MHC class II molecules, in the presence of IL-12, stimulate CD4+ cell differentiation into Th1 (T-helper) cells that subsequently release IFN-γ which activates macrophages (Salyers and Whitt 1994; Unane 2000).

### 1.6.2.4 Brucella Inhibition of Phagocyte Cytokine Signaling

The importance of both IFN-γ and TNF-α to macrophage activation and the control of *Brucella* infection have been shown in numerous studies (Jones and Winter 1992; Stevens, G. W. Pugh et al. 1992; Baldwin et al. 1993; Jiang and Baldwin 1993; Jiang, Leonard et al. 1993; Baldwin and Winter 1994; Caron, Peyrard et al. 1994; Murphy, Parent et al. 2001). Moreover, *Brucella* survival within human macrophage-derived cells appears to be at least partially dependent on its ability to inhibit the secretion of TNF-α and thus avoid macrophage activation via IFN-γ (Caron et al. 1994; Caron, Gross et al. 1996). Precedent for this virulence factor is found in other pathogens
such as *Yersinia pestis, Listeria monocytogenes, Bacillus anthracis, and Mycobacterium avium* all of which inhibit TNF-α production by their hosts (Furney, Skinner et al. 1992; Ugalde 1999). Interestingly, *Rhodopseudomonas sphaeroides*, a purple, nonsulfur bacteria found in α-Proteobacteria produces a lipid A that also inhibits TNF-α production by macrophages (Takayama, Qureshi et al. 1989; Madigan et al. 2000). Studies by Caron et al. (1994, 1996) found that *B. suis* infected human-derived macrophages could not accumulate TNF-α mRNA, and that *Brucella* LPS was not responsible. They also concluded that inhibition of TNF-α production was the result of an active process by the bacterium since chloramphenicol treated and killed *B. suis* did not trigger human macrophages to secrete TNF-α. Furthermore, Caron et al. (1996) have tentatively identified the *Brucella* inhibitor of TNF-α as a protein, based on its protease and heat sensitivity, of 45 to 50 kDa.

Research by Zhan, Liu et al. (1996) has reevaluated the centrality of TNF-α to macrophage activation and brucellacidal activity. Using a mouse model, they determined that the most important cytokine for macrophage activation is not TNF-α, but rather IL-12. Zhan et al. (1996) assert that although both TNF-α and IFN-γ are necessary for complete control of *Brucella* infection, TNF-α contributes to brucellacidal activity by an IFN-γ independent pathway, whereas IL-12 is mainly responsible for the activation of NK cells and secretion of IFN-γ leading to macrophage activation. This view is supported by work from Zhou, Miller et al. (1998), who also suggest TNF-α induced macrophage activation is independent of IFN-γ. Additionally, recent work proposes that monocyte programmed cell death is inhibited by brucellae through a TNF-α and LPS-independent mechanism (Gross, Terraza et al. 2000). Specifically, Gross et al. (2000)
assert that the apoptotic affect of IFN-\(\gamma\) towards human phagocytic monocytes is blocked after \(B.\ suis\) infection. An opposing view, however, is held by Zhan and Cheers (1998) and Flesch et al. (1995) (as reviewed by Zhan and Cheers 1998) who advocate a direct relationship among TNF- \(\alpha\), IL-12 and IFN- \(\gamma\).

1.6.2.5 \textit{Brucella} Outer Membrane

The \textit{Brucella} outer membrane has no pili, fimbriae or capsular components, but simply contains phospholipids, outer membrane proteins (Omp), and LPS, of which only the O-polysaccharide component of the LPS is considered a true virulence factor (Smith and Ficht 1990; Cloeckaert, Verger et al. 1996a; Elzer 2002a). Brucellae also have a high content of ornithine lipids (17-32\% of total lipid) in their outer membrane (Martin and Hancock 1990). Ornithine lipids are neutral lipids made up of ornithine, ethylene glycol and fatty acids, they resist hydrolysis by phospholipases and possibly serve as a stabilizing agent for \textit{Brucella} conveying resistance to EDTA and polycations by shielding negatively charged groups in the LPS (Cherwonogrodsky, Dubray et al. 1990; Moreno and Moriyon 2002).

1.6.2.5.1 \textit{Brucella} LPS

The lipopolysaccharide of \textit{Brucella} contains the main components of extracellular toxicity, lipid A, and one of the major \textit{Brucella} antigens, the O-polysaccharide. The presence or absence of the O-polysaccharide/antigen in the LPS layer is the molecular basis of \textit{Brucella} smooth and non-smooth (mucoid or rough) morphology (Caroff, Bundle et al. 1984; FAO/WHO 1986; Perry and Bundle 1990; Smith and Ficht 1990; Corbel 1997; Ugalde, Czibener et al. 2000). The \textit{Brucella} smooth phenotype is naturally more virulent, while non-smooth brucellae are normally avirulent (Riley and Robertson 1984b, a; Young, Borchert et al. 1985; FAO/WHO 1986; Harmon et al. 1988; Smith and Ficht
1990; Roop II 1991; Rasool, Freer et al. 1992; Thoen et al. 1993; Baldwin and Winter 1994; Kulakov et al. 1997). According to Riley and Robertson (1984a), *B. abortus* smooth and non-smooth strains elicit similar amounts of degranulation from bovine and human PMN cells, however, the smooth strains, unlike the non-smooth versions, are resistant to the phagocyte’s microbicidal action. The difference in intracellular success between non-smooth and smooth strains suggests that intracellular survival is dependent on cell surface components (see previous references). But exceptions to this rule do exist, as seen with *B. canis* and *B. ovis*, which are naturally occurring, virulent non-smooth strains, and *B. neotomae*, which is an avirulent smooth strain (FAO/WHO 1986; Smith and Ficht 1990; Baldwin and Winter 1994; Sangari and Aguero 1996; Kulakov et al. 1997). The methods by which smooth and non-smooth strains of *Brucella* differ in their protection from intracellular bactericidal mechanisms appear to be complex, and not solely dependent on bacterial surface structure. Evidence to this point is given by Kulakov et al. (1997), who found that *B. suis* and *B. melitensis* non-smooth mutants are able to withstand acidic pH as efficiently as non-mutated smooth strains.

The O-polysaccharide/antigen in smooth strains of *Brucella* may be found in either an A or M epitope, both of which can be expressed on the same organism, but in different quantities. The A epitope, examined from *B. abortus*, is a linear molecule composed of 96-100 residues of α-1, 2-linked 4,6-dideoxy-4-formamido-D-mannopyranose, also known as D-Rhap4 NFo (Cherwonogrodsky et al. 1990; Perry and Bundle 1990). The M epitope from *B. melitensis* is identical to the A epitope except that it is a series of repeating pentasaccharide units containing one α-1,3-linked and four α-1,2-linked monosaccharide residues (FAO/WHO 1986; Bundle, Cherwonogrodzky et al.
Brucellae of the smooth type that mutate and lose this O-polysaccharide chain exhibit the non-smooth phenotype (Sola-Landa et al. 1998). Unlike other Gram-negative, enterobacterial LPS endotoxins, *Brucella* LPS is non-pyrogenic and much less toxic to its host, especially in the non-smooth (rough) form (Moreno, Berman et al. 1981; Cherwonogrodsky et al. 1990; Rasool et al. 1992). In a study using human neutrophils, Rasool et al. (1992) found that 100 times more *Brucella* LPS and 1000 times more lipid A were required to elicit an equivalent lysosome release from phagocytes compared to LPS from *Salmonella typhimurium*. In addition, *Brucella* LPS and lipid A were several orders of magnitude less toxic than *Salmonella* LPS. Rasool et al. (1992) speculated that the low biological toxicity of *Brucella* LPS might be a factor allowing the intracellular survival and persistence of brucellae inside phagocytes.

### 1.6.2.5.2 *Brucella* Outer Membrane Proteins (Omp)

*Brucella* spp. possess three classes of outer membrane proteins (Omp). The first class, group 1, contains two Omps of 88 and 94 kDa; group 2 includes proteins of 35-43 kDa, and is composed of two Omps, 2a (41 kDa) and 2b (43 kDa); group 3 incorporates proteins of 16.5-34 kDa and contains Omp25 (25 kDa), and Omp31 (31 kDa) (Verstreate, Creasy et al. 1982; Douglas, Rosenberg et al. 1984; Mayfield, Bricker et al. 1988; Cherwonogrodsky et al. 1990; Bowden, Cloeckaert et al. 1995; Cloeckaert, Jacques et al. 1995; Cloeckaert et al. 1996a; Lindler, Hadfield et al. 1996; Vizcino, Cloeckaert et al. 1996; Edmonds, Cloeckaert et al. 2001; DelVecchio et al. 2002). The group 1 Omps are believed to be minor components of the cell envelope, while group 2 Omps are assigned porin structural properties (Douglas et al. 1984; FAO/WHO 1986; Smith and Ficht 1990). Membrane proteins in group 3 are structural in nature; Omp31 is complexed to the peptidoglycan whereas Omp25, which is highly conserved among all six *Brucella*
species, is bound to the peptidoglycan and LPS (Madkour 1989). It should be noted that while some Omgs are considered major membrane components in one species, they may only be a minor membrane constituent of another species, i.e., Omp 31 is a major protein in \textit{B. melitensis} but is absent in \textit{B. abortus} (Vizcino et al. 1996). Research has confirmed that every group of \textit{Brucella} outer membrane protein elicits an antigenic response from the host (Dubray and Bezard 1980; Mayfield et al. 1988; Limet, Cloeckaert et al. 1993, 1995, 1996a; Bowden et al. 1995; Lindler et al. 1996; Vizcino et al. 1996; Bowden, Cloeckaert et al. 1998). Research by Edmonds et al. (2001) has confirmed the importance of Omgs to \textit{Brucella} intracellular survival. They demonstrated that \textit{B. abortus} Omp25 mutants exhibited lower levels of survival in bovine macrophages and trophoblasts compared to wild type strains.

1.6.3 Other \textit{Brucella} Virulence Genes and Gene Products

Although brucellae exhibit many of the same characteristics as other intracellular pathogens, i.e., survival and multiplication within the phagosome and inhibition of cell signaling, the majority of genes involved in \textit{Brucella}-related virulence have not been discovered (Smith and Ficht 1990; Robertson, Kovach et al. 2000). One large step towards identifying and confirming the presence of virulence factors and other essential genes comes from a sequence analysis of the entire \textit{B. melitensis} 16M genome by DelVecchio et al. (2002).

Although their true importance to \textit{Brucella} pathogenicity has not yet been established, the genes, proteins and pathways listed in Table 2 likely play a role in virulence. In common with most other bacterial species, brucellae also employ a number of specialized enzymes, which are not necessarily thought of as virulence factors, for
Table 2. Putative virulence-related genes, proteins or pathways in *B. melitensis* 16M.

<table>
<thead>
<tr>
<th>Protein, Gene or Pathway Identified in <em>B. melitensis</em></th>
<th>Homologous to:</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysin, α-hemolysin, hemolysin III</td>
<td></td>
<td>DelVecchio et al. 2002</td>
</tr>
<tr>
<td>Adhesin protein precursors AidA-I</td>
<td></td>
<td>Sanchez, Zandomeni et al. 2001; DelVecchio et al. 2002</td>
</tr>
<tr>
<td>Invasin precursor</td>
<td>InvA of <em>Mesorhizobium loti</em> and <em>Burkholderia cepacia</em></td>
<td>DelVecchio et al. 2002</td>
</tr>
<tr>
<td><em>acvB</em></td>
<td>VirJ of <em>Agrobacterium tumefaciens</em></td>
<td>DelVecchio et al. 2002</td>
</tr>
<tr>
<td><em>mviN</em></td>
<td>Virulence protein of <em>Salmonella</em></td>
<td>DelVecchio et al. 2002</td>
</tr>
<tr>
<td>Lipid IV synthesis</td>
<td></td>
<td>DelVecchio et al. 2002</td>
</tr>
<tr>
<td>UDP-<em>N</em>-acetlymuramoyl pentapeptide synthesis</td>
<td></td>
<td>DelVecchio et al. 2002</td>
</tr>
<tr>
<td>Ortholog of <em>cydB</em></td>
<td><em>cydB</em> of <em>B. abortus</em></td>
<td>Endley et al. 2001; DelVecchio et al. 2002</td>
</tr>
<tr>
<td>Symbiosis protein</td>
<td>Protein E of <em>Bradyrhizobium japonicum</em></td>
<td>DelVecchio et al. 2002</td>
</tr>
<tr>
<td><em>entD</em></td>
<td></td>
<td>DelVecchio et al. 2002</td>
</tr>
<tr>
<td>Adaptive acid tolerance regulatory protein</td>
<td>ActR of <em>Bradyrhizobium</em></td>
<td>DelVecchio et al. 2002</td>
</tr>
</tbody>
</table>

...
Mayfield 2000; Robertson et al. 2000; Teixeira-Gomes, Cloeckaert et al. 2000). Phillips et al. (1995) and Elzer et al. (1996) have shown that mutation of the htrA gene results in decreased resistance of B. melitensis and B. abortus to oxidative damage and phagocyte killing.

In addition to the identification of coding regions for cold and heat shock proteins in B. melitensis (DelVecchio et al. 2002), several studies have revealed increased production of the molecular chaperones DnaK and GroEL by B. abortus and B. melitensis under conditions of macrophage phagocytosis, acid and heat stress (Rafie-Kolpin et al. 1996; Teixeira-Gomes et al. 2000). To complement this, Kohler et al. (1996) have shown decreased B. suis survival under conditions of macrophage phagocytosis and acid stress when dnaK is mutated. Robertson et al. (2000) studied the activity and function of the stress response protease Lon in B. abortus and found it may play a critical role in turnover of heat-damaged proteins in the initial stages of infection and colonization.

With the exception of B. neotomae and B. suis biovar 2, all brucellae encounter and scavenge highly reactive oxygen species within the phagosome primarily with a catalase and two types of superoxide dismutases (SOD), a Fe-Mn SOD and a Cu-Zn SOD (Bricker, Tabatabai et al. 1990; Sriranganathan, Boyle et al. 1991; Tatum, Detilleux et al. 1992; Rafie-Kolpin et al. 1996; Kim and Mayfield 2000; Teixeira-Gomes et al. 2000; DelVecchio et al. 2002). Disabling B. abortus catalase expression through mutation of the catalase promoter, oxyR, causes increased sensitivity of B. abortus to hydrogen peroxide (Kim and Mayfield 2000). Moreover, virulence and level of catalase expression appear to be positively correlated (Huddleson and Stahl 1943). And after mutation of the
Cu-Zn SOD, Tatum et al. (1992) found that BALB/c mice were better able to control the growth and progression of *B. abortus* during early stages of infection.

### 1.7 Limitations of the Murine and Bovine Models of Brucellosis

The ability of brucellae to survive within phagocytic cells is also a function of the mammalian host infected, i.e., mouse, human or cow. A large difference exists between mouse and human macrophage susceptibility to *Brucella* infection and persistence (Cheers and Pagram 1979; Caron et al. 1996; Gross et al. 2000). As supported by several studies (Zhan and Kelso 1993; Caron et al. 1994, 1996; Zhan and Cheers 1995, 1998; Liautard, Gross et al. 1996), murine macrophages readily produce TNF-α upon infection with live *Brucella* whereas human cell lines do not. It has been postulated that the secreted TNF-α inhibitory factor is species specific, and mouse cell lines are resistant to inhibition (Caron et al. 1996; Liautard et al. 1996). This may help to explain some contradictory results regarding TNF-α production and/or inhibition seen in the literature. In addition, while *Brucella* avoids the bactericidal activity of IFN-γ-induced human monocytes, it is regularly cleared in mouse macrophages induced in the same manner (Stevens et al. 1992; Zhan and Cheers 1995; Gross, Spiesser et al. 1998; Gross et al. 2000).

Bovine phagocytes also are markedly different from their human counterparts. In studies conducted with *B. abortus* smooth strain 45/0, Riley and Robertson (1984a) found that human PMN cells were not as effective in killing phagocytized brucellae when compared to bovine PMN cells. The difference in brucellacidal activity appeared to be due to more extensive degranulation in bovine as compared to human PMN cells (Riley and Robertson 1984b, a). This decreased ability to control a *Brucella* infection may help explain the recurring nature of untreated human brucellosis.
1.8 *Brucella* Type IV Secretion and Virulence - The *vir* Family

1.8.1 *vir* General Characteristics

In contrast to typical type I, II, III and V secretion systems which only export proteins, Gram-negative bacterial type IV secretion systems are believed to be specialized for the secretion of nucleoprotein complexes in addition to straight protein products such as multisubunit bacterial toxins (Christie and Covacci 2000). As proposed by Christie and Covacci (2000), the type IV secretion family is thought to have originated from a system originally designed for conjugative transfer of DNA between bacterial cells. Through horizontal transfer and association with other types of genes, different classes of bacteria may have adapted the type IV secretion machinery to their environments, or exploited new niches. Specifically, the type IV secretion system is used for the exchange of protein-DNA complexes between bacteria, as seen in *Escherichia* F plasmid transport, between bacteria and eukaryotic cells, as with *Agrobacterium* T-complex transfer to plant root cells, and for extracellular secretion of polypeptide toxins, as in *Bordetella* toxin export (Christie and Covacci 2000; Sieira et al. 2000).

*Brucella* do not contain typical, complete operons for type I, II or III secretion, however, they do encode components for *sec*-dependent, *sec*-independent, flagellar-specific type secretion systems, and type IV and V secretion systems (DelVecchio et al. 2002; Moreno and Moriyon 2002). The *Brucella* type IV secretion system is similar to secretion systems in other symbionts and pathogens within *α-Proteobacteriacae* (Christie and Covacci 2000; DelVecchio et al. 2002). Type IV secretion in all *Brucella* species, including *B. melitensis*, is encoded by *virB* located on chromosome II (O'Callaghan et al. 1999; DelVecchio et al. 2002). *Brucella* *virB* genes are sequentially related to genes in type IV secretion systems found in other closely related bacteria; e.g., the *vir* system of

The virB regions of B. suis and B. melitensis are composed of 12 linearly arranged open reading frames (ORF), whereas B. abortus contains an additional 13th ORF that precedes and overlaps ORF12 (O'Callaghan et al. 1999; Sieira et al. 2000; DelVecchio et al. 2002). There exist 2 palindromic, repeat sequences (BruRS1 and BruRS2) in the virB region, the first is between virB1 and virB2, while the second is after virB12 (Halling and Bricker 1994; O'Callaghan et al. 1999; Sieira et al. 2000; Boschiroli et al. 2002). Two researchers have proven that despite this first palindrome, Brucella virB genes are transcribed as an operon starting from virB1 (Sieira et al. 2000; Boschiroli et al. 2002). However, some disagreement exists between these two studies as to whether the virB operon is turned on during early stationary or early exponential phase. A possible explanation for this discrepancy may be that the previous studies employed different species of Brucella, namely B. abortus (Sieira et al. 2000) and B. suis (Boschiroli et al. 2002). Using B. suis, Boschiroli et al. (2002) were the first to show virB operon induction in response to environmental stress. By mimicking the macrophage intracellular environment, they demonstrated that acid stress, elevated temperature (37°C)
and nutritional deprivation were all positive regulators of \textit{virB} expression. Additionally, translation of each \textit{virB} gene is thought to occur independently since possible ribosome binding sites are found upstream of all \textit{B. suis virB} genes (O’Callaghan et al. 1999).

Very little is known about the actual structure encoded by the \textit{Brucella virB} operon. However, between genera, the \textit{Brucella virB} operon is closely related to the \textit{tra} system of the broad-host-range plasmid pKM101 found in \textit{E. coli} and to the \textit{virB} genes of \textit{Agrobacterium tumefaciens}, from which it’s name was taken (Stachel and Nester 1986; Lessl, Balzer et al. 1992; O’Callaghan et al. 1999; Christie and Covacci 2000; Sieira et al. 2000; DelVecchio et al. 2002; Moreno and Moriyon 2002). O’Callaghan et al. (1999) have revealed that at the protein level, the \textit{B. suis virB} operon is more closely related to the \textit{ptl} operon of \textit{Bordetella pertussis} than to \textit{virB} of \textit{Agrobacterium} or the \textit{tra} system of \textit{E. coli}. But since 11 genes of the \textit{Brucella melitensis virB} share sequence and organizational similarity with the \textit{virB} operon of \textit{Agrobacterium}, structural and functional characteristics of the \textit{Brucella} system can be speculated (O’Callaghan et al. 1999; DelVecchio et al. 2002). Like the \textit{Agrobacterium} type IV system, the \textit{Brucella virB} probably codes for proteins that form a gated, pilus-like structure that spans the inner and outer bacterial membranes as well as the eukaryotic cell membrane (Ward, Akiyoshi et al. 1988; Kulda, Vos et al. 1990; Christie and Covacci 2000). While the \textit{Agrobacterium} pilus penetrates the eukaryotic plant cell envelope from the outside, it is reasonable to suspect the \textit{Brucella} pilus would gain access to the mammalian host cell cytoplasm by spanning the phagosome where it has been localized (Christie and Covacci 2000; Rittig et al. 2001).
Using functional studies of the *Agrobacterium virB* encoded proteins as a guide, *Brucella virB* protein characteristics have been predicted (Table 3). The exact function of the *virB* encoded secretion system in *Brucella* is unknown, although knockout studies have shown the *virB* operon is essential for *Brucella* intracellular survival. Construction of polar mutants of the entire *virB* operon or non-polar mutants of single *virB* genes, i.e., *virB1, 2, 4, 5, 8, 9, 10 and 12*, have resulted in an attenuated phenotype in every case and almost complete elimination of brucellae from HeLa cells, human monocyte-like cell lines and BALB/c mice in some instances (O’Callaghan et al. 1999; Ugalde 1999; Foulongne, Bourg et al. 2000; Hong et al. 2000; Sieira et al. 2000; Comerci et al. 2001; Delrue et al. 2001; Boschirolı et al. 2002). Comerci et al. (2001) and Delrue et al. (2001) have shown that a functional *virB* operon in *B. abortus* and *B. melitensis* is necessary for

### Table 3. Predicted function of *Brucella virB* operon components (adapted from O’Callaghan et al. 1999; Christie and Covacci 2000; DelVecchio et al. 2002).

<table>
<thead>
<tr>
<th><em>virB</em> Gene</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VirB1</td>
<td>Trans-glycosylase</td>
</tr>
<tr>
<td>VirB2</td>
<td>Pilus formation</td>
</tr>
<tr>
<td>VirB3</td>
<td>Inner membrane protein</td>
</tr>
<tr>
<td>VirB4</td>
<td>Cytoplasmic protein, ATPase</td>
</tr>
<tr>
<td>VirB5</td>
<td>Pilus component</td>
</tr>
<tr>
<td>VirB6</td>
<td>Inner member protein</td>
</tr>
<tr>
<td>VirB7</td>
<td>Lipoprotein, forms complex with VirB9</td>
</tr>
<tr>
<td>VirB8</td>
<td>Inner membrane protein</td>
</tr>
<tr>
<td>VirB9</td>
<td>Periplasmic protein, forms complex with VirB7</td>
</tr>
<tr>
<td>VirB10</td>
<td>Periplasmic protein</td>
</tr>
<tr>
<td>VirB11</td>
<td>Inner membrane protein, ATPase</td>
</tr>
<tr>
<td>VirB12</td>
<td>Function unknown (no homology to other type IV proteins)</td>
</tr>
</tbody>
</table>
manipulation of phagosomal trafficking, including delay or prevention of phagosome-lysosome fusion and establishment of a replication niche. *VirB* is not thought to play a role in *B. abortus* entry into the host cell since no reduction in adherence or internalization has been observed with any *virB* mutation (Sieira et al. 2000; Comerci et al. 2001).

The *Brucella* type IV secretion system is thought to be responsible for the export of a virulence factor, as yet unknown, which mediates intracellular survival and multiplication (O’Callaghan et al. 1999; Sieira et al. 2000; Comerci et al. 2001). Materials exported by bacteria with homologous type IV secretion systems include: interleukin-8-inducing factor by *Helicobacter pylori*, the T-complex by *Agrobacterium tumefaciens*, pertussis toxin by *Bordetella* and plasmid DNA by *Escherichia* (Christie and Covacci 2000). Yet, unlike *Escherichia* and *Agrobacterium*, brucellae do not contain plasmids, and exchange of naturally occurring genetic material has never been observed (O’Callaghan et al. 1999).

Recently, three *Brucella* products have been identified which are potential candidates for *virB*-mediated export. The first two are non-protein products of *B. abortus* identified by Canning et al. (1985, 1986) mentioned previously as GMP and adenine, which act as inhibitors of the PMN myeloperoxidase (MPO)-H₂O₂ halide antibacterial system. The second possibility, identified by Caron et al. (1996), is a protein, based on its protease and heat sensitivities, of approximately 45 to 50 kDa released by *B. abortus* that inhibits TNF-α production in a human, macrophage-like, neoplastic cell line, U937. Either one or both of these inhibitory products may be exported by the *virB* system of *Brucella*. However, it should be noted that O’Callaghan et al. (1999) also found a
Brucella protein inhibitor of TNF-α release, but they acknowledged that virB mutants were still able to inhibit TNF-α production.

1.8.2 virB1 and Virulence

This body of work demonstrates the inactivation of the first virB gene transcribed in the virB operon, virB1. Efficient knockout of this gene was established by assessing the survival and virulence of virB1 mutated strains of B. abortus 2308 and B. melitensis 16M in macrophage and in-vivo murine models. Little is known about the Brucella virB1 gene and gene product, except what has been predicted by nucleotide and protein sequence analysis and comparison to virB1 of Agrobacterium (Christie 1997; O'Callaghan et al. 1999). The virB1 genes of B. melitensis and B. abortus are located on chromosome II, are 717 nucleotides long with a GC content of 57 % (DelVecchio et al. 2002). By homology to VirB1 of A. tumefaciens and TraL of E.coli, the Brucella VirB1 is suspected of being a trans-glycosylase, playing a role in the formation of a pore for extrusion of macromolecular complexes from the bacterial cell (O'Callaghan et al. 1999; Christie and Covacci 2000; Sieira et al. 2000; DelVecchio et al. 2002). However, whether VirB1’s role is essential to virB mediated export is under suspicion after a study by Berger and Christie (1994) proposed that VirB1 of A. tumefaciens is supportive but not essential for virulence (Christie and Covacci 2000).

1.9 Brucella Genetic Manipulation

Members of the genus Brucella are not naturally competent, however, they will take up plasmid DNA after chemical treatment or electroporation (Lai, Schurig et al. 1990; Halling, Detilleux et al. 1991; Elzer, Kovach et al. 1995). In addition, brucellae do not contain any naturally occurring plasmids (Rigby and Fraser 1989; Smith and Ficht 1990), yet, conjugative transfer of artificially introduced broad-host-range plasmids
between brucellae, from *E. coli* into brucellae and from brucellae into *E. coli* has been demonstrated (Rigby and Fraser 1989; Verger, Grayson et al. 1993). Genetic complementation of all *Brucella* species is possible using the broad-host-range plasmid pBBR1MCS and its derivatives (Kovach, Phillips et al. 1994; Elzer et al. 1995), which is not integrated into the genome but stably maintained within all *Brucella* spp. at low copy number (Elzer et al. 1995). Also, gene replacement by homologous recombination in *Brucella* has been well established as an important method for the creation of specific in-vivo gene mutations (Lai et al. 1990; Halling et al. 1991; Tatum et al. 1992; Elzer, Phillips et al. 1994; Drazek, Houng et al. 1995; Elzer et al. 1995; Phillips et al. 1995; Sola-Landa et al. 1998; Kohler, Ouahrani-Bettache et al. 1999; O'Callaghan et al. 1999; Sieira et al. 2000; Ugalde et al. 2000; Alvarez-Martinez, Machold et al. 2001; Boschiroli et al. 2002).

One particular technique employed in the process of *Brucella* gene replacement is gene disruption via deletion or insertion mutagenesis, a gene knockout. This process involves cloning the gene of interest into the appropriate plasmid vector, followed by in-vitro mutation of the gene (Fig. 2). The mutation may be accomplished by the deletion of a portion of the gene or insertion of a positive selectable marker, i.e., antibiotic resistance gene (Halling et al. 1991; Madigan et al. 2000), at a particular restriction site within the gene. Once the mutation in the gene of interest is confirmed, the mutagenized plasmid is introduced into *Brucella* where it undergoes a double crossover event, replacing the wild-type gene with the mutagenized one via homologous recombination. Both gene deletion and insertion result in a loss of gene function.
Fig. 2. Gene disruption by homologous recombination between the wild type (virB1) and the mutant gene (virB1::kan). The wild type gene was cloned into a suitable suicide vector, which is not stably maintained in Brucella, and mutagenized by insertion of a positive selectable marker (Kan). Transformation of the suicide vector into Brucella results in recombination between the homologous regions of the mutated and wild type genes leading to gene replacement, in-vivo loss of function and expression of the selectable marker.
As demonstrated by Halling et al. (1991), narrow host range ColE1-derived plasmids (i.e., pBA31-R7) are incapable of replication in *Brucella* spp. and become suicide vectors when they contain *Brucella* DNA. In some instances, homologous recombination occurs between the vector and the *Brucella* genome enabling gene replacement studies. One important note regarding ColE1-derived plasmid nomenclature is the common practice of referring to ColE1-based plasmids as pMB1-derived, and vice versa. The exact basis for this confusion is unknown, however, it is likely that since both types of *E. coli* based plasmids share many of the same properties, i.e., colicin production and immunity, mobilization via the F-factor, mode of replication and same incompatibility group, they are considered similar enough to be used interchangeably (Bolivar 1979; Bhagwat and Person 1981; Sambrook, Fritsch et al. 1989). In line with this uncertainty, many studies have successfully employed pMB1-based suicide vectors in *Brucella* gene replacement studies, i.e., pGP704, pUC18, pBluescript II, pUC19 and pUC4k (Sola-Landa et al. 1998; Kohler et al. 1999; O'Callaghan et al. 1999; Ugalde et al. 2000; Alvarez-Martinez et al. 2001). In addition, at least two such gene replacement studies have found that the cloning vector pGEM-T, created by Promega Corp. (Madison, WI), also functions as an efficient suicide vector in brucellae (Sieira et al. 2000; Boschirolı et al. 2002). PGEM-T is a pMB1-based cloning vector with a mutation in the origin of replication RNAI binding region, thereby making it neither like pMB1 nor ColE1 (Promega Corp., personal communication). Research in the present work employs pGEM-T as a *Brucella* gene-cloning vector and suicide plasmid for in-vivo gene replacement.
Another technique that has been widely used for Brucella gene disruption is in-vivo transposon mutagenesis, mainly employing a Tn5-based transposon (Smith and Heffron 1987; O'Callaghan et al. 1999; Foulongne et al. 2000; Hong et al. 2000; Lestrate, Delrue et al. 2000; Endley et al. 2001). This type of mutagenesis, although not directed at a specific locus, has been employed in the discovery of Brucella genes responsible for virulence and pathogenesis. However, because the B. melitensis 16M genome has been sequenced and potential virulence factors identified, a targeted, systematic approach to gene knockout may now be more productive.

1.10 Transposition

Transposition is the process by which a piece of DNA, a transposable element, is translocated from one site to another by an enzyme known as a transposase (Madigan et al. 2000). Three types of transposons are currently known to exist: insertion sequences (IS), composite transposons (Tn), and certain types of viruses such as Mu (Bennet 1991; Maloy, Cronan et al. 1994; Atlas 1997). Transposition, as described above, involves the use of a transposon, which is a gene or set of genes that code for structural proteins, usually 2000-20000 base pairs long, flanked by short inverted terminal repeats, as seen in ISs (Atlas 1997; Madigan et al. 2000). Examples include the transposons Tn5 and Tn7, which confer resistance to kanamycin and neomycin, and Tn9, which contains a chloramphenicol resistance gene (Smith and Heffron 1987; Atlas 1997; Madigan et al. 2000). Transposition of a transposable element can occur by either a conservative or replicative manner. As its name implies, replicative transposition is the translocation of a copy of a transposable element to a new location, resulting in two copies of the element, one at the original site and one at the new site. Conservative transposition, however,
involves the excision of the transposable element from its original location and
reinsertion at a new site (Madigan et al. 2000).

Minimally, transposition requires the presence of inverted or direct repeats that
flank transposable elements, and the transposase, which recognizes and cleaves the
flanking sequences and then ligates the transposable element at it’s new location. At
least two models exist which explain the conservative mechanism of transposition. In the
first model, proposed by Arthur and Sherratt, and Shapiro, transposase makes single
stranded cuts in both the donor and recipient DNA (as reviewed by Bennet 1991). A
transposition cointegrate is then formed, known as a “Shapiro intermediate,” that may
resolve through the cleavage of the second donor strand and gap repair.

The second model, proposed by Douglas Berg, known as the cut-and-paste
mechanism (Fig. 3) involves the complete release of the transposon from the donor DNA
by transposase enzymes without a cointegrate intermediate (as reviewed by Bennet
1991). The Berg, cut-and-paste model of conservative transposition, is now widely held
as the correct mechanism for the simple insertion of many composite transposons and
insertion sequence elements, including $Tn7$ and $Tn10$. The cut-and-paste model of
transposition is believed to involve the formation of a nucleo-protein complex, where
donor and target DNA and several transposase enzymes interact to carry out a strand
transfer reaction (Bainton, Gamas et al. 1991; Bainton, Kubo et al. 1993; Craig 1995,

1.11 In-Vivo Versus In-Vitro Transposon Mutagenesis

The basic principals behind both in-vivo and in-vitro transposon
mutagenesis are fundamentally the same: a transposable element is translocated from one
Fig. 3. \textit{Tn7}-based transposon mutagenesis. For simplicity, the transposase enzymes are not shown. Within a DNA-protein complex, transposase nicks the donor and target DNA (solid arrows pointing to gaps), releasing the transposon flanked by insertion sequences (IS) and mediates the insertion into the donor and strand transfer reaction. The cell’s DNA repair mechanisms fill-in and ligate the gap left by strand transfer, which results in the formation of a 5 base pair repeat on both sides of the inserted transposon (Maloy et al. 1994).

location to another (with or without replication). But, as their names imply, the major difference between the two lies in whether the transposition reaction occurs intracellularly. With in-vivo transposition, a transposable element is introduced into a microorganism, via plasmid, phage, etc., where it either uses the host’s transposition
machinery or it codes its own transposase enzymes for the process of transposition. In this system, the transposon may theoretically choose to insert anywhere in the host’s available genome. In-vivo transposon mutagenesis of *Brucella* has been used successfully for at least 15 years in search of *Brucella* virulence genes. Smith and Heffron (1987) first mutagenized the *Brucella abortus* genome using a Tn5-based system encoded on a plasmid and on a bacteriophage. Numerous studies of *Brucella* spp. followed which also employed an in-vivo Tn5-based system (Jumas-Bilak et al. 1998a; Fouloungne et al. 2000; Hong et al. 2000; Lestrate et al. 2000; Delrue et al. 2001; Endley et al. 2001).

In contrast to the in-vivo system, in-vitro transposition is a cell-free process. In-vitro transposition was first developed by Bainton et al. (1991) to insert a Tn7-based transposon into the genome of *E. coli*. In order to mimic the intracellular environment, the Tn7-based, in-vitro method requires the addition of a target DNA, Mg$^{2+}$, ATP, and the appropriate transposase proteins, TnsA, TnsB, TnsC, and TnsD (Bainton et al. 1991, 1993; Craig 1996). Originally, given the appropriate enzymes, Tn7 would preferentially insert at specific sites in a genome (attTn7 sites). However, an artificial mutation in the TnsC enzyme has allowed random targeting of a given genome with Tn7 (Stellwagen and Craig 1997). This type of in-vitro mutagenesis system is important for *Brucella* genetics because it now allows targeted transposition of genes that have been cloned into suitable vectors.

### 1.12 The Current Study

This thesis describes the application of a rapid, simplified method of gene disruption in the genus *Brucella* known as in-vitro transposition. The bacterial transposon, Tn7, found on a donor plasmid, pGPS3, created by New England Biolabs
(Beverly, MA), was used, in-vitro, to insert a selectable marker expressing kanamycin resistance into the \textit{virB1} gene of \textit{B. abortus} 2308, which had been cloned by PCR into a \textit{Brucella} suicide vector, pGEM-T. This construct was then used for the in-vivo replacement of the wild type \textit{virB1} gene from virulent strains of \textit{B. melitensis} 16M and \textit{B. abortus} 2308, resulting in null mutations. The two \textit{virB1} mutant strains of \textit{Brucella} created, BM29-106, which was derived from \textit{B. melitensis}, and BA29-6, which was derived from \textit{B. abortus}, were used to infect murine macrophages and BALB/c mice. An analysis of \textit{Brucella} mutant survival compared to wild type at several time points was then performed. Reliable gene disruption of the virulence gene \textit{virB1} was examined in the present study to scrutinize the efficacy of in-vitro, \textit{Tn7}-based transposition in the evaluation of \textit{Brucella} genes that may hold promise for the development of vaccine strains of \textit{Brucella}.
2.1 Bacterial Strains, Plasmid Vector Construction and Growth Conditions

Original Brucella strains used in this study include *B. melitensis* 16M and *B. abortus* 2308 obtained from Dr. Elzer of the Department of Veterinary Science at Louisiana State University (Baton Rouge) (Table 4).

Table 4. Bacterial strains and plasmids used in this work.

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristic(s)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brucella melitensis</em> 16M</td>
<td>Wild type, virulent, smooth</td>
<td>Elzer 2002b</td>
</tr>
<tr>
<td><em>Brucella abortus</em> 2308</td>
<td>Wild type, virulent, smooth</td>
<td>Elzer 2002b</td>
</tr>
<tr>
<td>BA29-6</td>
<td><em>B. abortus</em> 2308 <em>virB1</em> mutant, Amp&lt;sup&gt;S&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>BM29-106</td>
<td><em>B. melitensis</em> 16M <em>virB1</em> mutant, Amp&lt;sup&gt;S&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5α-MCR</td>
<td>Host for pGEM-T and its derivatives</td>
<td>Invitrogen (Carlsbad, CA)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBBR1MCS</td>
<td>Broad-host-range cloning vector, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Elzer 2002b</td>
</tr>
<tr>
<td>pBBR1MCS::kan</td>
<td>Tn&lt;sup&gt;7&lt;/sup&gt;-based, kanamycin transposon from pGPS3 transposed into pBBR1MCS</td>
<td>This work</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Gram-negative host cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEMT-<em>virB1</em></td>
<td>0.75 kb <em>virB1</em> PCR product from <em>B. abortus</em> 2308 cloned into pGEM-T</td>
<td>This work</td>
</tr>
<tr>
<td>pBA<em>virB1</em>::kan-29</td>
<td>Tn&lt;sup&gt;7&lt;/sup&gt;-based, kanamycin transposon from pGPS3 transposed into the <em>virB1</em> gene of pGEMT-<em>virB1</em></td>
<td>This work</td>
</tr>
</tbody>
</table>

All brucellae were grown in one of the following media: 1) *Brucella* broth (Difco Laboratories, Detroit, MI), 2) Tryptic Soy Broth (TSB) (Difco Laboratories), or 3) SOC-B media (6% TSB, 10mM NaCl, 2.5mM MgCl<sub>2</sub>, 10mM MgSO<sub>4</sub>, 20mM glucose, pH to
6.8-7.0) at 37°C with constant shaking. Solid media used for Brucella culture included Schaedler Agar (SBA) (BBL/Becton Dickinson, Cockeysville, MD) plates supplemented with 5% defibrinated bovine blood and incubated at 37°C in a 5% CO₂ atmosphere.

Inoculation doses for each strain were prepared as previously described (Elzer et al. 1994). Escherichia coli DH5α-MCR (Table 4) were grown on Luria Butani (LB) media (10g tryptone, 5g yeast extract, 5g NaCl per 1000ml H₂O) + 1.5% agar at 37°C or in LB broth at 37°C with constant shaking. When appropriate, the following antibiotics or chemicals were added to the following final concentrations: kanamycin 50 µg/ml (Sigma, Chemical Co., St. Louis, MO); ampicillin 100 µg/ml (Sigma); chloramphenicol 30 µg/ml (Sigma); IPTG 0.5mM (Amresco, Solon, OH); X-Gal 80 µg/ml (Amresco). The E. coli compatible cloning vector pGEM-T (Table 4) was used to construct plasmids containing the virB1 gene of B. abortus 2308 as per the manufacturer’s instructions. The broad host range vector pBBR1MCS (Table 4) that carries a chloramphenicol resistance gene (Cmᴿ) was also obtained from Dr. Philip Elzer of LSU (Kovach et al. 1994).

2.2 Isolation and Preparation of Brucella DNA

For Brucella chromosomal DNA isolation, 5-10ml TSB was inoculated with the appropriate species of Brucella and grown overnight with shaking at 37°C. The culture was pelleted and resuspended in 300µl of phosphate buffered saline (PBS). An equivalent volume of chloroform was added and the entire tube was mixed on a rocker for 30-60 minutes. One hundred seventy five microliters of 50mg/ml lysozyme (Invitrogen) was added and allowed to sit, with occasional mixing, for 30-60 minutes at 37°C. Three microliters of 20mg/ml proteinase K (Invitrogen) was added and incubated for 30-60 minutes at 37°C. Ten percent sodium dodecyl sulfate (SDS) (Sigma) was
added to a final concentration of 1% and incubated at 65°C for 5 minutes with occasional mixing. An equal volume (500ul) of phenol:chloroform (25:24 ratio) (Sigma) was added and mixed well but gently. This mixture was centrifuged at 10,000 x g for 5-10 minutes. The upper layer was removed with a wide bore 200µl pipette tip and extracted a second time with an equal volume of phenol:chloroform. After the second phenol:chloroform extraction, an equal volume of chloroform was added, the mixture was centrifuged at 10,000 x g for 2 minutes and the chloroform was removed and discarded. A 1/10 volume of 3M sodium acetate (Sigma) plus a 2X volume of 95% ethanol was added and allowed to precipitate overnight at –20°C. The mixture was centrifuged and the liquid was decanted or pipetted out and discarded. The Brucella DNA was resuspended in 500µl Tris-EDTA (TE), pH 8.0, and treated with 10µl of 50mg/ml RNaseA (Invitrogen) for 1 hour at 37°C. A 1/10 volume of 3M sodium acetate plus a 2X volume of 95% ethanol was added and the DNA was allowed to precipitate overnight at –20°C. The mixture was centrifuged and the liquid was decanted or pipetted out and discarded. The DNA was resuspended in 300-500µl TE. Brucella DNA yield was typically 150-300 mg/ml per isolation.

2.3 Transposon Mutagenesis and Creation of Suicide Plasmid

The Tn7 based GPS-Mutagenesis System (New England Biolabs) was used, according to the protocol provided, to insert a KanR cassette randomly into pBBR1MCS. Mutagenized pBBR1MCS (pBBR1MCS::kan) was transformed into E. coli DH5α-MCR cells as described in the Invitrogen transformation protocol. Transformants carrying the plasmid pBBR1MCS::kan were selected on LB + kanamycin (50 µg/ml) + chloramphenicol (30 µg/ml). Plasmid DNA was isolated by the alkaline lysis method.
(Sambrook et al. 1989) and by using a plasmid miniprep kit (Qiagen-Operon, Valencia, CA) and suspended in sterile, deionized H2O. The \textit{virB1} gene fragment from \textit{B. abortus} 2308 was generated by the polymerase chain reaction (PCR) using \textit{virB1F} and \textit{virB1R} primers (Table 5) and ligated, with DNA ligase, into the plasmid vector pGEM-T at a 1:1 molar ratio of \textit{virB1} PCR product to pGEM-T vector following the protocol provided by Promega.

Table 5. Primers used for PCR and sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{virB1F}</td>
<td>5’-AGGAGACGATCCTATGGTGCT-3’</td>
</tr>
<tr>
<td>\textit{virB1R}</td>
<td>5’-GTCCTGATCGGATGATGG-3’</td>
</tr>
<tr>
<td>N-primer</td>
<td>5’-ACTTTATTTGATCAGTTTATCTTTTG-3’</td>
</tr>
<tr>
<td>S-primer</td>
<td>5’-ATAATCCTTAAAAACTCCATTTCCACCCCT-3’</td>
</tr>
<tr>
<td>AmpF</td>
<td>5’-TCATCCATAGTTGCCTGACTCC-3’</td>
</tr>
<tr>
<td>AmpR</td>
<td>5’-GTATTCAACATTTCCGTGTCGC-3’</td>
</tr>
</tbody>
</table>

The PCR parameters for \textit{virB1} amplification using \textit{virB1F} and \textit{virB1R} are given below (Table 6). The \textit{virB1} PCR product plus vector, pGEM-T + \textit{virB1} (pGEMT-\textit{virB1}), was transformed into \textit{E. coli} DH5α-MCR cells and 100-200 µl of the transformant mixture was plated on LB + 1.5% agarose plates containing ampicillin (100µg/ml) + IPTG (0.5mM) + X-Gal (80µg/ml). After incubation for 10-24 hours at 37°C, white colonies were chosen as successful recombinants which were inoculated into LB broth + ampicillin (100 µg/ml) and grown overnight. One and a half milliliters of each broth culture was centrifuged for 30 seconds at 10,000 x g and plasmids were isolated using the Qiagen-Operon plasmid miniprep kit. Purified plasmids were suspended in sterile, deionized H2O. Correct \textit{virB1} insertion into pGEM-T was confirmed by restriction
endonuclease digestion of purified plasmids using the restriction enzyme Rsal (New England Biolabs).

Table 6. PCR conditions used with the \textit{virB1} and Amp primers

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°C)</th>
<th>Repeat</th>
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<td>1X</td>
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<tr>
<td>∞</td>
<td>4</td>
<td>Holding Temp</td>
</tr>
</tbody>
</table>

Correct forward or reverse insertion gave the following size bands when run in a 1% agarose gel for one hour at 90 V and stained with ethidium bromide (0.3 µg/ml) (Sigma): Forward insertion = 1414 and 2336 base pairs; Reverse orientation insertion = 1686 and 2064 base pairs (Fig. 4).

Ten plasmids were chosen and reconfirmed by restriction with BsgI (New England Biolabs); correct banding pattern after restriction, indicating a forward insertion = 3381 and 370 base pairs. Confirmed pGEMT-\textit{virB1} plasmid DNA concentration was measured with a Pharmacia Biotech, UV/Visible spectrophotometer. One microgram of the confirmed pGEMT-\textit{virB1} plasmid was mutagenized using the GPS-M transposon system according to the protocol provided with the kit. One microliter and 10µl of the mutagenesis reaction were transformed into \textit{E. coli} DH5α-MCR cells, plated on LB + kanamycin (50 µg/ml) and incubated overnight at 37°C. Transformants carrying the plasmid pGEM-T + \textit{virB1} + pGPS3 (pBA\textit{virB1}:kan) were selected on LB agar plates +
kanamycin (50 µg/ml). Plasmid DNA was isolated by the alkaline lysis method and by using the Qiagen-Operon plasmid miniprep kit and suspended in sterile, deionized H₂O.

![Diagram of restriction products](image)

**Fig. 4.** Possible restriction products after virB1 insertion into pGEM-T.

Plasmids were screened for the correct restriction pattern by restriction endonuclease digestion indicating a possible 1700 base pair Tn7 cassette insertion into virB1 using the restriction enzymes Nco I and Pst I (New England Biolabs). The restriction pattern for a possible Tn7 insertion into virB1, after electrophoresis in a 1% agarose gel and staining, consisted of two bands, 2485 and 2964 base pairs, while Tn7 insertion into pGEM-T would have been seen as bands of 785 and 4664 base pairs (Fig. 5). PCR was performed on plasmids that gave the correct restriction pattern using the virB1F and virB1R primers under the previous PCR parameters (Table 6). PCR reactions giving the correct band size of 2450 base pairs, after being run in a 1% agarose gel and ethidium bromide staining (0.3 µg/ml) were used as templates for a sequencing reaction.

Sequencing was carried out by the di-deoxy method using a premixed sequencing reagent (United States Biochemical/Amersham Life Sciences, Cleveland, OH). Primers N and S,
which are provided by New England Biolabs with the pGPS3 mutagenesis system, were used for sequencing under the PCR conditions given in Table 7.

Fig. 5. Possible restriction products after Tn7 insertion into pGEMT-\textit{virB} 1.

Primers N and S anneal at the ends of the pGPS3 Kan\textsuperscript{R} transposon and face out into the gene of interest. The result of sequencing for a successful Tn7 insertion was plasmid #29 (pBA\textit{virB}1::kan-29).

Table 7. Sequencing PCR conditions used with the N and S primers.

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<th>Temperature (\textdegree C)</th>
<th>Repeat</th>
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</tr>
<tr>
<td>(\infty)</td>
<td>4</td>
<td>Holding Temp</td>
</tr>
</tbody>
</table>
2.4 Electroporation and Gene Replacement

In preparation for electroporation, brucellae were grown in TSB overnight at 37°C with constant, gentle shaking, centrifuged and washed 3X in ice cold, sterile deionized H₂O. The plasmids pBBR1MCS::kan and #29 were introduced into B. abortus 2308 and B. melitensis 16M by electroporation at 2500 volts with an Eppendorf, Corp. electroporator (Hamburg, Germany; model 2510). For most transformations, 1µg plasmid DNA was added to 33µl (1x10^8 cfu/ml) of brucellae. Immediately after electroporation 500µl of SOC-B was added to the transformation broth and the brucellae were allowed to recover overnight at 37°C with constant, gentle shaking. Successful transformants were chosen after plating on SBA + kanamycin (50 µg/ml) and incubation at 37°C in a 5% CO₂ atmosphere.

Brucellae that underwent successful crossover between the mutated virB1 region found on the electroporated plasmid #29 and the Brucella wild type virB1 gene, exhibited ampicillin sensitivity (Amp$^S$) and Kan$^R$. This was confirmed by isolating chromosomal DNA from the Kan$^R$ brucellae and performing PCR using the previous virB1 primers. Visualization of this PCR product from transformed brucellae on a 1% agarose gel gave only the long product of 2450 base pairs (virB1+pGPS3 Kan$^R$ transposon). The restriction endonuclease Hind III was used to digest the amplified virB1 fragment giving the expected band sizes of 1280 and 1170 base pairs after electrophoresis in a 1% agarose gel (Fig. 6). A sequencing reaction was run of the mutant virB1-amplified fragments from both suspected Brucella mutant strains, B. melitensis 16M mutant BM29-106, and B. abortus 2308 mutant BA29-6. The virB1 portion of both mutant sequences matched the existing virB1 sequence of B. abortus 2308 in Genebank.
PCR of *Brucella* chromosomal DNA using *virB* 1F and *virB* 1R

![Diagram](image)

**Fig. 6.** Confirmation of *virB*1::*Tn*7 insertion by the amplification of *virB*1::*Tn*7 from *Brucella* chromosomal DNA and restriction with HindIII.

### 2.5 Murine Phagocyte Killing Assay

Murine macrophages were harvested in the following manner. Twenty week-old BALB/c mice were euthanized by a halothane overdose and the peritoneal body cavity was exposed by peeling back the skin on their abdomens. Using a 6cc syringe with a 1.5”, 22 gauge needle, 4 mls of media + 1ml of air was injected into the peritoneum at the mid-belly region. The injected media was a combination of Minimum Essential Eagle Media (MEM) (Sigma) + 5% Fetal Calf Serum (FCS)(Sigma) + Heparin (5U/ml)(Invitrogen). After media injection, the mouse was shaken vigorously for 1 minute and the media containing peritoneal macrophages was drawn out. The macrophages were centrifuged for 5 minutes at 1100 rpm and 4°C and the resulting pellet
was resuspended in 1ml of MEM media per mouse used for harvest. Viability counts were performed by staining 100µl of macrophages with 100µl trypan blue in 800µl of MEM media and visual counting with a hemocytometer leading to a final stock dilution of 5x10^6 cells/ml. One hundred microliters of macrophages were placed in each well of a 96 well plate (5x10^5 cells/well) and allowed to adhere (differentiate) overnight. The next morning, macrophages were washed 3X with 200µl of warm PBS + 0.5% FCS to remove non-adherent cells. After the last washing, 200µl of warm MEM + 5% FCS was added to each well. For opsonization, FCS + mouse antibodies were added at 1µl per ml of cells, to 1x10^8 Brucella in MEM and incubated for 30 minutes at 37°C. Two hundred microliters of opsonized brucellae were added to each well containing macrophages and incubated for 2 hours at 37°C. The macrophages were washed once with MEM media and twice with PBS-0.5 % FCS. Two hundred microliters of MEM + gentamycin (50µg/ml) (Sigma) was added to each well and incubated at 37°C for 45 minutes to 1 hour. Macrophages in each well were washed 2X with PBS/FCS and reincubated with MEM + gentamycin (12.5µg/ml). For each time point investigated, T₀, T₂₄ and T₄₈ hours, the gentamycin was washed out and deoxycholate (Sigma) was added to a final concentration of 0.1% to lyse all macrophages. Each well was diluted with 20-180µl PBS and plated on SBA or SBA + kanamycin (50 µg/ml). Plates were incubated for 2-3 days at 37°C in a 5% CO₂ atmosphere.

2.6 Mouse Infection Assay

Both male and female BALB/c mice at 6 weeks of age were challenged intravenously with 5x10⁴ cfu of parental strain (B. abortus 2308 or B. melitensis 16M) or mutant (BA29-6 or BM29-106). Infectious doses were confirmed by serial dilution followed by plating on SBA for wild type brucellae and SBA + kanamycin (50 µg/ml) for
Brucella mutants. Brucella in-vivo survival was assessed at 1 week and 4 weeks when half the mice were euthanized at each time point by halothane overdose and the whole spleen was aseptically removed. The spleen was weighed, homogenized, serially diluted and plated on SBA or SBA + kanamycin (50 µg/ml). Plates were incubated at 37°C in a 5% CO₂ atmosphere for 3 days.

2.7 Primers

Primers for amplification of virB1 from Brucella chromosomal DNA include virB1F and virB1R that were supplied by Qiagen-Operon. The B. abortus and B. melitensis virB1 sequences differ by only 1 base pair, therefore, virB1 primers were chosen based on the published sequence of virB1 of B. abortus in GeneBank (Genebank accession number AF226278). Primers AmpF and AmpR were also supplied by Qiagen-Operon. Primers N and S were provided by New England Biolabs for use with the GPS-M mutagenesis system.

2.8 Biochemical Identification Tests

Brucella virB1 mutants and parental strains were characterized with standard biochemical identification tests (Table 8). All virB1 Brucella mutant strains were biochemically identical to their original parental species. Biochemical identification tests on BM29-106, BA29-6, B. abortus 2308 and B. melitensis 16M included the following:

<table>
<thead>
<tr>
<th>Test</th>
<th>B. abortus 2308</th>
<th>B. melitensis 16M</th>
<th>BA29-6</th>
<th>BM29-106</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Gram Stain</td>
<td>-</td>
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</table>

Table 8. Results of biochemical identification tests on BM29-106, BA29-6, B. abortus 2308 and B. melitensis 16M.
oxidase (BBL/Becton Dickenson), urease (LSU School of Veterinary Science Media Lab, Baton Rouge, LA), and Gram stain (Becton Dickson, Sparks, MD).

2.9 Statistical Analyses

For statistical comparison of two independent samples at a single time point the student two-sample, paired t-test was used (Graphpad Instat, version 3.05). This test was utilized in the analysis of *Brucella virB1* mutant versus non-mutant survival in both the macrophage-killing assay and the in-vivo mouse assay examining *Brucella* survival. In addition, the two-sample t-test was employed for statistical assessment of *virB1* mutant versus non-mutant splenic weight due to *Brucella* burden. For all statistical tests, a confidence interval of 95% (P<0.05) was considered significant.
CHAPTER 3. RESULTS

3.1 Complementation of Brucella with pBBR1MCS::kan

The ability of brucellae to exhibit resistance to the antibiotic kanamycin after complementation with a kanamycin resistance gene has been established previously in several studies (O'Callaghan et al. 1999; Foulongne et al. 2000; Hong et al. 2000; Sangari, Aguero et al. 2000; Sieira et al. 2000). However, it was necessary to confirm that the promoter, which precedes the kanamycin resistance cassette on the transposon in pGPS3, demonstrates reliable expression within Brucella. The broad host range vector pBBR1MCS, which is stably maintained within all Brucella species, was mutagenized with the GPS-Mutagenesis system giving the plasmid pBBR1MCS::kan. This construct was then transformed by electroporation into B. melitensis 16M and B. abortus 2308 (Kovach et al. 1994; Elzer et al. 1995). The pGPS3 donor backbone contains a gene conferring ampicillin resistance while pBBR1MCS contains a chloramphenicol resistance gene. Successful mutagenesis, without pGPS3 backbone integration, transformation of the plasmid into Brucella, maintenance and antibiotic gene expression was confirmed when B. abortus 2308 and B. melitensis 16M were plated on SBA and the appropriate antibiotics. Stable maintenance was displayed by colonies that exhibited sensitivity to ampicillin (Amp\textsuperscript{S}), resistance to chloramphenicol (Cm\textsuperscript{R}) and Kan\textsuperscript{R} (data not shown).

3.2 Creation of \textit{virB1} Brucella Mutants

A suicide plasmid was created to replace \textit{virB1} in Brucella species. This plasmid is composed of the B. abortus 2308 gene \textit{virB1} that has been ligated into the cloning vector pGEM-T, and mutagenized by insertion of a \textit{Tn7} based kanamycin resistance cassette generating the plasmid pBA\textit{virB1}::kan-29 (Fig. 7).
Fig. 7. *Brucella* suicide vector, pBAvirB1::kan-29, constructed by inserting a kanamycin resistance cassette into the *virB1* gene of *Brucella* which had been cloned into the vector pGEM-T.

The *virB1* gene contained on this plasmid is that of *B. abortus* 2308, which is almost identical (one base pair difference) in sequence to the *virB1* gene of *B. melitensis* 16M. Transformation of *B. melitensis* 16M and *B. abortus* 2308 by electroporation with pBAvirB1::kan-29 resulted in the isolation of Kan<sup>R</sup>, *virB1* mutant colonies which were named BM29-106 and BA29-6 respectively. The designation of “29” in strain nomenclature serves only to further classify which mutant plasmid was employed in gene replacement, while the numbers “-106” and “-6” give the particular mutant colony from which the strain was cultured. These two strains exhibited Amp<sup>S</sup>, verifying that the
pGEM-T backbone, which contains an ampicillin resistance gene, was not incorporated into the *Brucella* genome.

To confirm a double cross-over event and replacement of the *Brucella* wild type *virB*1 gene with *virB*1::kan-29, PCR was performed on chromosomal DNA isolated from the suspected *Brucella* mutants, BM29-106 and BA29-6. The size of these PCR products was then determined by electrophoresis on a 1% agarose gel, staining with ethidium bromide and exposure to UV light (Fig. 8). The mutant *virB*1 PCR amplification products were then compared to the amplification products for wild-type *virB*1 from *B. melitensis* 16M and *B. abortus* 2308 using the previously employed *virB*1 primers. When amplified with the *virB*1 primers, the wild type *virB*1 genes of *B. melitensis* and *B. abortus* are 750 base pairs in length (lanes 6 and 7 respectively). Once the wild type *virB*1 gene of *Brucella* has been mutagenized with the *Tn*7-based transposon, which is 1700 base pairs long, the *virB*1 amplification product increases in length by 1700 base pairs to 2450 base pairs. As seen in Fig. 5, the resulting bands of 2450 base pairs for the *virB*1 mutant strains, BM29-106 and BA29-6, (lanes 3 and 4 respectively) correspond to the disrupted *virB*1 genes containing the additional 1700 base pair transposon.

Lane 2 contains a *virB*1 PCR product of 2450 base pairs from the suicide plasmid used for gene replacement, pBA*virB*1::kan-29, which serves as a positive control indicating the correct length of a *Tn*-7-mutated *virB*1 gene. Nucleotide sequencing of both 2450 base pair, mutant *virB*1 PCR products from BM29-106 and BA29-6 confirmed their identity as *virB*1 containing a *Tn*7-based transposon (Data not shown). Primers used for sequencing included primer N and S, which were supplied by New England Biolabs.
with the GPS-Mutagenesis system. These primers anneal at the ends of the Tn7-based transposon and extend out into the gene that has been disrupted.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
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</table>

Fig. 8. One percent agarose gel showing PCR amplification of virB1 from BM29-106 (lane 3), BA29-6 (lane 4), B. melitensis 16M (lane 6), B. abortus 2308 (lane 7), and pBAvirB1:kan-29 (lane 2). The virB1 gene of both mutant strains and pBAvirB1::kan-29 is 2450 base pairs while both parental strains contain the wild type virB1 of 750 base pairs. A 1 kb plus DNA ladder is provided in lanes 1, 5 and 8 (Invitrogen).

Sequencing, employing the N and S primers, reveals that the pGPS3 transposon was inserted between base pairs 909 and 910 of the B. abortus 2308 wild type virB1 gene (Genebank accession number AF226278).
To further ensure the ampicillin containing, vector backbone of pBA\textit{virB1}::kan-29 was not integrated into either the \textit{B. melitensis} or \textit{B. abortus} genome with the \textit{virB1} gene, PCR was performed on chromosomal DNA isolated from the mutants BM29-106 and BA29-6, using primers for the ampicillin resistance gene, AmpF and AmpR, as well as a combination of N or S primers and \textit{virB1F} and \textit{virB1R} primers (Fig. 9).

<table>
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<tr>
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<th>3</th>
<th>4</th>
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<td>800bp</td>
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</tbody>
</table>

Fig. 9. One percent agarose gel showing PCR amplification of ampicillin gene from: pBA\textit{virB1}::kan-29 (lane 1) using ampicillin primers (Amp product size 800bp), \textit{B. abortus} 2308 \textit{virB1} mutant (lanes 2, 4 and 5) and \textit{B. melitensis} 16M \textit{virB1} mutant (lanes 6, 7 and 8) using both Amp primers (lanes 2 and 6) and one Amp primer with either N or S primer (lanes 4-5 and 6-7). Lane 9 shows PCR of pBA\textit{virB1}::kan-29 using one Amp primer (reverse) and the N primer (2400bp). Lanes 3 and 10 contain a 1kb+ DNA ladder (Invitrogen).
No resulting bands were seen in lanes 2, 4, 5, 6, 7, and 8, which would be indicative of vector integration using genomic DNA from BM29-106 and BA29-6. As positive controls, the original suicide plasmid pBA\textit{virB}1::kan-29, which contains an ampicillin resistance gene, was PCR amplified using the AmpR and AmpF primers (lane 1) and using the AmpR primer and N primer in conjunction (lane 9). All PCR products were run on a 1% agarose gel, stained with ethidium bromide and exposed to UV light.

3.3 Increased Brucellacidal Activity of Murine Macrophages Against \textit{virB}1 Mutants

To assess whether a nonfunctional \textit{virB}1 gene will cause \textit{B. abortus} and/or \textit{B. melitensis} to become more susceptible to the bactericidal activities of the murine macrophage, a series of murine phagocyte killing assays were run. Isolated murine macrophages were infected with BM29-106, BA29-6, \textit{B. abortus} 2308 and \textit{B. melitensis} 16M at a ratio of 100 brucellae per macrophage in 96 well plates. At time points of 0, 24 and 48 hours, infected macrophages were lysed, their intracellular brucellae were serially diluted, plated on SBA and incubated at 37°C in a 5% CO$_2$ atmosphere. Results of these experiments are represented graphically in Fig. 10. The differences between both parental and mutant strains of \textit{Brucella} were statistically significant at a confidence interval of at least 95% (P<0.05) for both time points (24 and 48 hours). The disparity between \textit{B. melitensis} 16M and BM29-106 intracellular survival increased steadily over 48 hours. Compared to wild type \textit{B. melitensis} 16M, BM29-106 survival decreased rapidly by 24 hours post-infection (t=24) to a level 73% lower than the wild type (P<0.01, n=6), and by 48 hours (t=48), BM29-106 intracellular survival had fallen >98% below wild type \textit{B. melitensis} (P<0.01, n=6).

A similar pattern was observed between \textit{B. abortus} 2308 and its \textit{virB}1 mutant, BA29-6, for which every mean comparison was statistically significant. By 24 hours
Fig. 10. Results of the murine macrophage killing assay assessing the survival of BM29-106 (box) and BA29-6 (star) versus their parental strains, *B. melitensis* 16M (diamond) and *B. abortus* 2308 (triangle) over a period of 48 hours.

Post-infection (t=24) the average wild type *B. abortus* survival rate was 10% greater than for its mutant, BA29-6 (P<0.05, n=6). By 48 hours post-infection (t=48), the average BA29-6 population had dropped to 98% of wild type *B. abortus* 2308 (P<0.01, n=6).

### 3.4 Virulence and Survival of *Brucella virB1* Mutants in the Mouse Model

To assess the virulence of *virB1 Brucella* mutants and the capacity of the *virB1* mutant strains to survive within a mammal host, 40 5-week old BALB/c mice were intravenously infected with *B. abortus* 2308, *B. melitensis* 16M, BM29-106, and BA29-6. Half the mice in each category (5 mice/category) were euthanized at 1 week while the other half were euthanized at 4 weeks. This resulted in two time points for each strain of *Brucella*, each containing 5 mice. Necropsy at both time points involved aseptic removal...
of the spleen, which was then weighed, homogenized, serially diluted and plated on SBA for *Brucella* viability counting.

### 3.4.1 Spleen Mass

The average change in mass of a mouse spleen as a function of time of necropsy for each strain of *Brucella* used for initial infection was calculated (Fig.11).

![Graph showing spleen mass over time](image)

Fig. 11. Average mouse spleen mass assessing the virulence of each *Brucella* strain as a function of the time post-infection. *B. abortus* 2308 (triangle), BA29-6 (star), *B. melitensis* 16M (diamond) and BM29-106 (box).

According to the data, spleen mass for mice infected with *B. abortus* 2308 and *B. melitensis* 16M wild types was greater than for mice infected with the *virB*1 mutants BA29-6 and BM29-106 at both 1 and 4 weeks. Student t-tests comparing the spleen mass of mice infected with wild type brucellae versus *virB*1 mutants for each time point were statistically significant at a confidence interval of 95% (P<0.05): *B. abortus* 2308 average spleen mass was 87% greater than BA29-6 at 1 week (P<0.01, n=5) and 135% greater at 4 weeks (P=0.001, n=5); average spleen mass for *B. melitensis* 16M was 78%
greater than BM29-106 at 1 week (P<0.0001, n=5) and 190% greater at 4 weeks (P<0.001, n=5). In addition, the average spleen mass for the *B. melitensis* 16M infected mice from 1 to 4 weeks increased 81% and was statistically significant, (P<0.01, n=5), while the spleen mass of BM29-106 infected mice did not significantly increase (an 11% increase). Spleen mass for *B. abortus* 2308 infected mice did increase 5% from 1 to 4 weeks, which was not statistically significant; however, BA29-6 spleen mass decreased 17% over the same period at a statistically significant level (P<0.05, n=5).

### 3.4.2 Spleen *Brucella* Burden

The average mouse spleen *Brucella* burden for each of the 4 *Brucella* strains is graphically represented in Fig. 12 as *Brucella* colony forming units (cfu) per spleen.

![Graph](image_url)

**Fig. 12.** Results of the in-vivo mouse model of *Brucella* infection assessing the survival of BM29-106 (box) and BA29-6 (star) versus their parental strains, *B. melitensis* 16M (diamond) and *B. abortus* 2308 (triangle) in-vivo over a period of 4 weeks.
The mean cfu/spleen for both *virB*1 mutants, BM29-106 and BA29-6, was considerably below (1.4 to 2 logs lower) their parental counterparts by 4 weeks post-infection and statistically significant (P<0.05, n=5) at both time points. As represented in the graph, a comparison between *B. melitensis* 16M and BM29-106 spleen colonization reveals that at 1-week post-infection, BM29-106 colonized the mouse spleen at a statistically significant lower level, 0.68 logs less than its parental strain (P<0.05, n=5). The gap between these two strains, in regards to spleen *Brucella* burden, increased at 4 weeks to 1.4 logs, which maintained statistical significance (P<0.05, n=5). The disparity between *B. abortus* 2308 and its *virB*1 mutant counterpart, BA29-6, was even greater at both time points. Analysis reveals that at 1-week post-infection the splenic colonization of the *B. abortus* 2308 *virB*1 mutant (BA29-6) was 0.82 logs less than its parental strain, which is statistically significant (P<0.01, n=5). By 4 weeks post-infection BA29-6 remained at a statistically significant lower level (2.06 logs lower) in the mouse spleen than wild type *B. abortus* 2308 (P<0.01, n=5).
CHAPTER 4. DISCUSSION

4.1 Experiment Summary

The mechanisms of Brucella survival and virulence are not fully understood. The success of Brucella, however, appears to be a product of its ability to endure and thrive in the normally bactericidal environment of the phagocyte. Many of the past studies into the novel mechanisms that allow Brucella this environmental niche have relied upon the creation of null mutations. At least two methods have been employed in the construction of null mutations leading to Brucella knockout strains: In-vivo, random transposition using Tn5-based transposons, and in-vitro gene disruption by insertion of a selectable marker at an endonuclease restriction site. A third method of gene mutation, performed in-vitro, is presented in the current study. GPS-M is a Tn7-based, in-vitro transposition system designed by New England Biolabs for the targeted inactivation of genes with a kanamycin resistance cassette that can be used in Gram-negative organisms. This thesis described the use of the GPS-M system for insertional mutation of a Brucella virulence gene, virB1.

The central aim of this study was to investigate the efficiency and utility of an in-vitro, Tn7-based transposition system for the inactivation of genes within members of the genus Brucella. Initially, the plasmid pBBR1MCS, which is stably maintained in all Brucella species (Elzer et al. 1995), was mutagenized by the insertion of a Tn7-based transposon containing a kanamycin resistance cassette producing the plasmid pBBR1MCS::kan. Expression of kanamycin resistance by Brucella indicated the reliable functioning of the foreign, Tn7-based promotor within brucellae. The virulence gene virB1, found in B. melitensis 16M and B. abortus 2308, was then cloned into a vector where it also was mutagenized by the insertion of a Tn7-based transposon, creating the
suicide plasmid pBA\textit{virB}1::kan-29. This construct was used successfully to replace the wild-type \textit{virB}1 gene in-vivo by homologous recombination, generating \textit{virB}1 mutant strains of \textit{B. melitensis} 16M (BM29-106) and \textit{B. abortus} 2308 (BA29-6). PCR and nucleotide sequencing confirmed disruption of the \textit{B. melitensis} and \textit{B. abortus \textit{virB}1} loci.

The efficacy of gene inactivation via in-vitro transposition and the pathologic significance of the \textit{Brucella \textit{virB}1} gene were evaluated by infection of murine peritoneal macrophages and BALB/c mice with the \textit{virB}1 mutants BM29-106, BA29-6 and their parental strains. Results of these experiments suggest that in-vitro \textit{Tn7}-based transposition is an effective method for the creation of null mutations. Both \textit{virB}1 mutant strains exhibited attenuated virulence and lower levels of survival compared to wild type \textit{B. melitensis} 16M and \textit{B. abortus} 2308 in both the macrophage and murine models. The survival of \textit{Brucella} mutants in both models and at all time points were significantly lower (P<0.05) than wild type.

4.2 \textit{virB}1 Mutation in Other Species Results in Attenuation of Virulence

Several studies have investigated the significance of the \textit{virB} operon to the pathogenicity of \textit{Brucella} (O'Callaghan et al. 1999; Ugalde 1999; Foulongne et al. 2000; Hong et al. 2000; Sieira et al. 2000; Comerci et al. 2001; Delrue et al. 2001; Boschirol et al. 2002), however, most of what is known about the \textit{virB} operon has come from work with other type IV secretion systems, especially the \textit{Agrobacterium \textit{virB} complex}. In mutational studies with \textit{Agrobacterium}, a plant parasite that causes crown gall disease, Berger and Christie (1994) discovered that \textit{virB}1 is a non-essential virulence factor or transport component for eukaryotic cell transformation (Christie 1997; Christie and Covacci 2000). While non-polar mutations of \textit{virB}2-\textit{virB}11 resulted in avirulent strains
of *A. tumefaciens*, mutation of only virB1 decreased the efficiency of DNA transfer from bacterium to plant by 1 to 3 orders of magnitude compared to bacteria with a wild type virB1 gene (Berger and Christie 1994; Fullner 1998). Similar results (10-100 fold decrease in conjugation or transfer frequency) were found in other virB1 relatives: “gene 19” of plasmid R1 (Bayer, Eferl et al. 1995) and *traL* of plasmid pKM101 (Winans 1985 as cited in Berger and Christie 1994; Pohlman et al. 1994; Baron, Llosa et al. 1997). As asserted by Baron et al. (1997), this decrease in virulence, as opposed to complete avirulence, may signal that transglycosylase-like activities are not essential for functional type IV machinery, or that there are redundant systems present (Boschirolì et al. 2002).

In support of this theory, the type IV secretion systems of *Bartonella henselae* (Schmiederer, Arcenas et al. 2001), *Legionella pneumophila*, *Bordetella pertussis*, *Rickettsia prowazekii*, IncW plasmid R388 and *Helicobacter pylori* (Bayer et al. 1995; Baron et al. 1997; O'Callaghan et al. 1999; Segal, Russo et al. 1999; Christie and Covacci 2000; Sieira et al. 2000) lack a virB1 homologue entirely. And in the case of *B. melitensis* 16M, a membrane-bound lytic murein transglycosylase is encoded on chromosome II immediately before the virB operon (DelVecchio et al. 2002).

Apart from sequence and protein similarity, it is not known to what extent the *Agrobacterium* and *Brucella* VirB1 proteins are homologous. For instance, VirB1 of *Agrobacterium*, after translational processing, produces 2 proteins, VirB1 and VirB1*, believed to have independent properties: 1) protein VirB1 is a transglycosylase which cleaves the glycosidic bonds of peptidoglycan to create a pore for VirB transporter assembly (Dijkstra and Keck 1996; Mushegian, Fullner et al. 1996; Baron et al. 1997; Christie 1997; Zupan, Ward et al. 1998; Christie and Covacci 2000; Llosa, Zupan et al.
2000), while 2) protein VirB1* is secreted outside the bacterial cell and associates with the bacterial membrane, VirB9, and possibly the plant cell wall to initiate or participate in pilus formation (Baron et al. 1997; Christie 1997; Chumakov and Kurbanova 1998, 1999; Llosa et al. 2000). A comparison of VirB1 homologues in Agrobacterium and E. coli reveals that Brucella VirB1 also contains an N-terminal signal sequence cleavage site required for export and amino acid motifs consistent with lytic transglycosylases (Mushegian et al. 1996; O'Callaghan et al. 1999). However, there is currently no literature that suggests Brucella VirB1 is processed, as is the Agrobacterium VirB1 homologue, into 2 independent proteins.

4.3 virB1 Mutation via Tn7-Based Transposition Results in Brucella Attenuation

In this study, mutation of virB1 with a Tn7-based transposon produced strains of Brucella that were attenuated in the murine macrophage model of infection. In this model, at 24 hours post-infection, the survival of the B. abortus virB1 mutant (BA29-6) was 90% of its wild type while the B. melitensis virB1 mutant (BM29-106) survival was only 27% of its corresponding parental strain. By 48 hours in the macrophage model both mutant survival rates had dropped to or below 2% of their wild types. The current work shows a statistically significant (at least P<0.05) 1-2 log decrease in Brucella intracellular survival at 48 hours in the murine macrophage model and by 4 weeks in the mouse model when the Brucella virB1 gene is disrupted. These results are in agreement with previous findings (Berger and Christie 1994; Pohlman et al. 1994; Bayer et al. 1995; Baron et al. 1997; Fullner 1998) that inactivation of other virB1 homologues (Agrobacterium virB1, “gene 19” of plasmid R1, and traL of plasmid pKM101) results in a 1-3 log decrease in microbial survival. This suggests that the B. melitensis 16M and B. abortus 2308 virB1 gene products, like the Agrobacterium VirB1 protein, may not be
essential for virulence or intracellular survival up to 4 weeks post-infection in the mouse model of infection.

Assuming VirB1 is not involved in cell-macrophage contact or entry but in intracellular survival and multiplication, as several studies have suggested (O'Callaghan et al. 1999; Hong et al. 2000; Sieira et al. 2000; Comerci et al. 2001; Delrue et al. 2001; Rittig et al. 2001; Boschirol et al. 2002), attenuated strains of *Brucella* would be more susceptible to the bactericidal mechanisms of the phagocyte, and thus give lower 24 and 48 hour macrophage intracellular survival counts and lower splenic burdens at 4 weeks post-infection. Evaluating both experimental portions of this series, it can be concluded that *Tn7*-based in-vitro transposition is an efficient means for creating mutated strains of *B. melitensis* 16M and *B. abortus* 2308. The current *virB1* mutant strains of *Brucella* are less virulent/replication efficient compared to their respective wild type species, evidenced by their decreased survival in both model systems of infection.

### 4.4 The Importance of the Macrophage and Murine Models of Infection

The macrophage model is important to the understanding of *Brucella* infection for two reasons: 1) *Brucellae* depend on the protection afforded them by their intracellular lifestyle within the relatively long-lived macrophage. In-vitro studies suggest that serum components are able to kill brucellae, particularly in cattle (Enright 1990). Outside the phagocyte, brucellae are exposed to antibodies, especially IgG, that may enhance phagocytosis and macrophage brucellacidal activity (Ralston and Elberg 1971 as cited in Nicoletti and Winter 1990). However, bacteremia is considered an important phase of *Brucella* dissemination and the value of complement to in-vivo brucellacidal action is not known or well understood. 2) *Brucellae* exploit macrophages as one of their main vehicles of distribution throughout the mammal host. *Brucellae* are non-motile, without
flagella, pseudopodia or fimbriae, and thus rely on their own phagocytosis and dissemination by macrophages. The murine model of *Brucella* infection has been demonstrated to be important because experimental results obtained with mice are frequently applicable to humans and especially to ruminants such as cows, goats and sheep, which are the main reservoirs of several *Brucella* species (FAO/WHO 1986).

### 4.5 Tn7-Based In-vitro Transposition and Vaccine Development

The underlying goal in the present study, with implications to human and ruminant health, is the exploration of *virB*1-mediated *Brucella* attenuation in the construction of a *Brucella* vaccine. Studies toward the discovery of candidates for an effective yet safe *Brucella* vaccine have dominated *Brucella*-related literature over the past 25 years. The problem has been approached by several angles, including investigations into live-vaccines, whole-killed vaccines, soluble extracts containing LPS and outer membrane proteins as vaccines, and increasing the population frequency of genes in the host responsible for natural resistance to brucellosis (Huddleson 1947b; Davies, Cocks et al. 1980; Dubray and Bezard 1980; Frenchick et al. 1985; FAO/WHO 1986; Nicoletti 1990; Price, Templeton et al. 1990; Zhan and Kelso 1993; Bowden et al. 1995; Drazek et al. 1995; Zhan and Cheers 1998). Although currently there is no safe human vaccine, several exist for use in ruminants and small mammals, such as *B. melitensis* Rev.1, *B. abortus* strain 19 and *B. suis* strain 2 (FAO/WHO 1986).

Lately, a promising method for vaccine development has been the creation of live, attenuated *Brucella* strains of low virulence by the inactivation of genes important for pathogenicity or survival. Three difficult hurdles on this track have been: 1) The identification and inactivation of a gene or genes which decrease or eliminate *Brucella* virulence enabling the host to produce antibodies while still effectively clearing or
reducing the number of bacteria, 2) keeping a high level of humoral immunity towards brucellae over a long period of time, and 3) developing a vaccine which, even if not intended for human use, is safe for man.

The current study focuses on part of the first hurdle to vaccine development, efficient inactivation of a virulence gene or genes. Based on the present set of experiments, successful null mutations can be created in different Brucella species using a Tn7-based, in-vitro mutagenesis system. While Tn5-based, in-vivo mutagenesis has been extensively carried out using Brucella, this is the first time, to the author’s knowledge, where a Tn7-based, in-vitro transposition system was employed with Brucella leading to mutant strains of B. melitensis 16M and B. abortus 2308 having attenuated virulence in the murine macrophage and mouse models.

4.6 Advantages and Applications of Tn7-based Transposition

Tn7 transposition of Brucella genes has several advantages over existing methods of in-vitro and in-vivo gene disruption. As adapted by New England Biolabs, Tn7-based in-vitro transposition using the pGPS3 mutagensis system inserts a transposon randomly into the genome and does not require the presence of a specific restriction endonuclease site within the gene of interest. Also, Tn7-based transposons exhibit target immunity, whereby only one insertion occurs over a distance of ~190 kilobases, which ensures single, rather than multiple gene knockouts in a cloned gene. Tn7-based transposition can be used in-vitro, to target a specific gene cloned into a vector, while Tn5-based, in-vivo systems cannot be targeted towards one particular gene. In addition, in-vitro transposition is an extremely rapid method for Brucella gene knockout. In-vivo, Tn5-based mutagenesis systems require the screening of hundreds and possibly thousands of recombinants, which is exceedingly time consuming. The entire process from
transposition to mutant strain confirmation, using targeted gene disruption via Tn7-based in-vitro transposition, can be performed in less than two weeks.

The in-vitro transposition system described here, in combination with the recently published nucleotide sequence of *B. melitensis* 16M, has great potential value for the study of open reading frames with no assigned functions that make up 22% of the *B. melitensis* genome (DelVecchio et al. 2002). Many regulatory and virulence-associated open reading frames, with homology in other bacterial systems, have also been identified within the *Brucella* genome. Most of these loci of *Brucella* have never been studied in the laboratory, some examples include: *virF* and *virJ* homologs, adhesins, invasins, incomplete sec-dependent, sec-independent, and flagellar type III and V secretion systems, thiamin synthesis genes, genes which code for heavy metal efflux pumps, enterobactin, BacA (a transport pathway gene), hemolysins, histidine kinase homologs, alkylation damage repair protein, stress-induced proteins similar to UspA of *E. coli*, a probable ABC transporter for copper uptake, and a heme catalase gene (DelVecchio et al. 2002; Moreno and Moriyon 2002). Creation of *Brucella* mutants lacking these genes, pathways or open reading frames could be swiftly accomplished with Tn7-based in-vitro transposition as it is described in this study. Additionally, in-vitro transposition can accomplish multiple *Brucella* gene knockouts in the same organism through the use of different antibiotic resistance cassettes. Possible targets could include inactivation of a putative or known pathway, such as lipid IV biosynthesis, at several important catalytic steps instead of one, or mutation of several outer membrane proteins at the same time.

In a further assessment of this in-vitro transposition system and to examine *virB1* for vaccine potential, the laboratory of Dr. Philip Elzer at Louisiana State University is
currently evaluating the efficacy of BM29-106 in the natural host of *B. melitensis*, the goat.
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