GENETIC AND IMMUNOLOGICAL ANALYSES OF A *BRUCELLA ABORTUS* PROTEIN EXHIBITING LECTIN-LIKE PROPERTIES

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Abstract

Brucella abortus is a facultative, intracellular zoonotic pathogen, which can cause undulant fever in humans and abortion in cattle. Despite all of the progress in brucellosis research, there are still many unanswered questions regarding the molecular mechanisms involved in the pathogenesis of *Brucella* infections. To better understand the *Brucella* antigens involved in virulence and/or immunity, genetic and immunologic characterization of a 16 kDa protein of *B. abortus* was performed. Using PCR methods, the gene encoding the 16 kDa protein was cloned and sequenced. PCR and Southern blot analysis revealed that the gene is conserved among the 6 nomen species of *Brucella*. Overexpression of this protein in *B. abortus* vaccine strain RB51 was achieved using *Brucella groE* and *sodC* promoters as well as its own promoter. Protection and clearance studies were performed in mice to determine the role of this protein in *Brucella* immunity and pathogenesis. Inoculation with either strain RB51 overexpressing the 16 kDa protein or a DNA vaccine encoding the 16 kDa protein gene failed to provide significant protection. No difference was noted between the splenic clearance of *B. abortus* strain 2308 and its recombinant overexpressing the 16 kDa protein. A mutant of strain 2308 (2308Δ16) was created by disrupting the 16 kDa protein's gene with a chloramphenicol resistance cassette. Western blot analysis indicated that the O antigen profile of strain 2308Δ16 differed from that of strain 2308. Mice cleared strain 2308Δ16 faster than strain 2308 indicating the potential attenuation of the disruption mutant. Purified 16 kDa protein was obtained by overexpressing it in *E. coli* via the pRSET expression system. Western blotting results initially identified this protein as an immunoglobulin-binding protein. Hemagglutination assay revealed that the 16 kDa protein exhibits lectin-like properties. Preliminary studies using hemagglutination inhibition identified mannose as a possible sugar to which the 16 kDa protein can interact. The lectin-like properties exhibited by the 16 kDa protein appears to influence smooth lipopolysaccharide production, and thereby may be involved in virulence.

DEDICATION

I would like to dedicate this work to my family in thanks for all of their loving support. To my parents, thank you for encouraging me to challenge myself both in academics and in life. To my brothers and sister, thank you for being there for me – always ready with support or a laugh when I needed it most. To my husband, Ramesh, thank you for always believing in me, for your emotional support in good times and in bad, and for being the best Daddy you can be. Finally, to my daughter, Maya, thank you for showing me with your childlike innocence why family is of paramount importance. I love you all.

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LIST OF ABBREVIATIONS

Δ	Deletion/disruption mutant
::gene	Name listed after :: indicates genetic sequence/gene inserted into the gene of interest
2308	*B. abortus* strain 2308
45/20	*B. abortus* strain 45/20
Amp	Ampicillin
APC	Antigen-presenting cell
BL-3	Biosafety Level - 3
BSA	Bovine serum albumen, fraction V
BvrR	***B****rucella* **v**irulence **r**elated regulatory protein
BvrS	***B****rucella* **v**irulence **r**elated sensory protein
CDC	Centers for Disease Control, Atlanta, GA
CFT	Complement Fixation Test
CFU	Colony forming units
Cm or CAM	Chloramphenicol
CMI	Cell-mediated immunity
dsDNA	Double stranded DNA
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
Fc	Crystalizable Fragment
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
H38	*B. melitensis* strain H38
HA	Hemagglutination assay
HI	Hemagglutination inhibition assay
HRPO	Horseradish peroxidase
IBP	Immunoglobulin-binding protein
ID	Intradermal
Ig	Immunoglobulin
IL-2	Interleukin-2
IM	Intramuscular
INF-γ	Interferon-gamma
IPTG	Isopropyl-β-D-thiogalactopyranosidase
IP	Intraperitoneal
KO mice	Knock-out
LB	Luria-Bertani broth
LPA	Lymphocyte proliferation assay
LPS	Lipopolysaccharide
Mab	Monoclonal antibodies
MCS	Multiple cloning site
MHC	Major Histocompatibility Complex
NK cells	Natural killer cells
OD	Optical density
OMPs	Outer membrane proteins
O-PS	O-polysaccharide
Ori	Origin of replication
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
Pi	Postinoculation
pRSET	for this and other plasmid names, see Tables 1 and 2
RB51	*B. abortus* strain RB51
RBC	Red blood cells; erythrocytes
RBS	Ribosomal binding site
Rev 1	*B. melitensis* strain Rev. 1
RFLP	Restriction fragment length polymorphisms
R-LPS	Rough LPS
rSODs	S19 recombinants containing the three peptides coding for SOD
RT-PCR	Reverse transcriptase-PCR
S19	*B. abortus* strain 19
SC	Subcutaneous
SDA	Serum dextrose agar
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S-LPS	Smooth LPS
SOD	Superoxide dismutase

Tc cells	Cytotoxic T cells
TE	Tris-EDTA
Th1	T-helper 1
Th2	T-helper 2
Tn5	Transposon 5
TNFα	Tumor necrosis factor alpha
TSA	Tryptic soy agar
TSB	Tryptic soy broth

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1. INTRODUCTION

Brucellosis is a chronic zoonotic disease resulting in undulant fever in humans and abortion and/or infertility in affected animals (2). *Brucellae* are small, non-motile, gram-negative coccobacilli, which cause disease in a variety of mammals (54). There are six species of *Brucella* that are currently recognized based on their host-specificity. They include *Brucella abortus* (cattle), *B. melitensis* (goats), *B. suis* (hogs), *B. canis* (dogs), *B. ovis* (sheep), and *B. neotomae* (wood rat) (53). Recently, *Brucella* has been recovered from a variety of marine mammals including cetaceans (e.g. dolphins), seals, and otters. Though the organisms recovered from these marine mammals do not fit into the nomen classification by the currently available laboratory identification scheme, they have been confirmed to be *Brucella*, but probably deserve a new species designation. There is at least one report of human exposure to *Brucella* isolated from a sea mammal (29).

Brucella organisms can be phenotypically categorized based on their colony morphology into smooth, rough, and intermediate/mucoid (54). Organisms characterized as smooth contain the O-antigen (O-polysaccharide composed of perosamine polymers) on their lipopolysaccharide (LPS); true rough organisms do not contain O-antigen. In general, smooth *Brucella* are more virulent than their rough counterparts (54). *B. canis* and *B. ovis* are the only species of *Brucella* that naturally occur in the rough form, and yet are still pathogenic in their host species (54). All other 4 nomen species naturally occur in the smooth form. The newly discovered marine isolates all appear to be smooth.

Despite all of the progress in understanding the biology and molecular mechanisms of pathogenesis over the years, there remain many unanswered questions in *Brucella* research. Reasons to continue to study *Brucella* are threefold: the zoonotic nature of *Brucella*, its potential

use as a biological warfare agent, and its economic impact on livestock. Species of *Brucella* known to cause zoonotic infections (in descending order of occurrence and virulence) are: *B. melitensis*, *B. abortus*, *B. suis*, and *B.canis* (54). They are also likely candidates for use as biological weapons, yet, to date, there is no commercial vaccine against brucellosis labeled for human use with which to protect our uniformed services or civilians (including lab personnel). Arguably, the economic impact of brucellosis within the livestock population (i.e. cattle) is presently almost non-existent in the United States, thanks to a national eradication program. However, worldwide brucellosis remains a serious threat to its livestock and remains a point of potential transmission to humans. This is especially true in Mexico, the Middle East, Indian subcontinent and other developing countries. In a time of increasing globalization of commerce, this potential source of brucellosis to the United States and other *Brucella*-free (or near free) countries cannot be ignored.

Protective immunity directed against *Brucella* depends on the induction of an effective and specific cell-mediated immune (CMI) response (171). In addition, antibodies directed toward specific antigens, like the O-antigen, may enhance protection against brucellosis in some animal species. Vaccination using live, attenuated strains of *Brucella* has been instrumental in the control and prevention of brucellosis in cattle and goats. Live, attenuated strains currently used in veterinary medicine, such as *B. abortus* S19 and *B. melitensis* Rev 1 can cause disease in humans (6, 101, 163, 172), while the fate of *B. abortus* strain RB51 in humans is unknown. Treatment is available to people exposed to or infected with *Brucella*. This treatment, consisting of a prolonged antibiotic regimen, is costly and won't always eliminate the infection. The economic impact of human brucellosis increases substantially when one looks at not only initial treatment costs, but also possible recrudescence and related costs such as lost productivity at

work, etc. This clearly demonstrates the need for the development of better, more efficacious vaccines, especially in the control of human brucellosis.

A non-pathogenic, recombinant vaccine inducing good CMI and effective protection would be very useful in the control and prevention of brucellosis. To accomplish this, we must identify antigens that could induce such an effective immune response. Currently, only two *B. abortus* antigens, L7/L12 and Cu-Zn SOD have been identified as having some degree of protective ability as demonstrated in the murine model (133, 171, 178). Other antigens with protective potential need to be identified and investigated so as to construct the best possible recombinant brucellosis vaccine. Such studies may also aid in understanding the functions of those antigens in the biology of *Brucella*.

The present study was undertaken to characterize a previously described immunoreactive protein of *B. abortus* involved in the immune response of experimentally and naturally infected hosts using genetic and immunologic methods (43). One important finding of my studies is that this 16 kDa protein possesses lectin-like properties. This is the first known published report of a *Brucella* protein exhibiting such properties.

2. REVIEW OF LITERATURE

2.1. Brucellosis.

Brucella is named after Sir David Bruce who, in 1886, first isolated the organism from the spleen of a soldier afflicted with what was then termed Malta fever (31). Brucellosis causes a variety of disease symptoms depending upon the species affected. In humans, *Brucella* infection results in a condition known as undulant fever. The condition is also often referred to as Bang's Disease (in cattle) or Malta fever (in humans) (31). The three *Brucella* species that are considered zoonotic to humans are (in descending order of severity): *B. melitensis*, *B. abortus*, and *B. suis* (54). In addition, *B. canis* has been known to infect humans, but this occurs at a substantially lower rate than the aforementioned species. In humans, the manifestation of particular signs and symptoms depends mainly on the target system(s) affected by the *Brucella* infection. The most prominent sign is that of a biphasic or "undulating" fever. Other signs and symptoms may include fever, weakness, and myalgia. This can lead to complications such as endocarditis, arthritis, meningitis, and osteoarticular problems. In chronic cases of human brucellosis, neurological disorders can also manifest themselves (54). There have even been reports relating to the psychological effects of brucellosis.

Transmission of brucellosis to humans occurs through contact between infective animal tissues/secretions and human mucous membranes. Foodborne brucellosis occurs when individuals consume infected raw milk or dairy products. Foodborne brucellosis from milk consumption has not been a major source of *Brucella* infections in the United States since the practice of pasteurization of milk came into the mainstream during the first half of this century. However, foodborne brucellosis has not been entirely eliminated in the US. A small number of foodborne cases of brucellosis occur every year and many have been linked to the consumption

of imported soft cheeses or cheeses where raw milk has been used. Most of these cases have yielded *Brucella melitensis* and/or *Brucella abortus* as the causative agents. Today in the US, the main mode of transmission comes from that which can be classified under occupational risk. Occupational risk of brucellosis occurs in the field and affects mainly livestock producers, veterinarians, and laboratory workers. This risk comes from handling infected tissues such as aborted fetuses, placental membranes or fluids, and vaginal discharges. At present, *B. melitensis* is the main cause of human brucellosis worldwide.

2.2. The Genus *Brucella.*

2.2.1. General Properties of the Genus *Brucella.*

The genus *Brucella* consists of polymorphic Gram negative cells in the shape of cocci, coccobacilli, or short rods. *Brucella* are 0.5-0.7 μm in diameter and 0.6-1.5 μm in length (54). They grow under aerobic conditions at an optimal temperature of 37°C, with many strains requiring supplemental CO_2 for growth. Optimal pH conditions are from 6.6-7.4. All strains lose viability at 56°C, however temperatures over 85°C may be required to insure complete killing of *Brucella*.

On serum-dextrose agar (SDA), smooth colonies appear transparent, raised, convex, with an entire edge, and have a smooth, shiny surface (54). On primary isolation using SDA, *Brucella* colonies are rarely seen prior to 48 hours. At 48 hours, colonies are approximately 0.5-1.0 mm in diameter. Under transmitted light, colonies appear pale yellow in color. In reflected light, colonies will appear slightly opalescent and bluish gray in color. Colony variants can be classified under four morphological categories: smooth, rough, smooth-rough intermediate, and mucoid. This classification is made based on characteristics of the bacterium's

lipopolysaccharide (LPS). At the microscopic level, smooth organisms have LPS molecules containing a polysaccharide O-side chain made from a homopolymer of perosamine on their surface, while rough organisms lack this chain on their LPS (34, 158). Smooth and smooth-intermediate colonies are indistinguishable macroscopically. Rough colonies are usually less transparent than smooth variants. They have a more dull, granular surface and appear matte white, yellowish white/buff, or brown in color. Mucoid colonies are similar to rough colonies except that they have a sticky glutinous texture.

In terms of antibiotic susceptibility, nearly all strains of *Brucella* are susceptible *in vitro* to gentamicin, tetracycline (and derivatives), and rifampicin. Additionally, many strains are also susceptible to ampicillin, chloramphenicol, erythromycin, kanamycin, novobiocin, spectinomycin, streptomycin, and sulfamethoxisole/trimethoprim. Susceptibility to antibiotics can differ among species, biovars, and even strains. These differences can aid in identification of specific strains of *Brucella*.

Genetically, *Brucella* has some striking features. First, the *Brucella* genome consists of two chromosomes of 2.1 and 1.5 Mbp, respectively (124). Both contain essential genes encoding for essential metabolic and replicative functions. Hence, both are considered chromosomes and not cosmids or plasmids. Naturally occurring plasmids have not been found in *Brucella*. *Brucella*, however, can maintain some plasmids introduced via electroporation or conjugative transfer (191). Observations at the DNA level are becoming more helpful in further classifying *Brucella* organisms beyond conventional methods. These genetic markers are becoming the definitive means by which to identify one *Brucella* isolate from another and will be further discussed in Section 2.2.2., below.

2.2.2. Classification Scheme.

Isolates of *Brucella* species share greater than 90% DNA homology with one another (3). As a result, some investigators have suggested that all isolates be grouped under a single species of *Brucella* (246). Currently, however, phenotypic characteristics are still used to divide the genus *Brucella* into 6 species. The primary characteristics that distinguish one species from another are host preference and pathogenicity. Because of this, some refer to the six subdivisions as "nomen" species. The phenotypic properties used to characterize *Brucella* include colony morphology, antigenicity, virulence, growth rate, CO_2-requirements, viability, phage susceptibility, salt sensitivity, antibiotic resistance, and resistance to dyes (3). These properties not only divide the genus into 6 species, but 3 species (*B. abortus, B. suis,* and *B. melitensis*) have been further divided into biovars. The biovars themselves are divided even further and contain individual strains of bacteria.

The first major phenotypic classification, beyond host specificity, is colony morphology. The details of colony morphology (i.e. smooth vs. rough) have been previously described in Section 2.2.1. Descriptive features can be used to determine if a colony is smooth or rough. Two highly definitive tests used in colony morphology determination utilize acriflavin and crystal violet (205). Auto-agglutination of colonies of bacteria in a 0.1% acriflavin solution indicates that the colonies are morphologically rough. A colony's ability to take up crystal violet stain further indicates a rough colony morphology.

Another major classification scheme is based on antigens dominant within an isolate's O-polysaccharide (O-PS). The two major antigens present in the O-PS are A- and M-antigens. A-dominant LPS is characterized as having 5 consecutive α-1,2 linked 4-formamido-4,6-dideoxymannose residues; M-dominant LPS is linked predominantly via α-1,2 with every fifth

residue linked via α-1,3 linkages (53). Smooth strains of *Brucella* are classified as either being A- or M-dominant based on the ability of the strain to auto-agglutinate in sera directed against either of these antigens (138). Today, it has become increasingly important to identify not only a particular species of *Brucella*, but more importantly, to pinpoint what particular biovar or strain the isolate falls under. This is especially important when trying to determine if an isolate represents a wild-type, laboratory, or vaccine strain. Given the highly conserved nature within the genus, biochemical methods are usually an insufficient means of achieving a definitive classification of a particular isolate. In addition to the standard classification scheme, molecular methods are becoming the diagnostic method of choice. Methods such as restriction fragment length polymorphisms (RFLP) (105, 198), polymerase chain reaction (PCR) (84), and the use of nucleic acid probes (45, 98) are being used to distinguish between strains of *Brucella*.

2.2.3. Marine Mammal Isolates.

In 1994, small, gram negative coccobacilli were isolated from the aborted fetus of a bottlenose dolphin (*Tursiops truncatus*) and were tentatively identified as a *Brucella* species (83). Since that time, *Brucella* species have been isolated from a variety of marine mammals. These include common, hooded, and gray seals; otters; porpoises; and common, white-sided, and bottlenose dolphins (86, 113, 194). Identification of the original 1994 dolphin isolate (as well as subsequent isolates) was based on the isolate's similarities to *Brucella* colony and cell morphology, staining characteristics, growth (CO_2) requirements, biochemical activity, protein profiles, agglutination by monospecific antisera, and lysis by specific *Brucella* phages (83). These new strains have been isolated from a variety of tissues and lesion that are characteristic for *Brucella*. Recommended sites for the recovery of *Brucella* species include the following: the

spleen, mammary gland, testes, blood, and the mandibular, sublumbar, gastric, external and internal iliac, and colorectal lymph nodes (86). This, and the fact that the original isolate was obtained from an aborted fetus, suggests that these strains may play a role in the pathogenesis of reproductive failure in marine mammals (113).

None of the isolates from marine mammals matches the identification patterns of any of the six, host-defined nomen *Brucella* species (83, 86, 113, 194). It is now recommended that a new nomen species be assigned to these newly discovered *Brucella* strains. Jahan et al. (1997) suggest that the new nomen species be named *B. maris*, and that the species be further subdivided into three biovars. They suggest that the biovars be defined using the relative differences in their CO_2 requirements, utilization of sugars, and LPS antigen (i.e. A- and M-antigen) profiles (113).

2.2.4. Virulence.

The basis for the virulence of *Brucella* can be attributed to the ability of these bacteria to escape the host defense mechanisms and to survive and replicate within the host cells.

2.2.4.1. Intracellular Survival of *Brucella*

Virulent *Brucella* organisms are capable of invading and residing in professional phagocytes(20), such as macrophages, as well as non-phagocytic cells (65, 66). The mechanism of attachment and entry into these cells by *Brucella* has yet to be clearly elucidated. Virulence mechanisms identified so far to be associated with the ability to reside within phagocytic and/or non-phagocytic cells are as follows: the ability to inhibit phagolysosome fusion, degranulation

and activation of the myelo-peroxidase-halide system, and the production of tumor necrosis factor (33, 35).

In both phagocytic and non-phagocytic cells, *Brucella* has the ability to replicate within membrane-bound compartments (184). In non-phagocytic cells, such as HeLa cells, virulent *B. abortus* 2308 has been documented to replicate in the endoplasmic reticulum by utilizing the autophagic machinery of the HeLa cell (184). In professional phagocytes, the membrane-bound compartment within which virulent *Brucella* organisms can replicate is the phagosome. By some unknown mechanism, *Brucella* is able to block phagolysosome fusion (89). It is now thought that the production of adenine and guanine monophosphate can inhibit phagolysosome fusion (53). The ability to produce these compounds is therefore considered a virulence factor of *Brucella*. In contrast, attenuated strains of *Brucella* are unable to prevent such fusion and are thereby destroyed by the lysosomal contents (185).

Research on intracellular survival and replication of *Brucella* within professional phagocytes has mainly focused on macrophages. Survival within macrophages is apparently associated with the production of many different proteins. These proteins tend to be stress-induced proteins such as heat shock or acid-induced proteins. They include the 17, 24, 28, 60, and 62 kDa proteins (141). Two of these proteins, the 17 and 28 kDa proteins, seem to be induced only during intracellular cohabitation of *Brucella* with macrophages.

HtrA, another stress-induced protein, has been examined for its possible role in virulence and intracellular survival. Using deletion mutants, HtrA has been shown to be involved in inducing a granulomatous reaction and reduced levels of infection during the early phase of infection (murine model). However, this does not result in reduced levels in the later phases of infection. In fact, overall, *htrA*-deficient mutants produce splenic bacterial loads comparable to

their wild-type counterparts (228). RecA mutants produce similar results as *htrA* mutants in early- and late-phase splenic load (230).

Two other types of proteins that have been put forth as possible virulence factors are siderophores and Cu-Zn superoxide dismutase (Cu-Zn SOD). Iron-sequestration by siderophores may be an integral virulence factor in intracellular survival of *Brucella* species. Low levels of iron *in vivo* aid the host's ability to restrict microbial growth (53). *Brucella* species do carry iron-sequestering proteins and other siderophores, but their role in pathogenesis has not been clearly elucidated (19, 69, 136, 142). Cu-Zn SOD may play a significant role in the early phase of intracellular infection, but contradictory results have been reported (134, 213, 229). Further studies are needed before the role of Cu-Zn SOD as a virulence factor of intracellular survival of *Brucella* can be accurately assessed.

An auxotrophic mutation encoding for an essential enzyme (5'-phosphoribosyl-5-amino-4-imidazole carboxylase) necessary for the *de novo* synthesis of purines has been demonstrated to be essential for the intracellular survival of *B. melitensis*. Deletion of the gene, *purE,* encoding this enzyme in virulent *B. melitensis* drastically reduced its ability to survive within macrophages (70) and demonstrated attenuated behavior in mice and goats (41, 57).

Recently, a two-component regulatory system has been discovered in *B. abortus*. The Bvr (***B****rucella* **v**irulence **r**elated proteins) system consists of a regulatory (BvrR) and a sensory protein (BvrS). This regulatory system, BvrR-BvrS, may play a critical role in the ability of *B. abortus* to invade and multiply within cells (212). *Bvr*R-deficient mutants were obtained by transposon mutagenesis. Morphologically, these mutants produced smooth-type LPS. They were found to be increasingly sensitive to polycations and surfactants and showed decreased *in vivo* replication and persistence in mouse spleens. This occurred even though no obvious *in vivo*

growth defects could be detected in the mutants. Complementation with the *bvrR* gene restores resistance to polycations and partially restored the ability of these mutants to multiply intracellularly. The results further suggest that restoration of full virulence requires both components of the regulatory system to be intact. Interestingly, LPS core and lipid A are known to be involved in polycationic resistance. Therefore, it is possible that these LPS features involved in polycationic resistance are under the BvrR-BvrS regulatory system. Analysis at the DNA level of the *bvrR* and *bvrS* genes reveal that they are highly homologous to other regulatory systems found within symbiotic plant pathogens such as *Rhizobium meliloti* (ChvI-ExoS system) and *Agrobacterium tumefaciens* (ChvI-ChvG system). It has been well established that *B. abortus*, *R. meliloti*, and *A. tumefaciens* are phylogenetically related. Therefore, this suggests that the BvrR-BvrS system coevolved with the other two systems listed above to aid in the ability of *Brucella* to survive intracellularly.

In *B. suis*, recently, genes encoding a type IV secretion system homologous to the *Agrobacterium tumefaciens* VirB and *Bordatella perutussis* Pt1 systems have been identified to be essential for the intracellular survival in HeLa cells and human macrophages (165). Further research is needed to clearly understand the actual role of this secretion system in the virulence of *Brucella* species. At present, there is no evidence to support a secretion system within *Brucella*. If *Brucella* is capable of secreting, it is probably in very small amounts.

Non-protein components of *Brucella* may also contribute to its ability to survive within cells. One such cellular component, lipopolysaccharide (LPS) will be discussed in the section below.

2.2.4.2. Role of Lipopolysaccharide (LPS) in Virulence.

The LPS of smooth strains of *Brucella* are comprised of a lipid A molecule, fatty acids, a core region, and a polysaccharide O-side chain. This O-side chain is made from a homopolymer of perosamine and is found on the surface of smooth strains, while rough organisms lack this chain on their LPS (34, 158).

Smooth *Brucella* organisms are better able to survive intracellularly than do their rough counterparts. Therefore, smooth lipopolysaccharide (S-LPS) probably plays a significant role in pathogenesis. The simple explanation of rough versus smooth morphology and virulence, however, does not explain how naturally occurring rough species *B. ovis* and *B. canis* retain their virulence.

Using Tn5 transposon mutagenesis, several genes necessary for the synthesis of S-LPS have recently been identified. *In vitro* and *in vivo* studies with the rough mutants derived from the deletion of these genes clearly established that S-LPS is necessary for efficient intracellular survival and virulence of *B. melitensis*, *B. abortus*, and *B. suis* (5, 95, 252).

B. abortus S-LPS is 100 times less potent than that of *E. coli* (96) and *Salmonella* (88) in inducing TNFα from macrophages as well as oxidative metabolism and lysozyme release by human neutrophils. This feature of S-LPS has been proposed to contribute to the survival of *B. abortus* within phagocytic cells. In addition, *Brucella* S-LPS is not susceptible to the actions of polycationic molecules, suggesting that smooth *Brucella* can resist the cationic bactericidal peptides of the phagocytes (151). S-LPS has also been found to confer anti-phagocytic properties to *Brucella* and does not activate the alternate pathway of the complement cascade (110).

2.3. *Brucella* Vaccines.

2.3.1. Live, Attenuated Vaccines.

Both killed and live, attenuated vaccines have been examined for their potential role in the control and eradication of brucellosis in cattle, goats, and swine. Live, attenuated vaccines carry several advantages above their killed counterparts. First, immunity derived from their use tends to be cell-mediated and long lasting. Also, as they are administered live, the organism is allowed to replicate within the host, thus making them less expensive. However, some live, attenuated vaccines may cause abortion in pregnant females and therefore their use is often relegated to males and non-gravid females.

The two main live, attenuated vaccines used in the control of *B. abortus* infection in cattle are *B. abortus* strain 19 and *B. abortus* strain RB51. A brief discussion of each, plus the use of *B. melitensis* strain Rev. 1 in goats, follows.

2.3.2. *Brucella abortus* Vaccines.

2.3.2.1. *Brucella abortus* strain 19 (S19).

B. abortus S19 is a smooth but attenuated strain. The molecular basis for the attenuation is not known. S19 has been shown to contain a deletion in the erythritol catabolic genes rendering it sensitive to erythritol (199). However, such a deletion in virulent strains has been shown not to result in attenuation (200).

Prior to the introduction of vaccine strain RB51 in 1996 (see below), *B. abortus* S19 was the official vaccine used in the brucellosis eradication program in the United States. S19 was quite effective in protecting cattle against subsequent infection with virulent strains of *B. abortus*. However, S19 did have several problems that restricted its use within the cattle

population. During protection studies, it was discovered that S19, when given to adult cattle (>1yr), often caused persistent titers which could not be distinguished from titers resulting from a natural infection using standard serological tests (108, 164, 218). These tests detect the presence of antibodies to the O-antigen and include, among others: the Card test (a plate agglutination test), complement fixation test (CFT), and tube agglutination test. This directly undermines the brucellosis eradication program that is dependent on a test and slaughter strategy to reduce numbers of infected cattle within the United States. Persistent antibodies could be detected for up to 10-11 months postvaccination when vaccinating adult cattle with the standard dose (3 X 10^{10} CFU) (108, 164). Although a rare finding, even some calves vaccinated with S19 produce persistent antibodies.

Use of S19 in pregnant cattle also resulted in a percentage of abortions. Even when a reduced dose of S19 (1/20-1/100 of the standard dose) was used to vaccinate pregnant cattle, abortions post-inoculation were still observed, although this reduced dose appeared to be less abortigenic (10, 56). The use of the reduced dose vaccine did not eliminate the problem of persistent titers (9, 26, 79). In fact, these titers lasted about the same amount of time as the full dose (79). For this reason, Erasmus and Erasmus (1987) recommended that vaccination of adults with (the reduced dose of) S19 be relegated to herds heavily infected with *B. abortus*. As a result of the overwhelming experimental evidence, S19 was designated for use as a calfhood vaccine. Under federal law, only calves between 4 and 12 months of age could be vaccinated with S19.

Calfhood vaccination with S19 is not completely without side effects. As with all other brucellosis vaccines, S19 cannot be administered to bulls or bull calves due to the resulting persistent orchitis that results (32). There have also been reports of an arthropathy (gonitis) linked to vaccination of female calves with S19 (55, 254). Immunological studies by Wyn-Jones

and colleagues (1980) indicated the presence of *B. abortus* strain 19 antigenic material within the cells of the stifle, synovial membrane and the drainage lymph nodes. With the discovery of *B. abortus* strain RB51, the benefits of S19 vaccination diminished and RB51 replaced S19 as the official vaccine of the brucellosis eradication program.

The use of S19 has also raised concerns about human exposure to brucellosis vaccines. There have been several reports of illness following accidental self-inoculation with the S19 vaccine (101, 163, 183, 247). This stresses the importance of safe-handling practices when vaccinating herds for brucellosis using the S19 vaccine. It also led investigators to try and develop a more efficacious cattle vaccine that would also be safer in terms of potential human exposure.

2.3.2.2. *B. abortus* strain RB51.

In February 1996, the USDA Animal Plant Health Inspection Service (APHIS) approved the use of *B. abortus* strain RB51 (RB51) as the official calfhood vaccine for protection against brucellosis (http://www.aphis.usda.gov:80/vs/nahps/Brucellosis/rb51.html). Vaccine strain RB51 is a stable, rifampin-resistant, rough mutant of *B. abortus* 2308. It was derived by serial passage of parental strain 2308 on Trypticase soy agar supplemented with varying concentrations of rifampin and penicillin. Colonies of RB51 are rough in morphology as indicated by their ability to absorb crystal violet as well as auto-agglutinate when in suspension. The LPS of RB51 is deficient in O-side chain, unlike its parental strain 2308. Biochemically, though, RB51 shares the ability to use erythritol with strain 2308. RB51 has proven to be an extremely stable rough mutant of *B. abortus*. Its stability and efficacy has been shown *in vitro* and *in vivo* (both in mice and in cattle) (42, 205). Like the strain 19 vaccine, under current federal regulation calves must

be vaccinated with strain RB51 between the ages of 4-12 months of age with the calf dose (1.0-3.4 x 10^{10} CFU) (239). In the US, it is recommended that only animals in high-risk areas receive the vaccine after 12 months of age.

Advantages of RB51 over other vaccines for protection against bovine brucellosis are numerous. It does not produce any clinical signs post-vaccination, nor does it produce a local reaction at the site of injection (42). It is rapidly cleared from the bloodstream, as early as 2 weeks post-inoculation (42). It is not shed in the nasal secretions, saliva, or urine. Therefore, the organism appears to be unable to spread from vaccinated to non-vaccinated animals through these routes. In immunosuppressed animals, no recrudescence of infection has been documented. In addition, vaccination with RB51 affords a high level of protection, characterized by good cell mediated immunity. In one study, vaccination of cattle at least one year prior to mating induced 100% protection against abortion caused by exposure to field conditions of high and low brucellosis levels (144).

The use of RB51 has also helped clear up the issue of *Brucella*-positive/"reactor" animals. Since RB51 lacks O-side chain, vaccination with the strain (unlike strain 19) produces no antibodies to O-side chain. This is particularly advantageous because all of the diagnostic tests used to screen for brucellosis in herds are directed toward the detection of O-antibodies in the serum or milk. Cattle vaccinated with RB51 are negative on all subsequent serological tests, including agar gel diffusion (144). This lack of interfering antibodies is even true in the face of calfhood vaccination with strain 19 and subsequent adult vaccination with RB51 (175). Although, sera from RB51 vaccinated cattle do not respond to standard diagnostic tests, they do contain antibodies that react to a dot-blot ELISA containing RB51 antigen (42). As these antigens are common to both RB51 and 2308, the dot-blot ELISA test cannot differentiate

between vaccinated and infected animals (177); it is, therefore, relegated to assessing the humoral, non-protective immune response of cattle post-inoculation.

In addition to serology, there are two molecular methodologies that may be used to differentiate RB51 from other isolates: pulse field gel electrophoresis (PFGE) and polymerase chain reaction (PCR). RB51 possesses a unique fingerprint using the pulsed-field gel electrophoresis patterns of genomic DNA digested with restrictive endonuclease *Xba* I. The fingerprint of RB51 contains a unique band at 104 kb, as opposed to a 109-kb fragment within genomic DNA samples of *B. abortus* isolates from naturally infected cattle, bison, and elk (115). In addition, there is a specific PCR test that can differentiate RB51 isolates from all other *Brucella* isolates tested (245). This PCR test is based on the interruption of the *wboA* gene by an insertion element (IS711), a unique mutation present only in RB51.

In a murine model, *B. abortus* strain RB51 has been shown to confer protection against challenge with *B. melitensis*, *B. suis*, and *B. ovis* (121). However, in rams this vaccine did not induce protection against *B. ovis* challenge (120). Field trials indicate that *B. abortus* strain RB51 is also protective against swine brucellosis (143).

In addition to domesticated species, *B. abortus* strain RB51 has also been used to vaccinate wild animals such as bison and elk (74, 116, 173, 176). Oral vaccination of mice and cattle with RB51 has been shown to be effective in inducing protective immune responses (75, 221). These results are encouraging and highlight the feasibility of oral vaccination of wild life on a large scale.

RB51 appears to be a safe vaccine with respect to human exposure. Since conditional licensure of the vaccine, the number of accidental self-injections or conjunctival exposure of the RB51 vaccine reported to either the Centers for Disease Control (CDC) in Atlanta, Georgia or to

the vaccine manufacturer is 32 (14). To date, there has been only one confirmed case of clinical (brucellosis) infection in humans as a result of accidental self-injection or ocular/mucosal exposure to RB51.

2.3.3. *Brucella melitensis* Vaccines.

2.3.3.1. *B. melitensis* Rev. 1.

B. melitensis Rev. 1 (Rev1) is currently the only approved vaccine available for protection against *B. melitensis* infection. In 1957, a smooth attenuated strain of *B. melitensis* was isolated from a streptomycin-dependent population that had been grown in a streptomycin deficient medium (71). In experimental challenge trials in goats, this strain was found to induce significant protection against the virulent challenge strain without shedding the organism (3). The organism was later designated *B. melitensis* Rev. 1.

Use of the Rev1 vaccine has both advantages and disadvantages. Vaccination with Rev1 induces significant protection in sheep and goats (7, 8, 11). Rev1 has been found to be much more protective in goats and sheep challenged with virulent *B. melitensis* than those animals vaccinated with S19 (122, 159). The Rev1 vaccine does have some disadvantages. It can cause abortions if used in pregnant animals (3). Vaccination with Rev1 can result in persisting agglutinins that can interfere with various serological diagnostic tests (3). Rev1 is pathogenic to humans via aerosol exposure (172) or self-inoculation (6) causing generalized brucellosis in affected individuals. Like all other *Brucella* vaccines, Rev1 can cause local hypersensitivity reactions in cases of accidental inoculation.

2.3.4. Killed Vaccines.

Killed vaccines can offer protection to a disease while still retaining safety for those animals that are young, immunosuppressed, or pregnant. Over the years, a variety of killed vaccines have been developed for protection against brucellosis. They have had limited success. None have approached the protection status afforded by the live, attenuated vaccines. Examples of vaccines in this category are *B. abortus* strain 45/20 and *B. melitensis* H38 (53). In addition to the lack of sufficient protection in the face of challenge, killed vaccines such as 45/20 and H38 can induce persistent antibody titers that can interfere with common serological tests used (44, 53).

2.4. Immune Response to *Brucella.*

2.4.1. Cell-Mediated Immune (CMI) Response.

2.4.1.1. Intracellular Parasites and CMI.

CMI is necessary for protection because intracellular pathogens survive within the host cell. Most of them are able to survive within macrophages, thus enabling them to evade the innate defense mechanism such as complement-mediated and phagocyte-mediated killing. They also evade specific defense mechanisms mediated by antibodies.

Th1 cells are central to the host response against intracellular pathogens. Th1 cells are responsible for the activation of macrophages, the recruitment of phagocytic cells, and the induction of general T-cell proliferation (thereby effectively increasing the number of effector cells) (114). Th1 cells coordinate these activities through the cytokines they produce. INF-γ produced by the T-cell and CD40 ligand expressed on the surface of said T-cell act in concert to activate macrophages which in turn destroy bacteria engulfed by them. Induction of the

expression of Fas ligand on the Th1 cell surface and the release of TNF-α by the macrophage cause chronically infected cells to be killed and thereby release the intracellular bacteria to the extracellular environment where fresh, activated macrophages can destroy them. T-cell mediated release of interleukins (IL) IL-2 and IL-3 with Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF) induces the proliferation of T-cells and the differentiation of macrophages, respectively (114). Some intracellular bacteria are able to chronically infect their host by localizing in macrophage vesicles such as the phagosome and, thereby, escape the host killing mechanisms. Others escape from these same cell vesicles and reside within the cytoplasm. Once in the cytoplasm, these bacteria are not susceptible to killing via macrophage activation by Th1 cells. They can, however, be detected by cytotoxic T cells (Tc cells). Upon detection, Tc cells kill the macrophage, which then releases the intracellular bacteria into the extracellular environment.

Specific mechanisms directed toward the immune response against *Brucella* infection have been elucidated and will be discussed in the next section.

2.4.1.2. Th1 Immune Responses Associated with *Brucella* Infection.

Cytokine profiles can be used to identify the particular type of immune response to a given antigen or organism. Cytokines associated with a humoral or Th2 response are largely IL-4, IL-5, and IL-10. The main cytokines associated with a CMI or Th1 response are IL-2, IL-12, and interferon gamma (INF-γ). Cytokine production (i.e. transcription) can be identified and their mRNA quantified by cytokine-specific ELISAs (171) and RT-PCR (255), respectively.

In *Brucella*, proinflammatory cytokines and T-cell associated cytokines play a role in the cell-mediated immune response. Proinflammatory cytokines involved in *Brucella* infection

include tumor necrosis factor alpha (TNF-α), IL-1 and IL-6, and GM-CSF; T-cell associated cytokines involved include INF-γ, IL-12, and IL-10 (85, 118). Experimentally, the role of these cytokines in *Brucella* infection has been examined through a variety of means. These include the injection of recombinant cytokines, blocking cytokine activity through cytokine-specific monoclonal antibodies, and the use of knock-out (KO) mice. By such means, it has been determined that IL-1, when present prior to infection, results in decreased colonization of the spleen and liver by *Brucella* (257). Cytokines IL-12 and TNF-α are released and serve to control *Brucella* infection early in the process. IL-12 stimulates natural killer- (NK-) and T-cells thereby producing INF-γ, which steers host CMI toward a Th1 response (256). TNF-α, however, appears to act via an INF-γ independent pathway (256).

The role of INF-γ is very important in the control of *Brucella* infections. INF-γ is responsible for macrophage activation, increased expression of the major histocompatibility complex (MHC) molecules and other antigen processing components as well as facilitating immunoglobulin (Ig) class switching (114). INF-γ is also responsible for the upregulation (within the macrophage/monocyte cell lines) of the production of oxidative metabolites and other molecules toxic to bacteria. Experimentally, INF-γ has been shown to reduce *Brucella* growth within macrophages, although it cannot totally eliminate the infection (118, 123). Factors contributing to INF-γ-mediated intracellular killing of *Brucella* include iron (119) and TNF-α (35). IL-2 has also been identified as a mediator of the inhibition of intracellular *Brucella* growth, however, this occurs independent of INF-γ (118). Other cytokines (i.e. IL-1, IL-6, TNF-α, GM-CSF, and IL-4) have been tested individually, but no consistent effect on the *in vitro* intracellular growth of *Brucella* could be found (118). Interleukin-10 (IL-10), however, has been

shown to downregulate the immune response to *Brucella* by affecting both macrophage effector function and the production of the protective Th1 cytokine INF-γ (85).

The role of Tc cells in *Brucella* infections has been investigated. Oliveira and Splitter (1995) have shown that specific CD8+ Tc cells lymphocytes of C57BL/6 mice can lyse *Brucella*-infected macrophages. They have also shown using genetically engineered mice lacking β-2-microglobulin, hence deficient in functional CD8+ cells, that MHC-I restricted T-cells play an important role in controlling *B. abortus* infection (168).

2.4.2. Humoral Immune Response.

Convincing evidence as to the role of antibodies in protection against *Brucella* infection comes from the passive immunization of mice with either monoclonal antibodies (Mab) or polyclonal immune serum. Passive immunization with immune serum from mice recovered from the infection or vaccinated with smooth strains of *Brucella* conferred protection against challenge with virulent *B. abortus* (15). A number of studies have investigated the protective effect of passive immunization with Mab directed towards *Brucella* LPS and outer membrane proteins (OMPs) (27, 28, 251). In *B. abortus*, Mabs to smooth LPS (S-LPS), specifically to O-antigen provided significant protection against challenge infection. Among these Mabs, IgM and IgG2a isotypes were reported to be better protectors (251). Protection afforded by a IgG3 Mab specific to rough LPS (R-LPS) was found to confer significant protection, although it did not reach the level of protection afforded by S-LPS-specific Mab (49). Some Mabs against OMPs have been reported to contain weak protective activity against challenge with smooth *Brucella* (48). Based on immunization experiments with *E. coli* extracts containing *Brucella melitensis* 25 kDa major OMP, Bowden et. al. (27) proposed that antibodies against well exposed

conformational epitopes of OMP25 can contribute to protective mechanisms against *B. melitensis* infections in mice. In naturally rough *B. ovis*, antibodies to OMPs and R-LPS have been demonstrated to be protective, at least in mice, against *B. ovis* infection (28). Even with all of the information gathered thus far, there is still no clear picture on the role of antibodies in protection against bovine brucellosis since few controlled experiments involving passive transfer of antibodies have been carried out.

2.4.3. Immunogenic Proteins of *Brucella.*

The construction of an efficacious vaccine starts with the identification of immunogenic proteins within the bacterial species of interest. Investigation of these potential immunogens has been done both at the phenotypic and genetic/molecular levels. Often, molecular manipulation, such as the creation of deletion mutants or strains overexpressing the immunogen of interest, is employed to enable the phenotypic and genotypic analysis of the immunogen. A review on important known immunogens of *B. abortus* and *B. melitensis* follows.

The overexpression of proteins and the use of deletion mutants are reviewed in greater detail in Sections 2.5 and 2.6, respectively.

2.4.3.1. Immunogenic proteins of *Brucella abortus*.

Many proteins of *B. abortus* have been investigated as having a possible role in the immune response against brucellosis. Previously, studies testing the immune response were performed with extracts that contained a variety of *B. abortus* antigens. However, such studies do not definitively examine individual proteins. With the advent of recombinant DNA technology, several *Brucella* genes have been cloned allowing for the purification of the

recombinant proteins. This, in turn, enabled investigators the ability to study the function and immunogenicity of the individual proteins to be studied.

B. abortus proteins investigated have included: Omp2a/b (50, 150), p39 (62), BCSP 31 (214, 215), RecA (228, 230), Pal (234), catalase (207), Omp25 (51), Omp10 (10 kDa) (233), 17 kDa (141), 18 kDa lipoprotein (233), HtrA (193, 228), GroEL/ES (139-141, 223), YajC (244), Cu-Zn SOD (178, 226), and L7/L12 (133, 169-171). The proteins RecA, GroEL/ES, HtrA, and Cu-Zn SOD are stress-induced proteins (30, 139, 193, 230). The 17 kDa protein has not been induced in any *in vitro* stress conditions and presumably represents a macrophage-specific induced protein (141). Most of the proteins are cell wall-associated proteins, while a few are found in the cytoplasmic or periplasmic space (62, 216).

The immune response to these immunogenic proteins has largely been tested using the mouse model (130, 133, 140, 167, 169, 178, 186, 214, 215, 221, 222, 237), however, some have also been tested in cattle (62, 224). Assessment of the humoral response to the various immunogens has been performed largely by Western blot using sera from naturally or experimentally infected animals (e.g. mice, cattle, and other *Brucella* hosts) (107, 140, 193, 228, 237) or by using sera from vaccinates (140, 237). The cell-mediated immune response has been demonstrated for a variety of these immunogens by *in vitro* methods such as the lymphocyte proliferation assay (LPA) (167, 170, 178, 215, 219, 220, 223, 224, 244) and/or ELISA and RT-PCR methods to identify and quantitate Th1-associated cytokines (167, 244). In addition, *in vivo* methods such as delayed type hypersensitivity (DTH) have been utilized to demonstrate the immunogenicity of *Brucella* antigens (167).

Several of the *B. abortus* proteins investigated have shown to be promising candidates for providing protection against brucellosis. Of particular importance are the Cu-Zn superoxide

dismutase (SOD) enzyme (178, 226) and the ribosomal protein L7/L12 (133, 169-171). These two antigens appear to be the most promising candidates to date.

Superoxide dismutases are responsible for the scavengering of superoxide radicals. In *B. abortus*, the Cu-Zn SOD protein has been examined for its ability to induce humoral and CMI responses. In 1994, Tabatabai and Pugh (226) vaccinated mice with 3 peptide vaccines based on the deduced amino acid sequence of the Cu-Zn SOD protein. Postchallenge sera contained SOD-specific IgG antibody, but only when SOD was present in the vaccine. The production of antibody was increased with the addition of the adjuvant monophosphoryl lipid A. Only one of the peptides (GGAPGEKDGKIVPAG) gave notable protection (2 logs). Also in 1994, Stevens et al. (224) used the 3 peptides to ascertain the antibody and CMI response in cattle vaccinated with *B. abortus* S19 or RB51. Cattle vaccinated with either the S19 or RB51 vaccine failed to produce antibodies to the purified SOD peptides. Neither were they able to respond to their respective S19 recombinants containing the three peptides (rSODs). Only S19 produced CMI responses, via LPA, to both forms of SOD (i.e. purified protein, specifically GGDNYSDKPEPLGG, as well as each of the rSODs).

L7/L12, the 50S ribosomal protein of *B. abortus*, is of interest for several reasons. This protein induces CMI as evidenced through its ability to induce lymphocyte proliferation by increasing the percentage of CD4+ T-cells (170). Further proof of CMI production is provided by the ability of L7/L12 to induce a cytokine profile consistent with a Th1 subset from CD4+ T-cells (171). There is also evidence that vaccination with L7/L12, both as a fusion protein and as a DNA vaccine, provides significant protection against challenge with virulent *B. abortus* (133, 169).

2.4.3.2. Immunogenic proteins of *Brucella melitensis*.

Like *B. abortus*, studies testing the immune response of potential immunogens were performed with extracts that contained a variety of *B. melitensis* antigens. Important investigations focusing on individual potential immunogenic proteins of *B. melitensis* include: CP24 (47, 231, 248), BP26 (46, 59, 231), Bfr (61, 63, 64), OMP31 (100, 127, 249) and OMP25 (27). Cytosoluble protein 24 (CP24), is a ribosomal releasing factor required for the release of the 70S ribosome from mRNA. This protein, when expressed as an *E. coli* recombinant, was reactive with sera from naturally infected sheep as well as sheep experimentally infected with *B. melitensis* H38 (248). BP26 is also a cytosoluble protein, and like CP24 is reactive to sera from naturally and experimentally infected sheep (46). The Bfr protein is a bacterioferritin of *B. melitensis*. A fraction of Brucellergene, a commercial allergen preparation of *B. melitensis*, containing the Bfr protein has been tested for CMI response via LPA, INF-γ assay, and DTH in guinea pigs (63). The protein fraction gave a good CMI response by all 3 methods. Like Bfr, OMP31 has been reported to induce CMI, and it also gave a good humoral response (100). In protection studies, however, it failed to give significant protection in the face of challenge. In fact, the only antigen of *B. melitensis* that has shown to provide significant protection is OMP25. In protection studies, OMP25 protected against rough *B. melitensis* B115, smooth-intermediate *B. melitensis* EP, and smooth *B. melitensis* H38 challenge; it did not provide protection against *B. melitensis* 16M (27). In addition, mice vaccinated with an OMP25 *E. coli* recombinant produced specific antibodies, although these antibodies were found to be more reactive toward rough or smooth-rough strains than strains with a predominantly smooth morphology (27).

2.5. Overexpression of Proteins within Bacteria.

The properties of a particular protein may be difficult to assess based on a low basal level of expression within the bacterium. This problem may be overcome by overexpressing the protein of interest. There are two methods of overexpressing genes encoding the protein of interest: heterologous and homologous overexpression.

Heterologous expression involves the expression of proteins from one species of bacteria within an unrelated species. Most often this involves the expression of foreign proteins within *E. coli*. However, other species of bacteria, such as *Salmonella*, are becoming popular expression systems (17, 161, 196, 203, 235). Often, these bacteria are being manipulated so that they can act as vaccine strains that afford protection against multiple bacterial and/or viral diseases.

Heterologous expression can be achieved in two ways. The protein can be expressed in its native form or as a fusion protein. In order for the protein to be expressed in its native form, several conditions must be met. The expression vector must have an origin of replication (Ori) that is recognized by the host species of bacteria (e.g. Col E1 for expression of proteins within *E. coli*). In addition, all of the genetic elements of the protein must be subcloned into the vector. This includes not only the entire gene sequence encoding for the protein, but also the upstream promoter element and ribosomal binding site (RBS). The host bacterial strain must be able to recognize the foreign promoter and RBS in order for correct transcription and translation of the protein to occur. Often, the host bacterium is not efficient in recognizing the foreign promoter and RBS. In those cases, a host bacterial promoter element and RBS are inserted just 5' to the foreign gene creating transcriptional and/or translational fusions.

In cases where large-scale purification of the protein of interest is desired, expression of the protein in fusion with the specific polypeptide sequences offers several advantages. Fusion

proteins are created using expression vectors designed for the host bacterial strain. The vector contains an Ori element, promoter, RBS, and start codon that are recognized by the host. It often contains a multiple cloning site (MCS) for ease of cloning. In addition, the vector often contains a protein "tag" encoded by a short piece of DNA located just downstream from the start codon. This tag is made up of amino acids that can be used to aid in the purification of the protein (often on an affinity column). For example, in the pRSET expression system (Invitrogen, San Diego, CA), the pRSET vector contains a short sequence encoding for six histidine amino acids. This histidine "tag" binds to a resin column preferentially at a slightly basic pH (pH 7.8) and can only be eluted off the column under acidic conditions (pH 4.0). Fusion proteins are not free from problems, however. Sometimes, the additional amino acids can interfere in the folding of the protein thereby altering or abolishing the phenotypic, functional properties of the protein, as well as its antigenic specificity. The purified protein should always be checked to insure that it maintains the phenotypic/functional properties of interest before proceeding with further studies.

Homologous overexpression involves the expression of bacterial proteins within the same species of bacteria. This expression strategy can be more problematic. In the case of *Brucella*, finding a compatible vector able to replicate within the species is the issue. *Brucella* species do not carry a plasmid under normal conditions. In fact, there are very few plasmids that can be artificially introduced into and replicate within *Brucella*. One such plasmid has been designated pBBR1MCS (77). For this reason, all homologous expression within *Brucella* has been achieved using this particular vector.

Both heterologous and homologous expression of proteins can enhance the immune response to pathogenic strains of bacteria. Heterologous expression of proteins has lead to the development of bacterial strains overexpressing antigens from a variety of different bacteria (1,

17, 38, 161, 178, 196, 210). Often, the aim is to make a vaccine strain that can target multiple bacterial/viral pathogens using a single species of bacteria. *Salmonella* Typhimurium has been used as the host bacterial strain in a number of protection studies (17, 161, 196, 203, 235). Overexpression of heterologous antigens has provided protection against a number of different pathogens such as *Plasmodium berghei* (malaria) (196), *Streptococcus pneumoniae* (161), and enterotoxigenic *E. coli* (17). In our laboratory, homologous overexpression of *Brucella* proteins has shown promise in enhancing the level of protection induced by the current brucellosis vaccine strain RB51. In particular, RB51 overexpressing the Cu-Zn superoxide dismutase (SOD) gene has been shown to confer a significantly higher level of protection than RB51 (243).

Heterologous or homologous overexpression can also be used as a tool to understand various phenotypic properties of the protein of interest. These may be biochemical properties such as the utilization of particular nutrients or substrates (e.g. overexpression of an enzyme) (24, 25) or the protein's role in the virulence of the organism (152, 157, 178, 180, 203). In the latter case, homologous overexpression of the antigen may enhance the organism's ability to evade the host immune system or increase its ability to attach and invade host tissues and cells.

In this study, both homologous and heterologous overexpression of the 16 kDa protein was undertaken for three main purposes. The first was to assess any differences in protection afforded between RB51 and RB51 overexpressing the 16 kDa protein against challenge. The second was to assess the effect of overexpression of the protein on virulence, in both strains 2308 and RB51, using a splenic clearance test. Third, a method with which to achieve production of the 16 kDa on a large scale was needed. This was accomplished via the creation of a fusion protein and its heterologous expression within *E. coli*. This fusion protein was purified and used in the performance of hemagglutination and hemagglutination inhibition assays.

2.6. Deletion Mutants.

There are two main types of deletion mutants. The first involves the removal of the entire gene sequence encoding the protein of interest. This is also known as a "true" deletion mutant. The second type of deletion mutant involves the insertion of foreign DNA into the gene of interest. Often, no or very little DNA from the gene of interest is actually deleted by this method. This second type of deletion mutant is known more accurately as a disruption mutant. In this case, "deletion" actually refers to the function of the protein that is abolished upon disruption of the gene encoding the protein. However, both are often referred to in the literature simply as deletion mutants. Notation for deletion or disruption mutants use the delta (Δ) symbol; insertion of genetic material is indicated by the "::" notation (e.g. *B. abortus* strain 2308Δ16::Cm^r, the 16 kDa gene has been disrupted by the insertion of a chloramphenicol resistance gene).

Deletion mutants can be achieved by a variety of means. However, most methods leading to the creation of a deletion or disruption mutants utilize a selectable marker, such as an antibiotic resistance gene, to knock out or disrupt the gene encoding the protein of interest. The use of insertional or transposable elements, such as the Tn5 "cassette", can create random deletion/disruption mutants. This is also known as transposon mutagenesis.

Knowledge gleamed from deletion mutants can be quite critical. Information about the protein's phenotypic properties, its possible role in virulence or on the immune response, and its potential use in a vaccine can be investigated by this means. Gene complementation experiments of the deletion mutant can also help establish that the change in phenotype noted is a result of the deleted gene (206).

In *Brucella*, deletion mutants have been used to investigate all of these possibilities (40, 41, 57, 61, 70, 76, 104, 134, 174, 181, 182, 192, 206, 228-230, 232).

Deletion mutants have helped gain information about a gene's role in the phenotypic properties of *Brucella*. Most notably, information on *Brucella* O-antigen synthesis has been obtained through the use of such mutants. Often these mutants were derived via transposon mutagenesis through the random insertion of a transposon into the genome and then screening for the interruption of the gene(s) coding for the phenotype of interest. Often, a particular transposon known as Tn5 was used(5, 95, 155, 252). Such mutagenesis has allowed for the discovery of genes involved in LPS biosynthesis of *Brucella*. These include the perosamine synthetase gene, phosphomannomutase, and a glycosyltransferase gene (5, 95, 155). Each of these genes, when deleted, resulted in mutants that were attenuated in mice indicating that the synthesis of O side chain is essential for the *in vivo* survival of *Brucella*.

Deletion mutants have been used to investigate the role of a protein(s) in *Brucella* pathogenesis. It has been demonstrated that an *htrA* deletion mutant is more susceptible to oxidative killing and is attenuated in mice (182, 192). In 1992, Tatum et al. deleted the Cu-Zn SOD gene from wild-type strain 2308 (229). The resulting mutants were introduced into HeLa cells and in the mouse macrophage-like cell line J774 as well as BALB/c mice. Although the mutants acted similarly to the wild-type *in vitro*, splenic counts (CFU/spleen) of the mutants was approximately ten-fold lower than that of the parental strain through 26 days postinfection indicating attenuation. Deletion of the *purE* gene in *B. melitensis* resulted in attenuation both in mice (57) as well as in human macrophages (70), and thus indicated the importance of this gene for survival in the host. Transposon mutagenesis has been used to create mutants in *B. suis* for the study of essential genes. Interruption in several genes encoding for the VirB proteins was

found to cause high levels of attenuation in human macrophages (165). Attenuation either *in vitro* or *in vivo* is an indicator that the genes being deleted encode for essential proteins or factors necessary for the survival of *Brucella* within the host. On the other hand, deletion of the *recA* or the BCSP31 genes from *B. abortus* did not result in attenuation *in vivo* or *in vitro*, respectively (104, 230). In *B. melitensis*, deletion of the bacterioferritin gene did not result in different survival or growth within human monocyte-derived macrophages when compared to the parent strain (61). These results indicate that neither RecA , BCSP31, nor bacterioferritin are essential for persistence of *Brucella* in mice.

Deletion mutants have also been used to attenuate strains of *Brucella* for their potential use as vaccines. In 1997, Philips et al. demonstrated that an *htrA* deletion mutant of *B. melitensis* was attenuated in goats (e.g. no abortions observed in vaccinated nannies) while still providing protection against challenge with strain 16M (181). Administration of a *B. melitensis purE* mutant to goats resulted in immune responses, was cleared from visceral tissues, and produced less pathology than the wild-type parent strain 16M (41). The gene for P39, a protein previously shown to be immunodominant, was deleted from vaccinal strains *B. abortus* S19 and *B. melitensis* Rev. 1 (232). Both deletion mutants were able to protect against challenge like their parent strains. Deleting the P39 gene, however, allows for the differentiation between vaccinated and diseased animals.

In order to further characterize the function of the 16 kDa protein, attempts were made to create deletion mutants. Deletion mutants have often been used in the investigation into a protein's function. By deletion of the gene encoding the protein of interest, information about the protein's function can be gathered.

2.7. DNA Vaccines.

DNA vaccines involve the direct inoculation of "naked" DNA into the vaccinant. Naked DNA refers to the fact that the only component of the organism being vaccinated with (e.g. a bacterium or virus) is the DNA. No other cellular or subcellular components are included in the vaccine.

DNA vaccines are a relatively new vaccine technology. In the March 1990 issue of Science, the first experiments using a new "naked" DNA vaccine in mice showing long term (greater than 2 months) *in vivo* expression of encoded proteins was published (253). The proteins expressed included the β-galactosidase and luciferase enzymes. Since then, there has been a vast proliferation of information concerning the mechanisms of antigen delivery, immunological response, and protection derived from various DNA vaccines.

DNA vaccines involve the injection of plasmid DNA into the host (128). This plasmid DNA contains the gene sequence of the antigen of interest. All currently used routes of administration can be utilized (e.g. intramuscular [IM], intradermal [ID], intranasal, oral, etc.). The DNA, once delivered to the host, is incorporated into the host cells that express the antigen of interest. Expression levels of the antigen are regulated by a constitutive eukaryotic promoter. In the case of the commercially available plasmids pCDNA3 and pSecTag2 (Invitrogen, San Diego, CA), the eukaryotic promoter is the human cytomegalovirus promoter. The antigen, once produced, may be excreted into the extracellular space where it becomes available for processing by antigen presenting cells. Conversely, the antigen may be expressed by the antigen-presenting cell (APC) itself. Through these routes, the antigen can elicit either a cell-mediated, humoral, or mixed (i.e. both CMI and humoral) type of immune response. The immune response to the

antigen can be characterized and the protective effect of the vaccine can be tested by challenge with virulent organism.

There are still many pieces missing in the elucidation of the mechanisms of action concerning DNA vaccines. There is little information on just how the eukaryotic cells take up the DNA. There are also questions surrounding the identity of the cells that can achieve this uptake of intact DNA. There is evidence that myocytes (90), dendritic cells (36, 149), and some mobile immune cells can perform this task, but what other cells are also capable is under conjecture. The method by which the antigen, once translated, is exported to the extracellular space and/or presented on the cell's surface is yet another area that needs further investigation.

The type of immune response elicited by the DNA vaccine depends on several factors including: the antigen's characteristics, the route of administration, and the presence of immunostimulatory DNA sequences. Comparing routes of administration, intramuscular administration of the DNA vaccine elicits primarily cell-mediated immune response of the Th1 type (153). Intradermal injection, however, can elicit either a Th1 or Th2 response. If the vaccine is delivered by classic ID route (i.e. needle and syringe), the response is typically that of a Th1 response; delivered via gene gun, the response is typically that of a Th2 response (153). Gene guns deliver the DNA vaccine directly into the nucleus of the host dermal cells using a helium propulsion system (91). The use of DNA adjuvants can also alter the immune response. DNA adjuvants can be genes for immunostimulatory compounds such as cytokines that are co-delivered with the DNA vaccine (23). They can also be short DNA sequences/motifs that are located upstream from the gene sequence of the antigen being tested (132). Much research effort is being spent on the alteration and improvement of the host immune response to such antigens using just these kinds of approaches.

Protection through the use of DNA vaccines has been attempted for many different human (22, 39, 60, 92, 145, 225, 238) and animal diseases (13, 37, 58, 93, 102, 103, 117, 137, 179, 204, 250). DNA vaccines for human diseases have primarily focused on three diseases: HIV infection (16, 22, 126, 148, 166, 208, 209), tuberculosis (145-147, 238), and malaria (68, 92, 135). The malarial DNA vaccine has shown the most promise as it has shown protection in the mouse model (92). The diseases under study for animal DNA vaccines are more varied. However, many of these vaccines have shown a protective effect in the host species. Protective immune responses have been elicited for pseudorabies (Aujesky's disease) (93, 94, 102), simian immunodeficiency virus (SIV) (103), foot and mouth disease (FMD) (111), canine parvovirus (CPV) (117), avian (H5N2) influenza (129), canine distemper virus (CDV) (211), and bovine herpes 1 virus (BHV-1) (241). To date, there has been only one published study utilizing DNA vaccine technology for protection against pathogens within the genus *Brucella* (133).

In assessing the presence of a protective immune response toward the 16 kDa protein, two vaccination methods were used: a conventional modified live vaccine and a DNA vaccine. Utilizing a DNA vaccine construct containing the 16 kDa protein, a pilot protection study in mice was initiated during this study.

2.8. Thesis Objectives.

After a thorough review of the literature, objectives for the investigation of the 16 kDa protein were drawn up.

Objective I: Physical characterization of the 16 kDa protein. This includes both genetic and phenotypic analysis of the 16 kDa protein. Genetic analysis was achieved by DNA sequencing of the antigen gene followed by computer analysis of the sequence. Examination of the phenotypic properties of the 16 kDa protein were performed using many different approaches. These included standard bacteriological methods (e.g. dyes, stains, and growth characteristics), homologous overexpression, and the creation of disruption mutants.

Objective II: Immunological characterization of the 16 kDa protein. To assess the immunogenicity of the 16 kDa protein, protection studies of the 16 kDa protein (overexpressed within *Brucella*) using both conventional and DNA vaccine technology were performed. In addition, splenic clearance studies in the mouse model were designed to examine the 16 kDa protein's role in the persistence of *B. abortus in vivo*.

In the pursuit of fulfilling these objectives, other (potential) properties were discovered serendipitously and attributed to the 16 kDa protein (e.g. immunoglobulin-binding and lectin-like properties). These properties will be detailed and discussed in the Results and the Discussion sections of this work.

3. MATERIALS AND METHODS

3.1. Bacterial Strains and Plasmids. *E. coli* DH5α cells were purchased from (Gibco BRL, Grand Island, NY) and were grown overnight in either Yeast-Tryptone (2X YT) or Luria-Bertani (LB) broth or on LB agar plates (Appendix 1). *B. abortus* strains RB51 and 2308 (our culture collection) were grown in tryptic-soy broth (TSB) or on tryptic-soy agar (TSA) plates (Difco Laboratories, Detroit, MI) (Appendix 1) for 4-5 days at 37°C in an atmosphere containing 5% CO_2. All *Brucella* strains were manipulated under Biosafety Level 3 (BL-3) conditions. Killing of *Brucella* strains was accomplished by incubation of the liquid culture at 68 °C for 2 hours. *E. coli* BL21(DE3) cells (Novagen, Milwaukee, WI) transformed with pRSET or subsequent recombinants were grown in 1L of 2X YT broth for 2.5-3 hours (OD_{600} = 0.4-0.6), induced thereafter by adding 1.0 mL 1M isopropyl-β-D-thiogalactopyranosidase (IPTG, 0.1 M final concentration), and allowing growth to continue for an additional 3 hours.

A list of the plasmids obtained or purchased for use in the investigation of the 16 kDa protein of *B. abortus* are summarized in Table 1. Plasmid constructs made by the author are summarized in Table 2.

3.2. Extraction of *Brucella* Genomic DNA.

Brucella cultures were grown on TSA plates as previously described. Extraction of *Brucella* genomic DNA was accomplished using the QIAamp DNA Mini Kit (Qiagen®, Valencia, CA). The protocol suggested by the manufacturer for the extraction of bacterial genomic DNA was followed using approximately 25 mg (wet weight) of *Brucella* cells.

Plasmid Name	Features	Source/Reference	Intended Use
pBBR1MCS	Plasmid capable of replication within *Brucella*	M. Roop (Baton Rouge, LA)/ (131)	Expression of 16 kDa protein in *Brucella*
pBBGroE	pBBR1MCS derivative; contains *Brucella groE* promoter	R. Vemulapalli (Blacksburg, VA)	Homologous overexpression of the 16 kDa protein
pBBSODss	pBBR1MCS derivative; contains *Brucella sodC* promoter	R. Vemulapalli (Blacksburg, VA)	Homologous overexpression of 16 kDa protein
pCR 2.1	3'-thymidine nucleotide overhangs at insertion site for easy insertion of PCR products	Invitrogen®	Cloning PCR products
pRSET B	T7 expression vector; Histidine tag for binding/protein purification	Invitrogen®	Expression of 16 kDa protein in *E. coli*
pRS44.8	pRSET derivative; contains 1.8 kb *Brucella* fragment; this fragment contains the 16 kDa –COOH terminal	M. Roop (Baton Rouge, LA)/(43)	Sequence data used in design of reverse primer
pSecTag2B	Eukaryotic expression vector; contains promoter from immediate early gene of human cytomegalovirus	Invitrogen®	DNA vaccine preparation

Table 1. Plasmids obtained or purchased for use in the investigation of the 16 kDa protein of *Brucella abortus*. Plasmids obtained from individual investigators are listed with literature references in parentheses where applicable. pRS44.8 was originally developed at our laboratory.

Plasmid name	Genetic Components
pBBGroE16	Contains the entire 16 kDa gene plus its upstream RBS; does not contain its own promoter; cloned into pBBGroE vector; 16 kDa gene under the control of *groE* promoter
pBBRBS16	Contains the entire 16 kDa gene plus its upstream RBS; does not contain its own promoter; cloned into pBBR1MCS vector
pBBSOD16	Contains the entire 16 kDa gene plus its upstream RBS; does not contain its own promoter; cloned into pBBSODss vector; 16 kDa gene under the control of the *sodC* promoter
pBBup16	Contains the entire 16 kDa gene plus 300 bp upstream sequence containing its RBS and its own promoter; cloned into pBBR1MCS vector; 16 kDa gene under the control of its own promoter
pRSETssL16	Contains 16 kDa gene without the DNA sequence coding for its putative signal sequence peptide; cloned into pRSET B vector
pSecTag16	Contains the entire 16 kDa gene without its promoter or RBS; cloned into pSecTag2B
pTA16mid	Contains the amino terminus of the 16 kDa gene; cloned into pCR 2.1
pTA16start	Contains the entire 16 kDa gene without its promoter or RBS; cloned into pCR2.1
pTAmp16::Cm^r	Contains the 16 kDa gene plus 300 bp upstream sequence containing its RBS and its own promoter; disruption of the 16 kDa gene just 3'- to the signal sequence peptide coding region by insertion of a chloramphenicol resistance gene into the 16 kDa gene's *Bss H* II site; cloning strategy detailed in Figure 3.
pTARBS16	Contains the entire 16 kDa gene plus its upstream RBS; does not contain its promoter; cloned into pCR2.1 vector
pTAup16	Contains the entire 16 kDa gene plus 300 bp upstream sequence containing its RBS and its promoter; cloned into pCR2.1 vector

Table 2. Plasmids constructed in this study for the investigation of the 16 kDa protein of *B. abortus*. Plasmid names are listed as well as the genetic components relative to the study of the 16 kDa gene.

3.3. PCR.

3.3.1. Primers. DNA sequences for the majority of the polymerase chain reaction (PCR) primers were constructed via computer-assisted analysis (Primer Select software, DNASTAR, Inc., Madison, WI). Sequences for primers P1 through P5 were obtained from the literature (84). A list of the primers constructed for use in the PCR reactions are summarized in Table 3 (Genosys Biotechnologies, Inc., The Woodlands, TX).

3.3.2. Amplification Protocol. PCR was performed per manufacture's instructions using Ready-To-Go® PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ). To each reaction tube, the following were added: 1-2 μL of template DNA (50-100 ng); 1 μL of each 10 μM primer (i.e. one forward and one reverse primer); sterile, distilled water was added to a total reaction volume of 25 μL. When brought to the final volume of 25 μL, each reaction mixture also contained the following: 1.5 units of *Taq* DNA Polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM of each dNTP and BSA. The reaction mixture was vortexed and then centrifuged to insure equal distribution of all reaction constituents. Finally, the reaction mixture was overlaid with 30 μL of sterile mineral oil. The following amplification protocol was performed on each reaction mixture: initial denaturation at 95°C - 10 min; 39 cycles of 95°C - 1 min, 58°C - 1 min, 72°C - 2 min; final extension at 72°C - 10 min. The Omnigene® Temperature cycler was used to perform all PCR amplifications (Hybaid, Woodbridge, NJ).

Primer Name	Primer Sequence (5'-3')	No. of Nucleotides
P1	**GGACTGCATAAAATTGGCAC**	**20**
P2	**CAGCAGCAGCAAGACCTTCA**	**20**
P3	**CGCCCACTGT**	**10**
P4	**CGGCCCCTGT**	**10**
P5	**CGGCCCCGGT**	**10**
16mid	**CCGTGCCAGTAACCGGGG**	**18**
16Start	**AGATCTATGAACAGCTTCAGGAAAACTTGCGCGGGG** ***Bgl* II**	**36**
16RBS	**AGATCTTTCGCGCCAATGCCCGGA** ***Bgl* II**	**24**
16upstream	**AGATCTACAATGCCCGCAAGGAGCTGATCG** ***Bgl* II**	**30**
16rev	**GGTACCTTAACGAGAATAAGGCGAACGGCACTGC** ***Kpn* I**	**34**
SPlessforw	**AGATCTGCCCCCATGAATATGGATCGCCC** ***Bgl* II**	**29**

Table 3. PCR primers used to amplify the 16 kDa gene for use in various constructs. Primers P1-P5 were used as the forward primers in attempts to amplify the amino terminus of the 16 kDa gene (84). Restriction enzyme sites added to the 5'-end of the primers are underscored.

A two-step PCR was performed to obtain the amino terminus, and thus the complete 16 kDa gene. The first PCR consisted of one cycle of low stringency PCR performed with only the forward primer. The reverse primer was then added to the reaction mixture and the PCR was allowed to proceed for an additional 39 cycles at high stringency. The cycle parameters are outlined below.

PCR, step 1 (low stringency):

Denaturation:	94°C - 1.5 minutes
Annealing:	35°C-2.5 minutes
Elongation:	72°C-5.0 minutes

PCR, step 2 (high stringency):

Denaturation:	94°C - 1.5 minutes
Annealing:	58°C-1.5 minutes
Elongation:	72°C-2.0 minutes

3.4. Cloning of PCR products.

3.4.1. Purification and Ligation. PCR products were cloned into pCR 2.1 using the TA Cloning® Kit (Invitrogen, San Diego, CA). The ligation mixture consisted of the following: 1 µL 10X Ligation Buffer; 1 µL (10 ng/µL) pCR 2.1 vector DNA; 1-3 µL PCR product; 1 µL (400,000 U/mL) T4 DNA ligase; sterile, distilled water, for a final reaction volume of 10 µL. The amount of PCR product (in µL) to be added to the ligation mixture was determined by estimating the amount of PCR product/µL available. This was done through gel electrophoresis

of a 5 μL sample of the PCR product in a 0.8% agarose gel containing 0.5 μg/mL of ethidium bromide and viewed under UV. The ligation mixture was allowed to incubate at 25°C overnight and thereafter used to transform *E. coli* DH5α cells as detailed below in Section 3.4.2.

Prior to plating the transformation mixture, the agar plates were supplemented by spreading 100 μL each of halogenated indolyl-β-D-galactoside (Bluo-gal®; Gibco BRL) and of 1M IPTG over the surface of the agar. Plates were incubated at 37°C overnight in a table-top incubator. The next day, disruption of the pCR 2.1 *lacZ* gene by the insertion of the PCR product was assessed via blue/white screening of colonies. Only the white colonies were chosen for plasmid isolation and restriction endonuclease analysis. The plasmid constructs containing PCR products were named for the forward primer used in their creation. The addition of TA prior to the primer name refers to the use of the TA Cloning® Kit (Invitrogen) (e.g. pTA16start contains the entire 16 kDa gene; the primer 16start was used to amplify the gene).

3.4.2. Transformation of *E. coli*. Transformation of *E. coli* DH5α competent cells with the ligation mixture was performed per the manufacture's instructions (Gibco BRL). The competent *E. coli* cells were transferred from -80°C and stored on ice just prior to use. To a 25 μL aliquot of competent cells, 2.5 μL of the ligation mixture was added and mixed by stirring with the pipette tip. This transformation mixture was immediately replaced on the ice to incubate for 30 minutes. After 30 minutes, the transformation mixture was incubated at 37°C in a waterbath for 35 seconds, whereupon it was immediately transferred back to the ice for an additional 2 minutes. Upon completion of this heat shock transformation, 500 μL of SOC media (Appendix 1) was added to the transformation mixture. The microcentrifuge tube containing the transformation mixture was then placed in a Lab line® shaking incubator (Lab line®

Instruments, Inc., Melrose Park, IL) and incubated at 37°C with 225 rpm shaking for 1 hour to allow for the recovery of the cells prior to plating. After the recovery period, 200 μL of each transformation mixture was plated onto either LB or 2X YT agar plates containing 100 μg/mL ampicillin.

3.5. Plasmid DNA Isolation. Screening of white colonies for true positive transformants required plasmid isolation. Each colony picked for screening was placed in 5 mL of LB or 2X YT media containing 100 μg/mL ampicillin. The cultures were placed in a shaking incubator and allowed to grow overnight at 37°C and 225 rpm. The next day, the cultures were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 12,000 rpm for 3 minutes in a TOMY® TX-160 High Speed Refrigerated Micro Centrifuge (Palo Alto, CA). The supernatant was then discarded and the pellet saved. This step was repeated again for a total volume of 3 mL of culture to be processed. The 2 mL of culture remaining were stored at 4°C and were later used to make frozen (-80°C) stock cultures for those cultures that were screened and found to be true positives.

One hundred microliters of ice-cold stabilization buffer (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA) was added to the saved bacterial pellet and the pellet was vortexed until a uniform suspension of cells was achieved. In order to obtain the plasmid DNA, cells were lysed by adding 200 μL of room temperature cell lysis solution (0.2 N NaOH, 1% SDS) and mixing thoroughly via inverting the microcentrifuge tube 4-6 times. Immediately thereafter, 150 μL of ice-cold 3M potassium acetate (pH 5.2) was added to the tube and mixed thoroughly by inverting the tube 4-6 times. After a precipitate formed, the tube was centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred to a clean microcentrifuge tube and centrifuged

again. The supernatant was transferred to a third and final microcentrifuge tube, whereupon 750 µL of ice-cold 200 proof ethyl alcohol was added to the tube to precipitate the plasmid DNA. Precipitation of the plasmid DNA was further assisted by placing the tube in the -80°C freezer for 10-30 minutes. Pelleting of the plasmid DNA was achieved by centrifuging the solution at 15,000 rpm for 15 minutes. The supernatant was carefully removed from the DNA pellet by aspirating it with light vacuum. The pellet was washed once by adding 700 µL of 70% ethyl alcohol (4°C) and centrifugating at 15,000 rpm for 3 minutes. The supernatant was again carefully removed and the DNA pellet was air dried.

3.6. DNA Sequencing. Sequencing of the 16 kDa gene was performed via the Sanger dideoxy method using an ALF Express DNA Sequencer (Amersham Pharmacia Biotech) (201). Sequence data was identified as containing the entire amino terminus by aligning the overlapping the known sequences of carboxyl terminus with the newly identified amino terminus (MegAlign Software, DNASTAR, Inc., Madison, WI). Evidence for a putative promoter was provided when sequence data was examined using the neural-network method of promoter prediction available at the Lawrence Berkley National Library (http://www.fruitfly.org/seq_tools/ promoter.html).

3.7. Determination of Protein and DNA Concentration.

3.7.1. Protein Assay. The Bio-Rad® Protein Assay (Bio-Rad, Hercules, CA) was used, according to the manufacturer's instructions, to quantify the amount of protein in each of the eluted fractions. Samples were analyzed using a 96-well flat bottom tissue culture plate (Nalge Nunc International, Rochester, NY). Samples were compared against concentrations of bovine serum albumen (BSA) ranging from 50-500 µg/mL (Pierce, Rockford, IL). The BSA was

diluted in 1X Denaturing Elution Buffer. The negative control consisted of 1X Denaturing Elution Buffer. To each well, 10 μL of sample/control was added followed by 200 μL of 1X Bio-Rad Protein Assay Dye Reagent; samples were mixed with the dye by pipetting up and down. Three replicates of each sample were used and the mean OD values of the triplicates were taken for each sample. The microtitre plate was read at OD_{450} (Kinetic Microplate Reader; Molecular Devices Corporation, Sunnyvale, CA). The OD values were analyzed and reported by SOFTmax® Pro Software (Molecular Devices Corporation). The negative control OD values were subtracted from the OD readings of the rest of the samples.

3.7.2. DNA Quantitation. The concentration of DNA was quantified by spectrophotometric methods using a Shimadzu UV-1201 UV-Vis Spectrophotometer (Shimadzu, Columbia, MD) and reading the transmittance at OD_{260}. The concentration of DNA (μg/mL) was determined by multiplying the OD reading by 50 (197).

3.8. Restriction Endonuclease Analysis. Purified plasmid preparations from recombinants were screened by restriction endonuclease analysis. Each restriction endonuclease reaction contained the following: 1 μL of each restriction endonuclease enzyme needed (either one or two different enzymes) (Promega, Madison, Wisconsin); 2 μL of the compatible 10X restriction enzyme buffer; 3 μL of the purified plasmid DNA; and sterile, distilled water was added to a final reaction volume of 20 μL. The mixture was vortexed and centrifuged briefly to allow for uniform dispersion of the reagents, incubated at 37°C for 2-3 hours in a waterbath, and the samples were run on a 0.8% agarose gel containing 0.5 μg/mL ethidium bromide and viewed under UV light. The size of the insert in each plasmid preparation was measured against a 1 kb

molecular weight marker and compared to the known, expected size of the insert (i.e. based on sequence data).

3.9. Southern Blotting.

3.9.1. Preparation of Non-radioactive Probe.

3.9.1.2. Extraction of dsDNA from Agarose Gel. Linearized double stranded DNA (dsDNA) was extracted from agarose gels in the following manner. To melt the gel slice and release the DNA, 300 μL of 6 M NaI was added to the gel slice and incubated in a 55°C waterbath for 10-15 minutes or until the gel slice had completely melted. Fifteen microliters (15 μL) of a silica suspension (Appendix 2) was added to the melted gel slice and incubated at 55°C for 10 minutes. Intermittent vortexing of the sample during incubation was performed to ensure maximal binding of the DNA to the silica beads. After incubation, the sample was centrifuged briefly at 12,000 rpm in a TOMY® TX-160 High Speed Refrigerated Micro Centrifuge (Palo Alto, CA) to allow the separation of the beads from the supernatant. The supernatant was removed by aspiration and the beads were washed twice with 600 μL of New Wash Buffer (Appendix 2) followed by centrifugation and aspiration of the supernatant. After the final wash, the beads were resuspended in 15 μL of sterile, distilled water and incubated at 55°C for 10-15 minutes to allow the DNA to dissociate from the silica. The sample was centrifuged a final time and the supernatant containing the DNA was carefully collected. This supernatant was then used in the preparation of a DNA probe specific for the 16 kDa gene.

3.9.2.2. Labeling of DNA Probe with Digoxinin. Digoxinin labeled DNA probes were prepared in the following manner using the DIG DNA Labeling and Detection Kit (Roche

Molecular Biochemicals, Indianapolis, IL). Once the linearized dsDNA had been extracted from the gel, it was denatured by heating in a waterbath for 10 minutes at 95°C and immediately placed in an ethanol/ice bath. The labeling reaction mixture consisted of the following: 1 μL denatured DNA; 2 μL hexanucleotide mixture; 2 μL dNTP labeling mixture; 13 μL sterile, distilled water; 1 μL Klenow enzyme (2U/μL). The labeling reaction mixture was allowed to incubate in a waterbath for at least 1 hour at 37°C. Freezing the mixture at -20°C stopped the reaction. The probe was stored at -20°C until just prior to use.

3.9.2. Hybridization Procedure. A 0.7-0.8% agarose gel was prepared for Southern blotting according to the protocol outlined in Supplement 21, Section 2.9.3 of the Current Protocols in Molecular Biology manual (18). Transfer of the DNA from the gel to a nitrocellulose membrane was achieved by downward capillary transfer using the protocol outlined in Supplement 21, Section 2.9.8 of the same manual. The DNA was then covalently bound to the membrane by UV irradiation using a UV Stratalinker 2400 (Stratagene, LaJolla, CA). Prehybridization and hybridization of the membrane was carried out using the DIG DNA Labeling and Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions.

3.10. SDS-PAGE and Western Blotting.

3.10.1. SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 15% gel as per the standard protocol (18) using the Mini-PROTEAN® II gel apparatus (Bio-Rad,). Twenty to 40 μL of sample were loaded onto each lane; 5 μL of MultiMark™ Multi-Colored Protein Standard (Novex, SanDiego, CA) was loaded onto one lane.

Gels were run for approximately 45-60 minutes at 50-65 mA/gel in SDS-PAGE electrophoresis buffer (Appendix 2).

3.10.2. Western Blotting. All protein samples were separated on a 15% SDS-PAGE and transferred to a nitrocellulose membrane (197). The membranes were blocked with TBS containing 2% BSA and reacted with hyperimmunized goat serum to strain RB51 (Goat 48). The membranes were reacted with HRPO-conjugated rabbit anti-goat IgG and developed using 1-chloro-4-naphthol/H_2O_2 (See Western blotting substrate, Appendix 2).

3.11. Expression of the 16 kDa Protein within *E. coli*.

3.11.1. Construction of Recombinants in *E. coli*. The 16 kDa gene without the sequence encoding the signal sequence peptide was amplified from *Brucella* DNA as described in Section 3.3 using SPlessforw and 16rev as the forward and reverse primers, respectively. Cloning of the PCR products and transformation into *E. coli* DH5α cells was performed in the manner previously described (Section 3.4.2). The sequence containing the 16 kDa protein (minus the sequence of the putative signal sequence peptide) was subcloned into the *Bgl* II and *Kpn* I sites of pRSETB. The recombinant, named pRSETssL16 (i.e. signal sequence-Less), was used to transform *E. coli* DH5α cells (Section 3.4.2). Subsequently, pRSETssL16 was used to transform *E. coli* BL21(DE3) cells. These recombinant DE3 cells were used in large-scale cultures for purification of the pRSET recombinant His-tag fusion protein (Section 3.11.2).

3.11.2. Purification of the Recombinant Fusion Protein. *E. coli* BL21(DE3) cells transformed with pRSETssL16 were grown as described in Section 3.1. Preparation of the

denatured *E. coli* cell lysate was performed as follows. The *E. coli* cells were harvested from a 250 mL culture by centrifugation at 5000 rpm for 10 minutes in a Sorvall GSA rotor (Sorvall, Washington, D.C.). The cells were resuspended in one-tenth (1/10) of the original culture volume with 6 M urea/Phosphate-buffered saline (PBS, see also Appendix 2) buffer. The cell suspension was then sonicated on ice at high intensity for 3 minutes to lyse the cells and shear the DNA and RNA. Insoluble debris was removed from the lysate by centrifugation at 3000 x g for 15 minutes. The supernatant (lysate) was then transferred to a fresh tube and stored either on ice or at -20°C until use.

Purification of the 16 kDa fusion protein (pRSETssL16) was achieved under denaturing conditions by metal affinity chromatography using ProBond™ nickel resin per the manufacturer's instructions (Invitrogen).

In addition, purification of the 16 kDa fusion protein was also accomplished using the SP (HiTrap) ion exchange column (Pharmacia Biotech). The column was prepared in the following manner. All reagents were added to and allowed to exit the column under gravity flow. The dry column was equilibrated with 5 column volumes (25 mL) of PBS, followed by 25 mL 1.0 M NaCl. Finally, a second application of 25 mL PBS completed the equilibration of the column. The sample containing the 16 kDa protein was loaded onto the column and allowed to drain by gravity flow. The column was washed with 5 mL PBS. Elution of the 16 kDa protein from the column was achieved by passing 25 mL of 0.5 M NaCl through the column and collecting 5 mL fractions. Further elution was achieved with 1.0 M NaCl in the identical manner. Samples (40 μL each) from both sets of eluted fractions were run on SDS-PAGE and Western blotting was performed to isolate fractions containing the 16 kDa protein. Goat-48 serum was used as the primary antibody. Concentration of the 16 kDa protein in each fraction was determined by the

protein assay (Section 3.7.1). Fractions containing high levels of the 16 kDa protein were pooled and dialyzed against PBS to remove the urea from the pooled fractions. The protein concentration of the dialyzed fractions was determined by protein assay (Section 3.7.1).

3.12. Overexpression of the 16 kDa Protein within *Brucella*.

3.12.1. Expression Under the *groE* and *sodC* Promoters. Concentration of the expressed 16 kDa protein under each of the promoters was standardized by pelleting the overexpressing *Brucella* strain and resuspending the pellet in 10 mM Tris, pH 8.0 to a transmittance of 10% (525 nm). After adjusting the transmittance, a 1.0 mL sample was spun down and the resulting pellet was resuspended in 100 µL Tris. This final cell suspension was used in Western blotting.

3.12.1.1. Construction of Plasmids. PCR products were cloned into pCR 2.1 as in Section 3.4. From the recombinant pCR2.1 vectors, the PCR product containing the 16 kDa gene with its ribosomal binding site was excised by a *Bgl* II/*Sac* I digestion and subcloned into *BamH* I/*Sac* I sites of pBBGroE and pBBSODss (Figure 1). Transformation of *B. abortus* strain RB51 was achieved via electroporation (Section 3.12.1.2).

3.12.1.2. Preparation of Competent Cells and Electroporation of *Brucella*. The preparation and electroporation of *B. abortus* competent cells (strains RB51 and 2308) was carried out as previously described (154).

3.12.2. Overexpression of the 16 kDa Protein Under its Own Promoter. PCR products containing the 16 kDa gene coupled with either the RBS or the 300 bp upstream sequence were

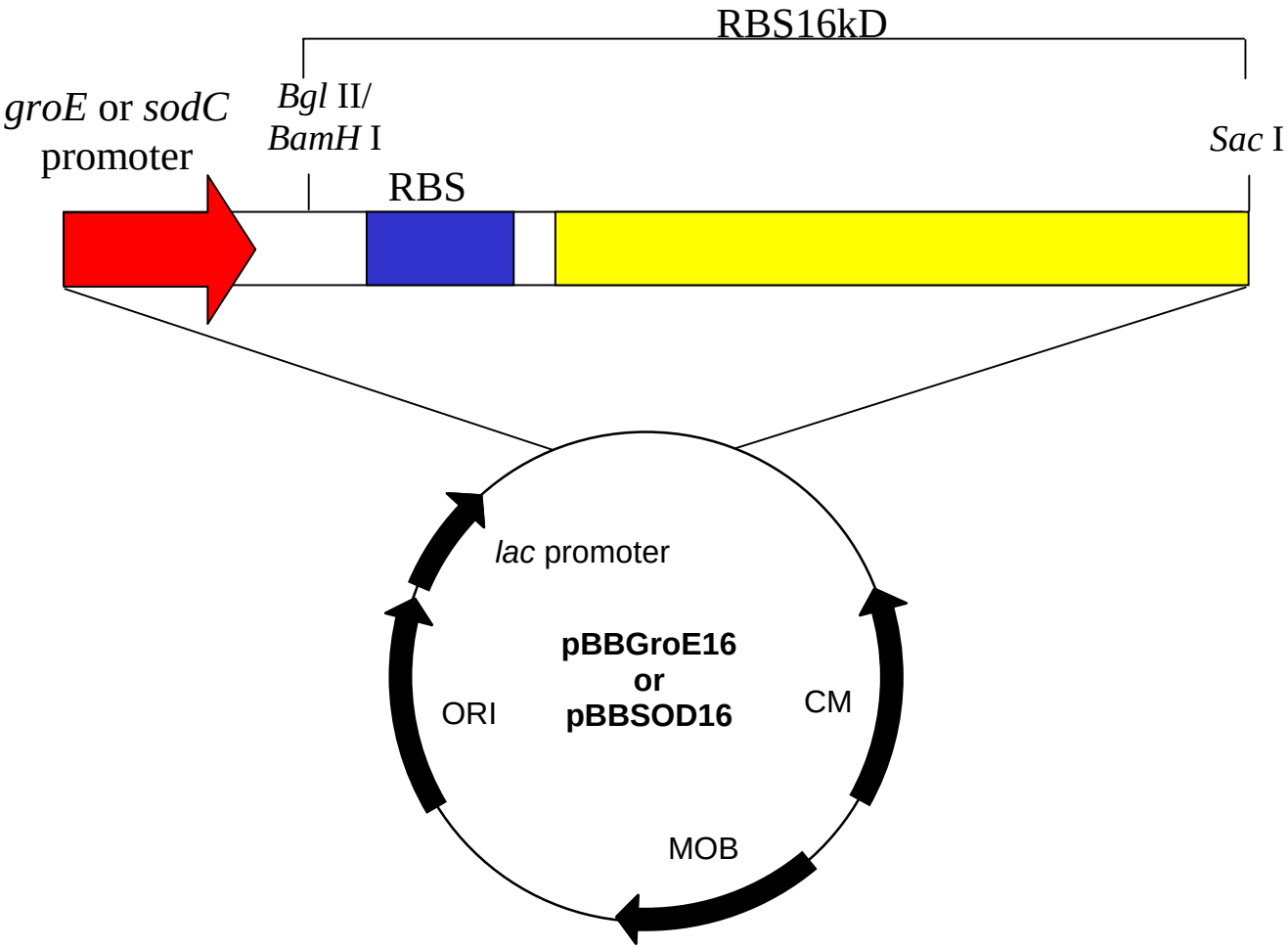

Figure 1: Schematic representation of the vectors used for overexpression of the 16 kDa protein under the *groE* and *sodC* promoters. The 16 kDa gene was subcloned into the *BamH* I/ *Sac* I sites of recombinant pBBR1MCS plasmids containing either the *groE* or *sodC* promoters; the resulting plasmids, pBBGroE16 and pBBSOD16, were used to transform *B. abortus* strain RB51 competent cells. The 16 kDa gene (yellow bar), its ribosomal binding site (blue box), and the *groE* or *sodC* promoter (red arrow) are shown. ORI – origin of replication; MOB – mobilizable element; CM – chloramphenicol resistance gene.

cloned into pCR 2.1 (see Section 3.4) and renamed pTARBS16 and pTAup16, respectively. These same PCR products were excised by a *Bgl* II/*Kpn* I digestion and then subcloned into *BamH* I/ *Kpn* I sites of pBBR1MCS (Figure 2) resulting in the plasmids pBBRBS16 and pBBup16, respectively.

3.13. Preparation of DNA and *B. abortus* strain RB51 for Immunization.

3.13.1. Genetic Immunization.

3.13.1.1. Construction of Plasmids. The 16 kDa gene was subcloned into pSecTag2B by excising the 16 kDa gene from pTA16start using a *Bgl* II/*EcoR* I restriction digest. The DNA fragment containing the 16 kDa gene was then subcloned into the *BamH* I/*EcoR* I site of pSecTag2B. The resulting construct was named pSecTag16. This construct, pSecTag16, was used to transform competent *E. coli* DH5α cells that were then plated on 2X YT agar plates containing ampicillin and grown overnight at 37°C. Recombinants were screened based on antibiotic susceptibility and via restriction digestion, as previously described (Section 3.8).

Plasmid pSecTag85 was obtained from R. Vemulapalli (Blacksburg, VA). Plasmid pSecTag85 contains a 2.7 kb fragment corresponding to the 85 kDa gene of *Ehrlichia risticii*. This plasmid was used as a vector control vaccine in the preliminary DNA vaccine protection study (Section 3.15.1).

3.13.1.2. Large-scale Purification of Endotoxin-free DNA. Large-scale purification of each of the constructs (pSecTag16 and pSecTag85) was accomplished using the Qiagen EndoFree™ Plasmid Mega Kit (Qiagen®, Valencia, CA). For each construct, a single colony was picked from a selective plate and used to inoculate a 5-10 mL starter culture of 2X YT broth containing

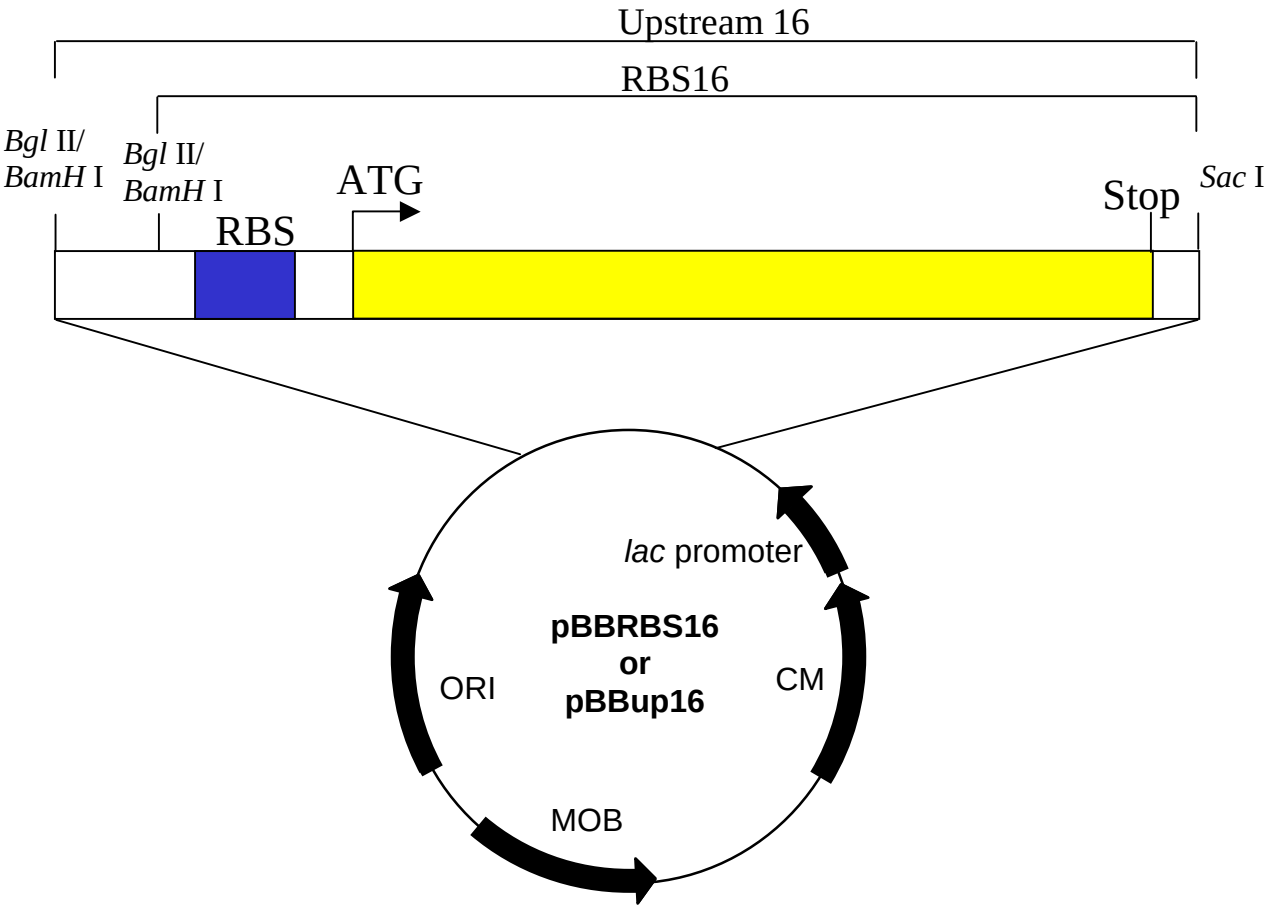

Figure 2: Schematic representation of vectors used for demonstrating overexpression of the 16 kDa protein under its own promoter. The 16 kDa gene (along with either its ribosomal binding site (RBS) or the 300 bp upstream region) was subcloned into the *BamH* I/ *Sal* I sites of pBBR1MCS plasmid. The resulting plasmids were named pBBRBS16 and pBBup16, respectively, and were used to transform *B. abortus* strain RB51 competent cells. Yellow bar indicates the location of the 16 kDa gene; RBS is shown in blue. ORI – origin of replication; MOB – mobilizable element; CM – chloramphenicol resistance gene.

ampicillin (100 μg/mL). The culture was grown for 8 hours at 37°C at 225 rpm in a shaking incubator. One milliliter (1 mL) of the starter culture was used to inoculate 500 mL of fresh 2X YT containing ampicillin. This new culture was allowed to grow overnight at 37°C at 225 rpm. The purification of plasmid DNA was performed per manufactures instructions with one modification. When resuspending the purified DNA, instead of using TE, the DNA pellet was resuspended in 25% sterile sucrose at a concentration of 1 μg/μL.

3.13.2. *B. abortus* strain RB51 and its Recombinants. The following recombinants of RB51 expressing the 16 kDa gene were used: pBBup16, pBBRBS16, pBBGroE16, and pBBSOD16. Construction of pBBup16 and pBBRBS16 were previously described in Section 3.12.2; construction of pBBGroE16 and pBBSOD16 was performed as described in Section 3.12.1.1. Transformation into *B. abortus* strain RB51 was performed in the manner described in Section 3.12.1.2. Stock cultures of RB51 and its recombinants were grown in TSB containing the appropriate antibiotic to late-log phase. The vaccine cultures were concentrated by centrifugation, resuspended in TSB at 1/100 of the original culture volume, and aliquotted before freezing them at -80°C for later use. Prior to inoculation, an aliquot of each of these vaccine stock cultures was thawed and the viable bacteria count (CFU/mL) was established so that the appropriate dose could be administered to each mouse in future experiments. After the dose of each vaccine was delivered, the remaining vaccine culture was plated so that the exact number of *Brucella* given to each mouse could be enumerated retrospectively. Enumeration of *Brucella* was accomplished via dilution plating as described in Section 3.15.1.1 for splenic cultures.

3.14. Construction of 16 kDa Deletion Mutants.

3.14.1. Construction of the Suicide Plasmid, pTAmp16::Cm^r. Creation of the suicide vector pTAmp16::Cm^r was accomplished by subcloning the chloramphenicol cassette from pBBR1MCS into pTAup16 using a *BssH* II digest (Figure 3). Transformation of *B. abortus* strain 2308 occurred via electroporation (154). Attempts were also carried out with strain RB51.

3.14.2. Screening and Characterization of the Deletion Mutants. After transformation *of B. abortus* strain 2308, transformants were plated on TSA plates supplemented with 10 μg/mL chloramphenicol. Colonies grown on the plates were further screened by patch-plating to TSA plates supplemented with 100 μg/mL ampicillin. Colonies that were identified as both chloramphenicol-resistant and ampicillin-sensitive were chosen for final screening. Final screening of positive colonies consisted of a *Hind* III digest of genomic DNA from positive colonies followed by Southern blotting (Section 3.9). Blots were hybridized with a digoxinin-label probe of the 16 kDa gene. In addition, phenotypic characterization of the disruption mutant colonies was performed via two methods: crystal violet staining and autoagglutination with acriflavin. These two methods were performed as previously described (12).

3.15. Mouse Experiments. All animal experiments utilized 4-6 week old, female, BALB/c mice (Charles River Laboratories, Wilmington, MA). All experiments were conducted and mice housed in Biosafety Level 3 (BL-3) facilities. Mice were housed in cages according to group number with 1 group/cage (up to 6 mice/cage). If there was more than 6 mice/group, the mice in that group were split between the requisite numbers of cages needed. All experiments were

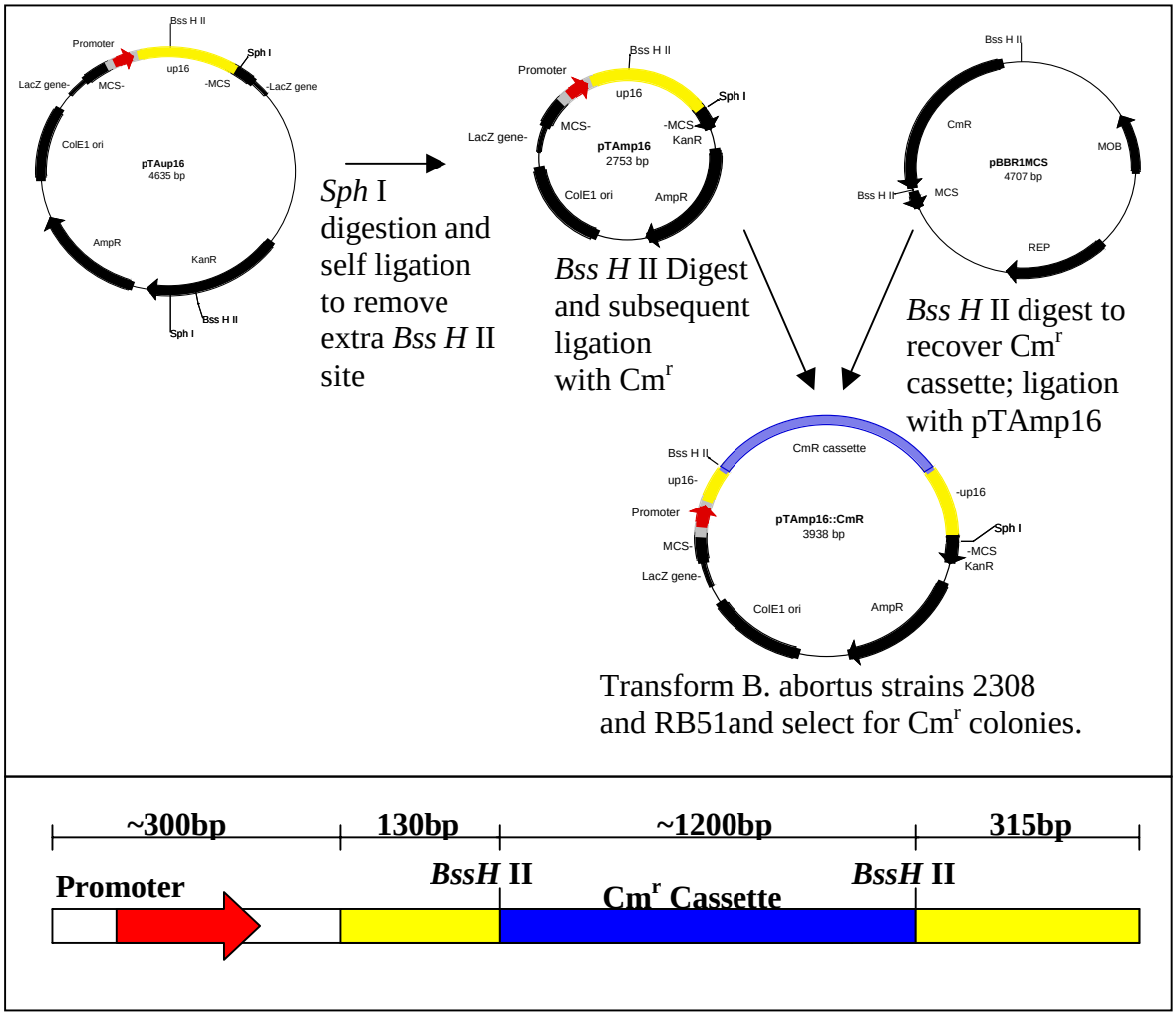

Figure 3: Cloning strategy for construction of the suicide vector, pTAmp16::Cm^r , used to create disruption mutants of the 16 kDa gene in *B. abortus* strain 2308. Top Panel: A *Sph* I digest of pTAup16 removed an extraneous *BssH* II site and was renamed pTAmp16. The suicide vector was created by the excision of the chloramphenicol (CM) cassette from pBBR1MCS via a *BssH* II digest and insertion into an identical site of the 16 kDa gene in pTAmp16. **Bottom panel:** Approximate number of base pairs (bp) of genetic material available for homologous double-crossover. CM cassette is denoted by the blue bar. The flanking yellow bars indicate the location of the 16 kDa gene. The white bar indicates the 300 bp upstream region containing the promoter (red arrow).

conducted under ACC #97-102-CVM.

3.15.1. Preliminary Protection Study after DNA Immunization. Three groups of mice were used in this pilot experiment. In group 1, all 4 mice were inoculated with pSecTag16 DNA (100 μg DNA/mouse). Group 2 consisted of 2 mice that received pSecTag85 DNA (100 μg DNA/mouse). Plasmid pSecTag85 contained an unrelated DNA sequence encoding the 85 kDa protein of *Ehrlichia risticii* and was used as the vector control (see Section 3.13.1.1). Group 3 (saline control) consisted of 4 mice. One day prior to the start of the experiment, the saline control mice were bled by puncturing the retroorbital plexus. Day 1, mice were injected with the appropriate preparation (see Experimental Groups, above). The preparations were administered by bilateral intramuscular (IM) injection of the gastrocnemius muscles (50 μL/leg). The second and third inoculations were given at 3 and 6 weeks, respectively. Serum samples were collected 2 days prior to the second and third inoculations and 2 days prior to challenging the mice. Challenging of the mice was performed on week 8 using a dose of 2.4 X 10^4 CFU/mouse of *B. abortus* 2308 delivered IP. Quantitation of the challenge strain from the spleen of mice was performed at 2 weeks post-challenge in the manner described in Section 3.15.1.1 (below).

3.15.1.1. Quantification of *Brucella* Recovered from Mouse Spleen and Statistical Analysis. Mice were euthanized by CO_2 asphyxiation and their spleens were removed aseptically and placed in individual plastic tubes containing sterile sand (~0.75 g/tube) and 1 mL TSB. Spleens were homogenized using a sterile pestle. Ten-fold serial dilutions of the homogenate were prepared in TSB. Five 10-μL drops from each dilution were plated on TSA plates and incubated at 37°C for 5 days. Plates were read on day 5. To minimize statistical error, lowest countable

dilution was used to quantify the *Brucella* per spleen (i.e. CFU/mouse). To determine the number of CFU/mouse the following formula was used:

Total CFU/dilution† **X** 20 **X** Dilution Factor

† Total CFU/dilution is obtained by adding the CFU from each of the 5 drops plated.

Statistical analysis of the data was performed as follows. For the protection studies (Section 3.15.1 and 3.15.2), the GLM procedure of the SAS system (version 7.0 SAS Institute, Inc., Cary, NC) was used to perform an analysis of variance (ANOVA) on the log transformed CFU counts. Post-hoc mean comparisons were done using Tukey's HSD (α=0.05). For the clearance data (Sections 3.15.3 and 3.15.4), the Student's t-test was performed using the Microsoft Excel 2000 Analysis Toolpak (Microsoft Corp., Redmond, WA) on the log transformed CFU counts (α=0.05).

3.15.2. Protection Study with Overexpressing *B. abortus* strain RB51. Three groups of mice were used in the protection study. Group 1, consisting of 5 mice, was given 2-4 X 10^8 CFU of RB51 containing the plasmid pBBGroE16. Group 2, consisting of 5 mice, was given 2-4 X 10^8 CFU of RB51 alone. Group 3, consisting of 4 mice, were designated the control group and given 0.5 mL saline. All 3 groups of mice were inoculated on day 1. All inoculations in the study were given IP. Mice were bled 4 weeks (day 28) and 6 weeks (day42) post inoculation by puncturing the retroorbital plexus. Serum obtained was stored at –20°C and later used to assess the humoral response of the vaccinated mice. Six weeks post inoculation, mice were challenged

with 2.4 X 10^4 CFU/mouse of *B. abortus* strain 2308. Eight weeks post inoculation, mice were euthanized by CO_2 asphyxiation and used to assess the CFU/spleen of the challenge strain (Section 3.15.1.1).

3.15.3. Persistence of *B. abortus* 2308 Overexpressing the 16 kDa Protein in mice. *B. abortus* 2308 was transformed with pBBGroE16 as described for RB51 (Section 3.12.1.2). Two groups of 9 mice each were used in clearance studies. Group 1 received 2 X 10^4 CFU of *B. abortus* strain 2308. Group 2 received 2 X 10^4 CFU of *B. abortus* strain 2308 containing the plasmid pBBGroE16. Both groups were inoculated IP with the appropriate preparations on day 1 of the experiment. Three mice per group were euthanized by CO_2 asphyxiation and used to quantify the clearance of the *B. abortus* strains at 1 day, 7 days, and 6 weeks post inoculation. Quantification of *Brucella* persisting in the mouse spleen was determined as described in a previous section (Section 3.15.1.1).

3.15.4. Persistence of *B. abortus* 2308Δ16 in mice. Two groups of 15 mice were used in clearance studies. Group 1 received 4 X 10^4 CFU of *B. abortus* strain 2308. Group 2 received 3.5 X 10^4 CFU of *B. abortus* strain 2308Δ16. Both groups were inoculated IP with the appropriate preparations on day 1 of the experiment. Five mice per group were euthanized by CO_2 asphyxiation and used to quantify the clearance of the *B. abortus* strains at 1, 7, and 14 days postinoculation. Quantification of *Brucella* persisting in the mouse spleen was determined as described in a previous section (Section 3.15.1.1).

3.16. Investigation of the Lectin-like Properties of the 16 kDa Protein.

3.16.1. Red Blood Cell (RBC) Blotting. RBC blotting was performed using a previously published protocol with modifications (190). Erythrocytes were prepared by collecting whole blood in citrate tubes. Erythrocytes were washed with PBS (1:10 ration of RBC:PBS) via centrifugation at low speed (1500 rpm; TOMY centrifuge). A total of 4 washes were performed. Finally, the erythrocytes were adjusted to 2% in PBS. Concurrently, protein samples were separated on a 15% SDS-PAGE and transferred to a nitrocellulose membrane (197). The membrane was incubated with the 2% erythrocytes suspension for 30-60 minutes at room temperature. The membrane was carefully washed by dipping it into PBS so as to eliminate non-specific binding without dislodging the RBCs from the lectin band (i.e. red agglutinated patch). As the carbohydrate content on the surface of erythrocytes differs among species, erythrocytes from a variety of species were used (e.g. mouse, cow, goat, sheep, and rabbit). Because of the fragile nature of this technique, blots were photographed immediately after they were developed and washed.

3.16.2. Hemagglutination Assay (HA). The ability of the 16 kDa protein to agglutinate RBCs was investigated in the following manner. Different suspensions (0.5-2%) of normal mouse erythrocytes were prepared as described above (Section 3.16.1). PBS was used to make serial dilutions of the purified 16 kDa protein. Purification of the fusion protein was previously described in Section 3.11.2. To each well of a U-bottom 96-well plate (Becton Dickerson Labware, Oxnard, CA), 25 μL of the serial dilutions were added. Next, 25 μL of the mouse erythrocyte suspension was added to each well and mixed with the 16 kDa protein by carefully pipetting up and down. The 96-well plates were incubated at room temperature for 1 hour.

Plates were visually examined after 1 hour for lattice (positive reaction, i.e. hemagglutination) or button (negative reaction) formation.

3.16.3. Hemagglutination Inhibition (HI) Assay. In order to determine the affinity of the 16 kDa protein for sugars, a seven different sugar solutions were used. They included: glucose, galactose, mannose, maltose, methyl-α-D-mannopyranosidase, D-galactosamine, and N-acetyl-D-glucosamine. Stock solutions (1 M, unless otherwise specified) were prepared in PBS. Serial dilutions of the sugar solutions were prepared. To each well of a U-bottom 96-well plate (Becton Dickenson Labware), 25 μL of each dilution was added. In the previous HA experiment (Section 3.16.2), the highest dilution of the 16 kDa protein still causing hemagglutination (i.e. the breakpoint) was determined. In the HI assay, we multiplied the breakpoint dilution by 4 (4X Breakpoint) and used this dilution for the HI assay. To each well of the HI assay, 25 μL of the 4X Breakpoint dilution was added and mixed with the sugar solution by pipetting up and down. The 96-well plate was then incubated for 30-60 minutes at room temperature. After incubation, 50 μL of 2% erythrocytes were added to each well and mixed by carefully pipetting up and down. The plate was then incubated for an additional 60 minutes at room temperature after which the plate was examined for button (positive reaction, i.e. hemagglutination inhibition) or lattice (negative reaction, i.e. hemagglutination) formation.

4. RESULTS

4.1. Nucleotide Sequence Analysis of the Upstream Region.

The DNA fragment encoding the carboxy terminus of the 16 kDa protein was originally isolated from a *B. abortus* genomic library and subsequently sequenced by Chirhart-Gilleland (43). To obtain the rest of the gene sequence by PCR, a reverse primer, 16mid (Table 3), was designed using the known sequence information. Five random primers, named P1-P5, were used as possible forward primers (Table 3). Using *B. abortus* 2308 genomic DNA as template, PCR amplification with primers P2 and 16mid yielded an approximately 500 bp fragment. In Southern blotting, a digoxinin-labeled probe containing the coding sequences for the carboxyl terminus of the 16 kDa protein hybridized with the PCR product (Figure 4). This indicated that the amplified fragment was most likely the upstream region of the gene containing at least part of the amino terminus coding sequences. The PCR product was subsequently cloned into pCR 2.1 (pTA16mid) and two clones containing the correct insert size were sequenced. Computer analysis of the resulting sequence data revealed that the DNA sequence of one of the clones overlapped with that of the region encoding the carboxy terminus described by Chirhart-Gilleland.

Analysis of the entire available nucleotide sequence revealed that the complete open reading frame was 444 bp in length capable of coding for a 16.82 kDa protein. A putative Shine-Dalgarno ribosomal binding site (RBS) was present immediately upstream to the start codon. Nucleotide sequences downstream to the gene's stop codon contained an inverted repeat that can potentially form a rho-independent transcriptional termination site. In addition, a potential prokaryotic promoter sequence was identified at 300 bp 5' to the RBS (Figure 5).

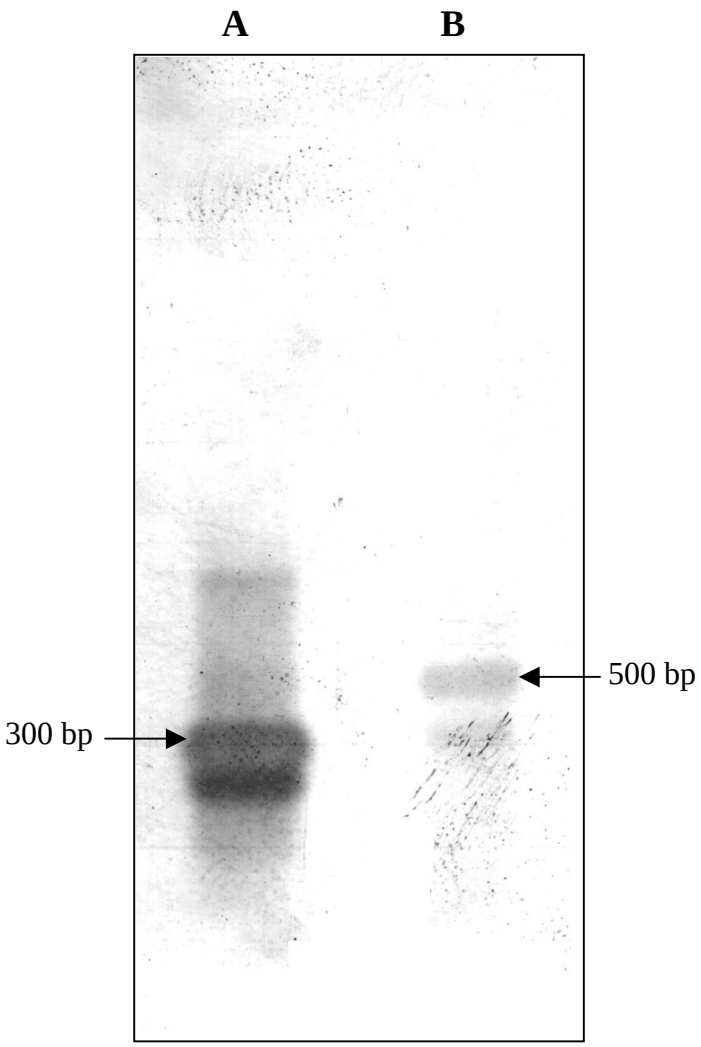

Figure 4: Southern blot analysis of the PCR product containing the gene fragment coding for the amino terminus of the 16 kDa protein. The DNA fragment obtained from PCR amplification using primers P2 and 16mid (lane B) was hybridized with a digoxinin-labeled probe prepared from the carboxyl terminus of the 16 kDa protein gene. PCR amplified product of the 16 kDa protein's carboxyl terminus encoding region from pRS44.8 was included as a positive control (lane A; see also Table 1). The approximate sizes of the hybridized products are indicated on either side.

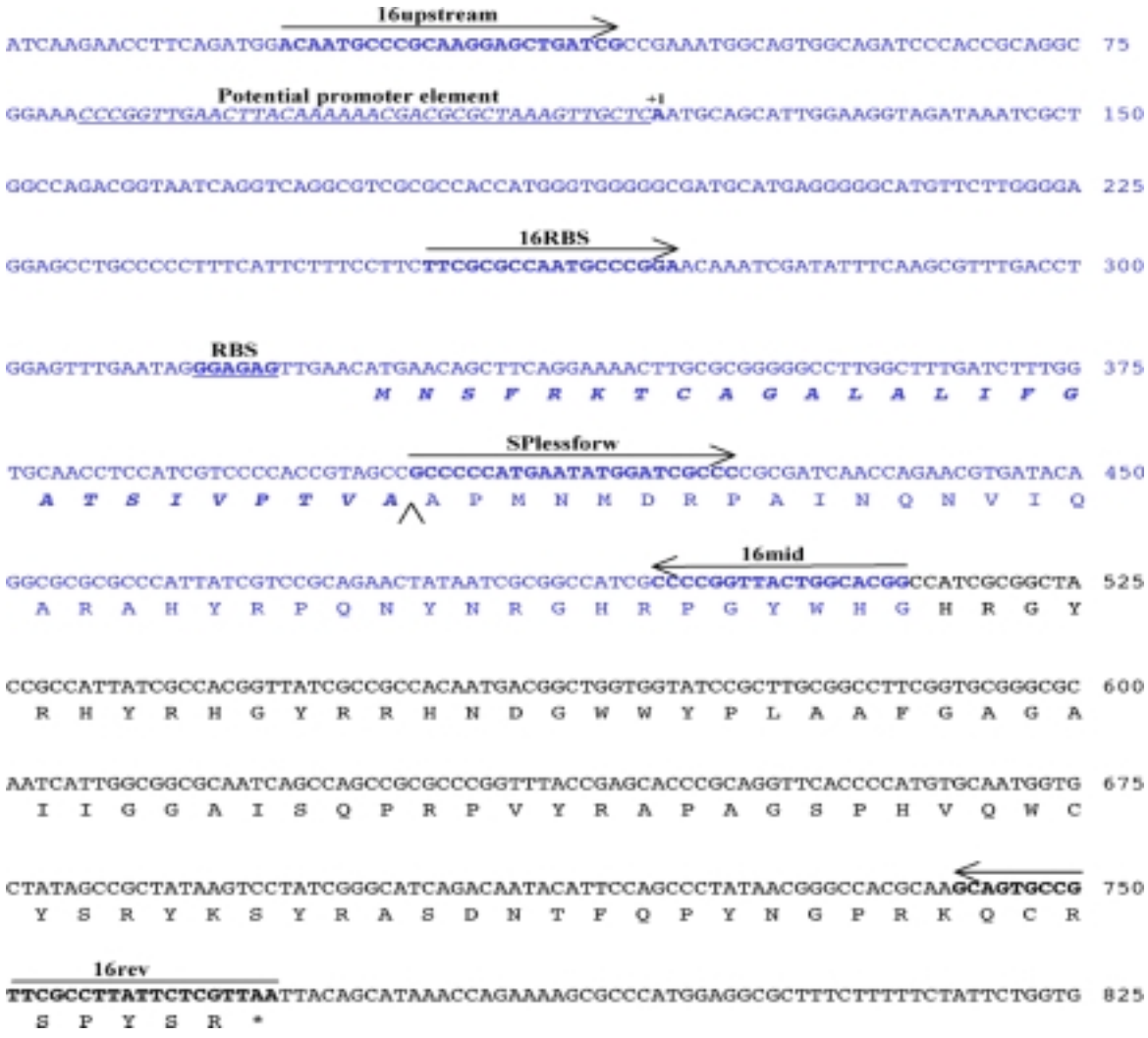

Figure 5. Nucleotide Sequence of the 16 kDa protein's gene and the 5' flanking region. Only the top strand's sequence is shown. Nucleotide sequence obtained from the studies presented in this thesis is shown in blue. The predicted amino acid sequence of the 16 kDa protein is depicted below the gene sequence. Sequences used for synthesizing the primers are indicated in bold-face; the direction of each primer is shown (arrow). The ribosomal binding site (RBS) and the potential promoter sequences are also indicated. The potential transcriptional start site is indicated with "+1". The putative signal sequence is shown in italics; the predicted signal peptidase cleavage site is indicated with an arrow head.

Preliminary analysis of the deduced amino acid sequence indicated that the 16 kDa protein is a highly basic protein with an isoelectric point of 11.47. The hydropathic profile predicted from the deduced amino acid sequence indicated that the protein contains two hydrophilic regions joined by a hydrophobic region of approximately 15 aa (Figure 6).

4.2. Conservation of the 16 kDa Antigen's Gene in *Brucella* Species.

The gene for the 16 kDa antigen was identified within various representative strains of the genus *Brucella* using two different methods: PCR amplification and Southern blotting of genomic DNA. PCR amplification of the 16 kDa antigen's gene from *Brucella* genomic DNA was performed using the 16start and 16rev primers. From all the strains tested, PCR resulted in the amplification of a ~440 bp fragment. In Southern blotting, the digoxinin-labeled probe of the entire gene's ORF hybridized to the 440 bp fragment indicating that the amplified product was indeed the gene for the 16 kDa antigen. In the second method, *Brucella* genomic DNAs were digested with the restriction enzyme *Hind* III and the resulting fragments were analyzed by Southern blot. In all the *Brucella* species and strains tested, the probe hybridized to a ~4 kb fragment (Figure 7).

4.3. Overexpression of the 16 kDa Antigen within *B. abortus* strain RB51.

Homologous overexpression of the 16 kDa protein in *B. abortus* strain RB51 was utilized as a method to provide preliminary evidence of the functionality of the promoter and also to examine the role of this protein's immunological role in protection against brucellosis. Overexpression was achieved using pBBR1MCS-derivative plasmids under different *Brucella* promoters (Figures 1 and 2). These promoters included the *groE* and the *sodC* promoters

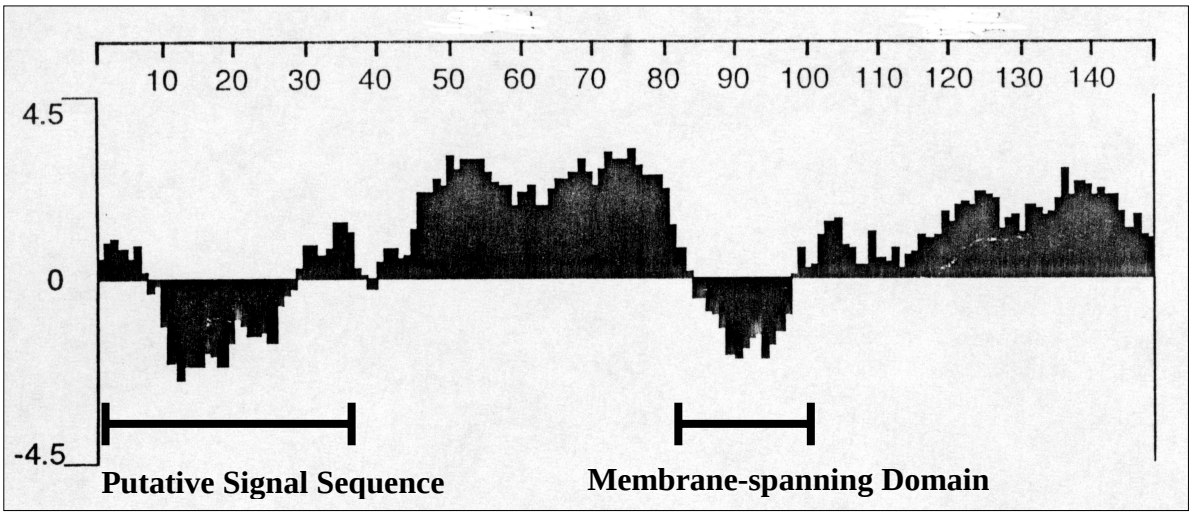

Figure 6. Hydrophilicity plot of the 16 kDa protein using the deduced amino acid sequence. Analysis was performed with the Kyle-Doolittle option of the Protean program of LaserGene Software (DNASTAR, Madison, Wisconsin). Positive and negative values indicate hydrophilicity and hydrophobicity, respectively. The numbers at the top are the amino acid positions. The putative signal sequence peptide and membrane-spanning domain are indicated by the closed bars.

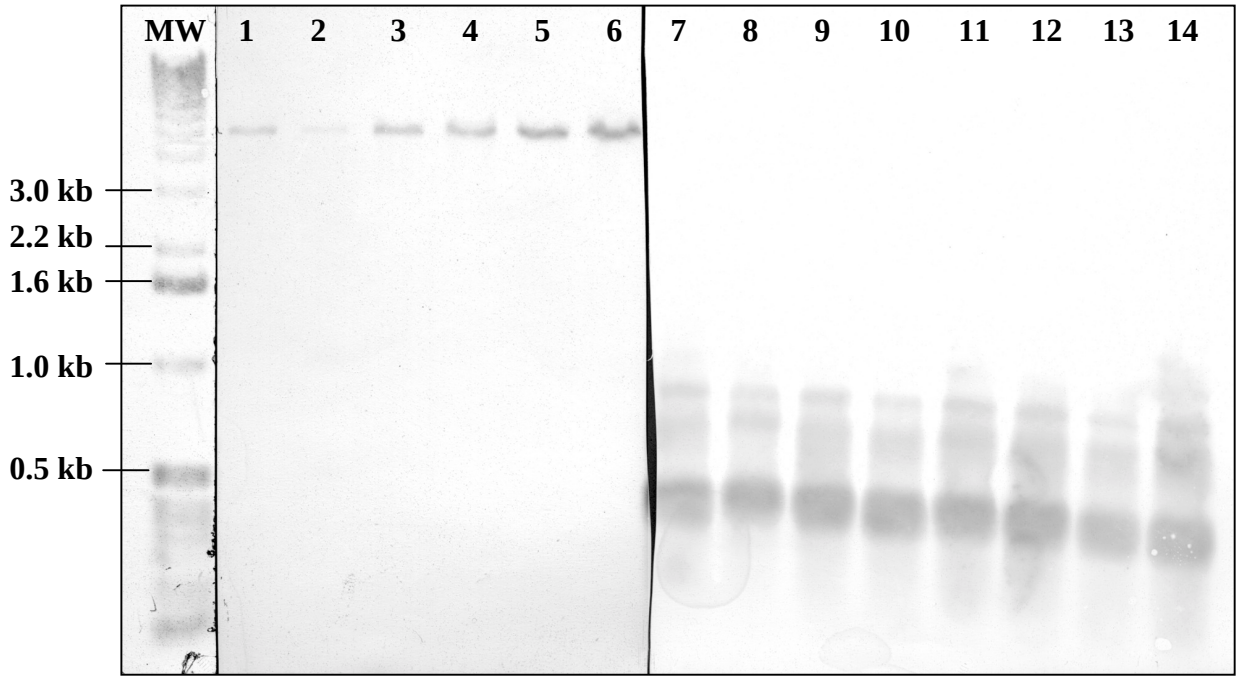

Figure 7. Demonstration of the presence of the 16 kDa protein gene within representative strains of the Genus *Brucella* by Southern blotting. The digoxinin-labeled probe of the 16 kDa gene was hybridized to either the DNA fragments resulting from a *Hind* III digest of genomic DNA (lanes 1-6) or to the DNA products obtained by PCR amplification using genomic DNA and the 16start-16rev primer pair (lanes 7-14). The *Brucella* strains used to obtain the genomic DNA were as follows (lane number, isolate): 1, *B. abortu*s strain 2308; 2, *B. abortus* strain RB51; 3, *B. abortus* biovar 2; 4, *B. suis* biovar 2; 5, *B. neotomae*; 6, *B. melitensis* biovar 3; 7, *B. abortus* strain RB51; 8, *B. abortus* strain 19; 9, *B. abortus* strain 45/20; 10, *B. melitensis* strain 16M; 11, *B. suis* 2; 12, *B. canis* strain RM666; 13, *B. ovis*; 14, *B. neotomae*. The lane labeled MW contains the DNA molecular size marker (i.e. 1 kb ladder).

as well as the promoter of the gene for the 16 kDa protein itself.

For examining the activity of the 16 kDa protein gene's promoter, two constructs were created by PCR amplification and subsequently cloned into pCR 2.1. One construct contained the 16 kDa protein's gene with its RBS, but no promoter (primer pair: 16RBS-16rev; plasmid name: pTA16RBS). The other contained the entire gene plus the 300 bp upstream region containing both the RBS and the promoter (primer pair: 16upstream-16rev; plasmid name: pTA16up). These two fragments were subsequently subcloned into pBBR1MCS as described in the Methods section and named pBBRBS16 and pBBup16, respectively (Figure 2). In addition, the 16 kDa protein's gene with its RBS was subcloned into pBBR1MCS-derived plasmids containing either the *groE* (pBB*groE*) or *sodC* (pBBSODss) promoters; the resulting plasmids were designated pBB*groE*16 and pBBsod16, respectively (Figure 1). The recombinant plasmids were electroporated into *B. abortus* strain RB51 and overexpression of the 16 kDa protein in the recombinant RB51 strains was examined by Western blot analysis using Goat 48 sera (a goat hyperimmunized with *B. abortus* strain RB51). As shown in Figure 8, overexpression of the 16 kDa protein was observed in recombinant RB51 strains containing pBBup16, pBBsod16, and pBB*groE*16 (Panel A: lanes 3 and 4; Panel B). No overexpression was detected in pBBRBS16 harboring recombinant RB51 strain; the levels of expression in this strain and RB51 containing pBBR1MCS were appeared to be similar to that of strain RB51 (Panel A: lanes 1 and 2). Based on a visual comparison, relative expression levels of the 16 kDa protein in the various recombinant RB51 strains were (in descending amount) as follows: Rb51/pBB*groE*16 > RB51/pBBsod16 > RB51/pBBup16.

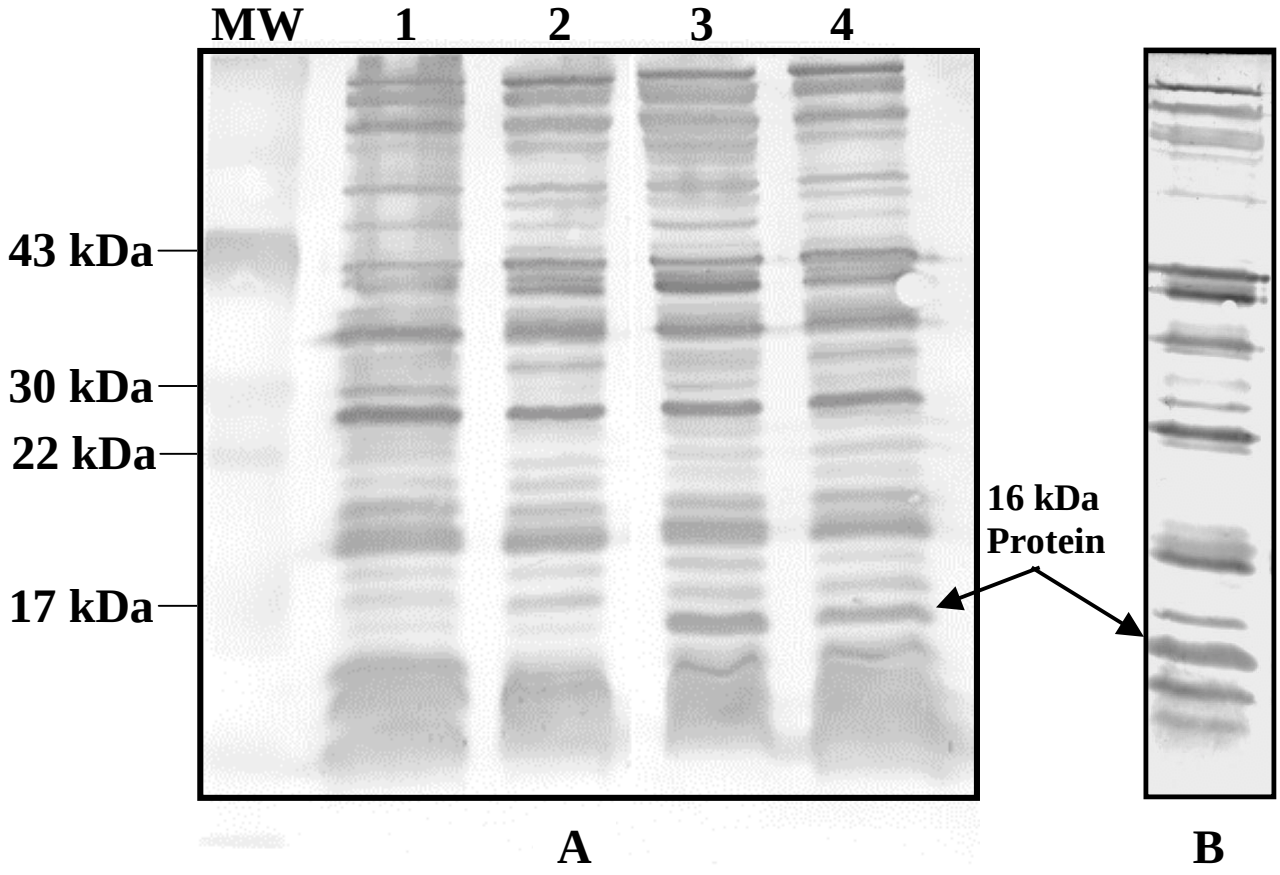

Figure 8: Overexpression of the 16 kDa protein in *B. abortus* strain RB51 under a variety of *Brucella* promoters. Total antigen extracts of the recombinant RB51 strains were subjected to Western blot analysis using serum from goat 48 as the source of primary antibodies. In panel A, Lane MW: Molecular weight marker; lanes 1-4: RB51 containing plasmids pBBR1MCS, pBBRBS16, pBBSOD16, and pBBup16, respectively. Panel B: RB51 containing plasmid pBBGroE16. The bands corresponding to the 16 kDa protein are labeled.

4.4. Overexpression of the 16 kDa gene within *B. abortus* strain 2308 and its Persistence using the mouse model.

Two groups of 15 BALB/c mice each were inoculated IP with either *B. abortus* strain 2308 or the 2308 recombinant overexpressing the 16 kDa protein (2308/pBBGroE16) at a dosage of 2.4 X 10^4 and 2.0 X 10^4 CFU/mouse, respectively. At day 1, 7, and 42 postinoculation, 3 mice per group were euthanized and the number of *Brucella* per spleen (CFU/spleen) were quantified (Table 4). No statistical difference in splenic clearance counts between the two groups was found for any of the 3 days using the Student's t-test, two-tailed (α=0.05). P-values for each of the days pi are listed in Table 4.

4.5. Purification of the Recombinant 16 kDa Fusion Protein.

To facilitate easy purification, the 16 kDa protein was expressed as a fusion with poly-histidine tag in *E. coli* using the pRSET expression system. The expressed recombinant fusion protein was present in insoluble form and buffers containing 8M urea as a denaturing agent were needed for the solubilization. Purification of the recombinant protein was achieved by either metal affinity chromatography on a Ni^{2+}-resin or ion-exchange chromatography on a cationic resin (Figure 9). When the fractions containing the purified 16 kDa protein were separated by SDS-PAGE and stained with Coomassie Blue, in addition to the major band corresponding to the recombinant fusion protein, several other minor bands were detected (Figure 10). On Western blotting, the purified recombinant fusion protein reacted with Goat 48 sera and the monoclonal antibody, T7 Mab, specific to the fusion part of the recombinant protein (Figure 11). The increase in the molecular size of the recombinant protein was because of the addition of a poly-histidine tag at the amino terminal by the vector sequences. Surprisingly, even normal

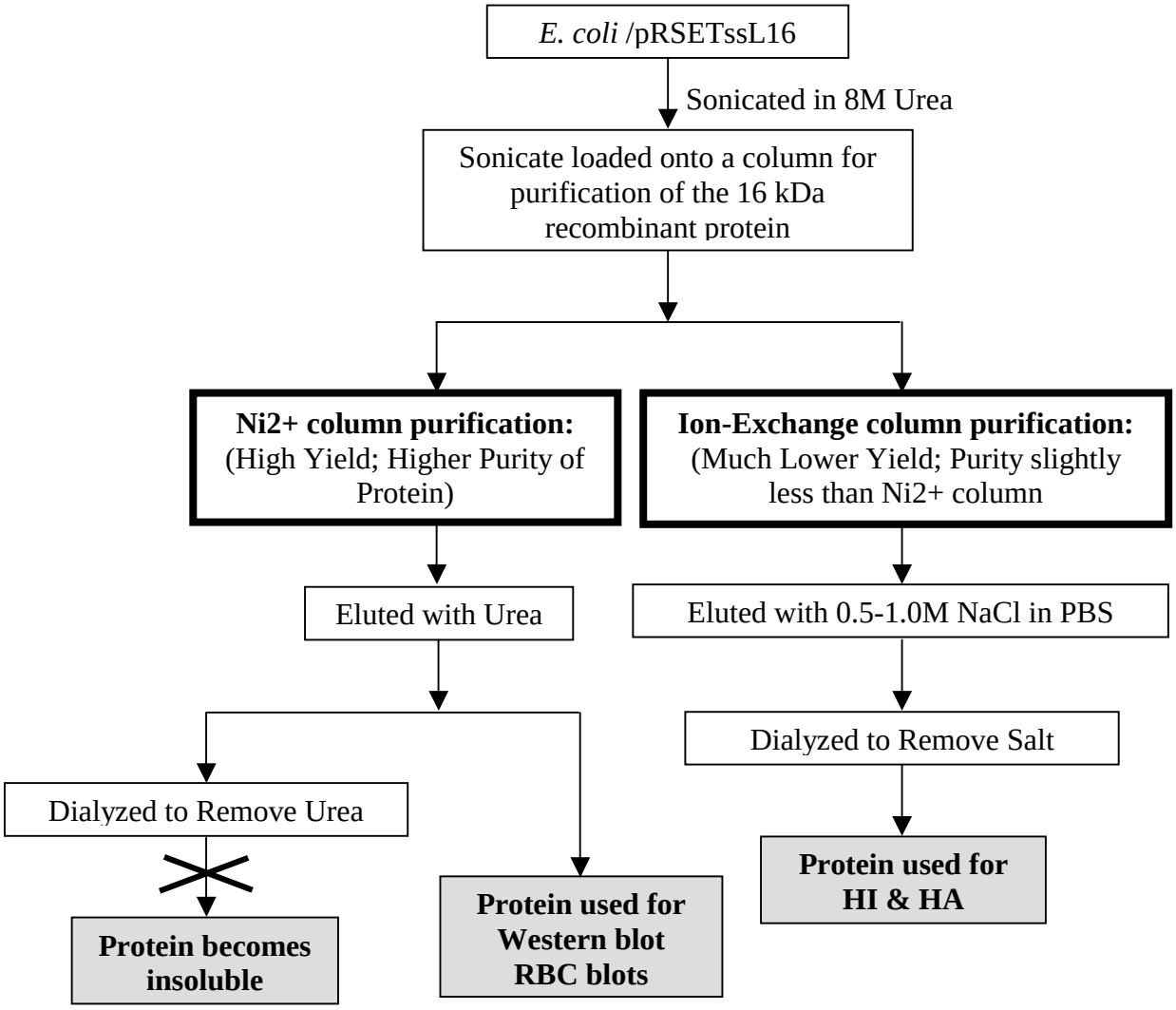

Figure 9. Flow chart depicting the strategy used in the purification of the recombinant fusion protein of the 16 kDa protein expressed in *E. coli*. Purification of the recombinant protein was achieved under denaturing conditions, either by Ni^{2+}-affinity chromatography or by Ion-Exchange column (bolded boxes). Use of protein derived from each method in subsequent experiments is outlined (shaded boxes).

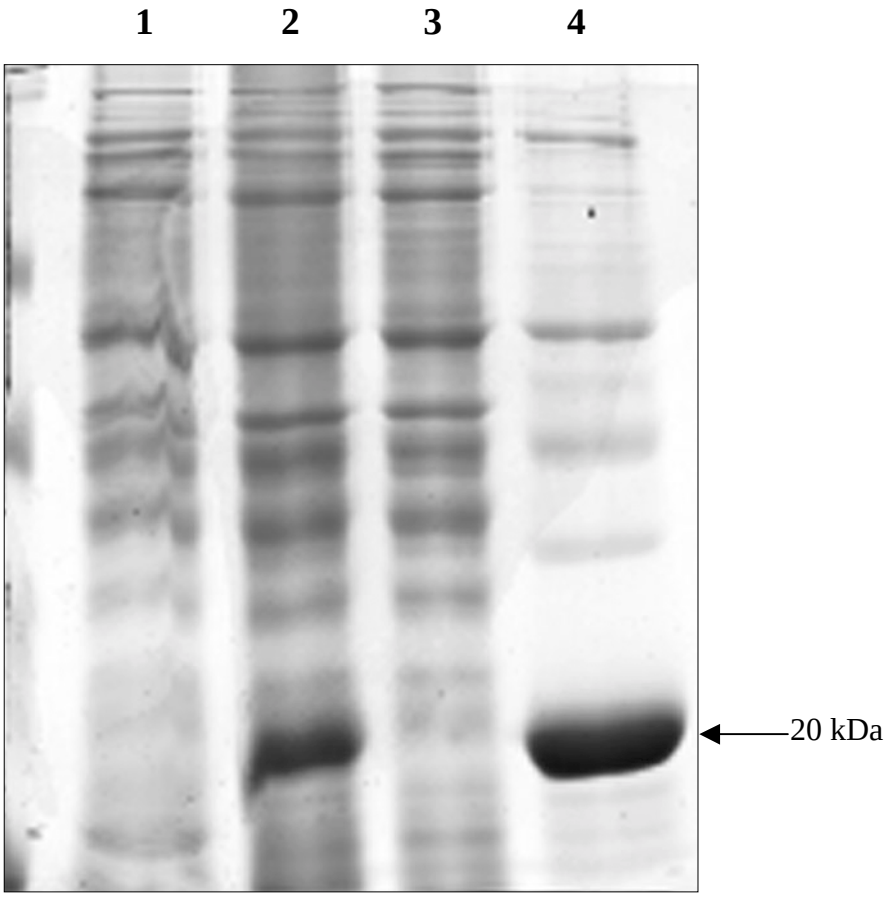

Figure 10: Coomassie Blue stained SDS-PAGE gel of the metal affinity purified recombinant fusion protein containing the 16 kDa protein. Lanes 1-4 contain samples of uninduced, induced, column unbound fraction, and the eluted fraction, respectively. The recombinant fusion protein of 20 kDa in size is indicated.

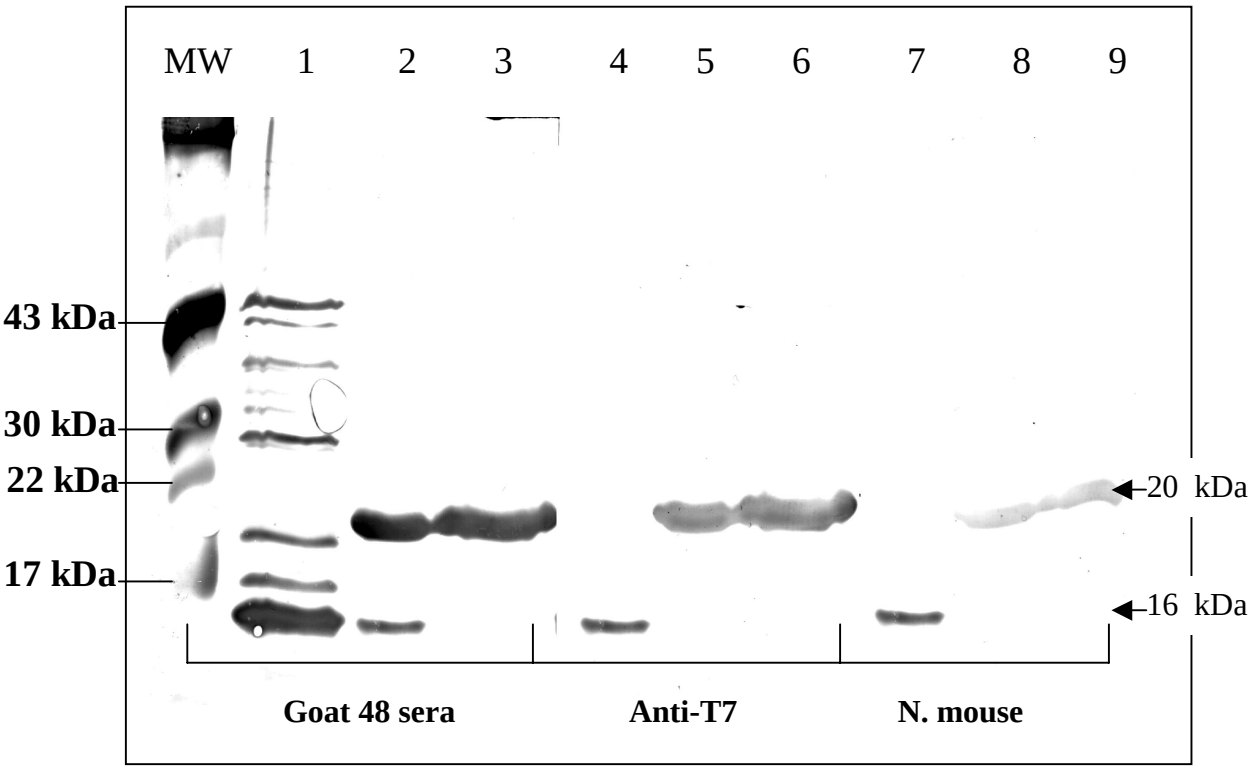

Figure 11: Western blot detection of the 16 kDa protein's reactivity with specific and non-specific immunoglobulins. Total antigen extract of RB51 overexpressing the 16 kDa protein under the *groE* (pBBGroE16) promoter (Lanes 1, 4, and 7) and the purified recombinant fusion protein (pRSETssL16) of the 16 kDa protein (Lanes 2,3,5,6,8, and 9) were reacted with either RB51 hyperimmunized goat sera (Goat 48 sera), monoclonal antibodies to the T7 tag of the *E. coli* fusion protein (Anti-T7), or to normal mouse sera; lanes are labeled accordingly. Lane MW contains molecular weight marker. The arrow indicating 20 kDa is the expected molecular weight of the fusion protein. Note: A band of the 16 kDa in size in lane 2 appears to be a result of non-specific binding and, therefore, considered to be an artifact.

				***P*-value: 2-tailed T-test**
Strain Used		**2308**	**2308/pBBGroE16**	N/A
Dose (Log_{10} CFU)		4.380	4.301	N/A
***Brucella* recovered Mean CFU/spleen (Log_{10})**	**1 day postinoculation (pi)**	3.939	4.270	0.503
	7 days pi	5.531	5.711	0.398
	42 days pi	5.733	5.711	0.763

Table 4. Persistence of the 2308 recombinant overexpressing the 16 kDa protein in mice. Two groups of 15 BALB/c mice each were inoculated IP with either wild-type 2308 or the recombinant overexpressing the 16 kDa protein (2308/pBBGroE16) at the dosages shown above. On day 1, 7, and 42, three mice per group were euthanized and the *Brucella* CFU/spleen (Log_{10}) were quantified. Statistical analysis used was the Student's t-test; *P*-values comparing the two groups for each of the days examined are given in the far right column (shaded).

mouse sera also reacted with the purified recombinant fusion protein (Figure 11: panel 3). In addition, the 16 kDa protein that was overexpressed in RB51/pBBGroE16 also reacted with T7 Mab and normal mouse sera. Based on these results, tests to determine the immunoglobulin-binding and lectin-like properties of the 16 kDa protein were undertaken (Section 4.6, below).

To obtain the 16 kDa protein in solutions devoid of denaturing agents, removal of urea from the fractions containing the affinity-purified recombinant protein was attempted. However, removal of urea below 2 M concentration resulted in the formation of insoluble aggregates of the protein. Nonetheless, initial attempts of extensive dialysis of the ion-exchange-purified recombinant protein against PBS yielded approximately 0.5 mg of the protein in soluble form. This fraction was used in hemagglutination (HA) and hemagglutination inhibition (HI) studies (see Section 4.6).

4.6. Lectin-like Properties of the 16 kDa Antigen.

Due to the apparent non-specific binding of the 16 kDa protein to mouse immunoglobulin G (IgG), the ability of this protein to bind IgG of other animal species was examined by Western blot analysis. The RB51/pBBGroE16 recombinant and the affinity-purified 16 kDa fusion protein were used for the Western blotting and were reacted with non-immune sera from a variety of species. These included: cattle, goat, rabbit, and chicken (Figure 12); mouse (Figure 11); rat and pig (data not shown). Appropriate secondary antibodies conjugated with horseradish-peroxidase (HRPO) were used to detect the bound IgGs. All the IgGs tested were able to bind to the 16 kDa protein. Interestingly, when reacted with secondary antibodies containing the HRPO, the 16 kDa protein failed to bind to these antibodies as determined by Western blot (data not shown). Since immunoglobulins are part of a larger superfamily of

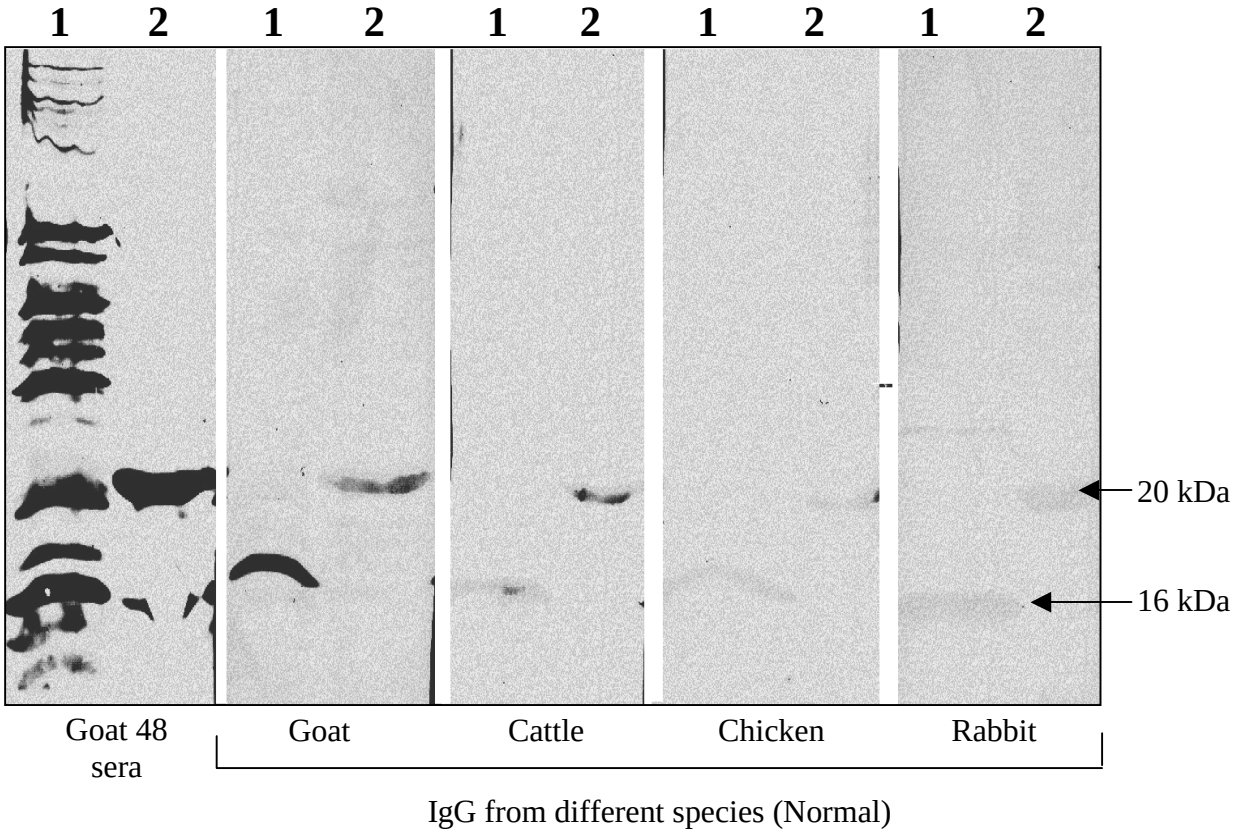

Figure 12: Western blot demonstrating the 16 kDa protein's reactivity to IgG from different species. Total antigen extracts of *B. abortus* RB51/pBBGroE16 (lane 1) and the fusion protein affinity purified from the recombinant *E. coli*/pRSetssL16 (lane 2)were reacted as indicated with either RB51 hyperimmunized goat sera (Goat 48 sera), or normal IgG immunoglobulins from goat, cow, chicken, or rabbit. The molecular sizes of the fusion (20 kDa) and non-fusion (16 kDa) proteins are indicated.

glycoproteins, the 16 kDa protein might actually bind, similar to lectin-like proteins, with some of the carbohydrate moieties of the IgGs.

To test this hypothesis, red blood cell (RBC) blotting was performed in which a 2% suspension of mouse erythrocytes was used to bind with the 16 kDa protein present in RB51/pBBGroE16 or with the affinity-purified recombinant fusion protein. Mouse RBCs bound to the 16 kDa protein as revealed by the presence of a distinct red-colored band corresponding to the appropriate molecular size (Figure 13). RBC blotting was also performed using erythrocytes from a variety of different species. The results are summarized in Table 5.

The lectin-like properties of the 16 kDa protein were further verified by hemagglutination assays (HA). Serial dilutions of the ion-exchange purified fusion protein were prepared in PBS and incubated at room temperature with a 2% suspension of mouse RBCs in a 1:1 ratio (protein:RBC suspension). Wells positive for hemagglutination showed lattice formation; negative wells showed button formation. The 16 kDa protein was able to hemagglutinate mouse RBCs at a protein concentration of 3.50 μg/mL (Figure 14: top panel).

To determine the specific carbohydrate(s) to which the 16 kDa protein was able to bind, hemagglutination inhibition (HI) assays were performed. Seven monosaccharides were chosen to perform the HI assays. The monosaccharides included: glucose, galactose, mannose, maltose, methyl-α-D-mannopyranosidase, D-galactosamine, and N-acetyl-D-glucosamine. These seven sugars were selected because of their presence in the carbohydrate chains present on the immunoglobulin Fc region. Only mannose, was able to cause HI at a minimum concentration of 62.5 mM (Figure 14: bottom panel).

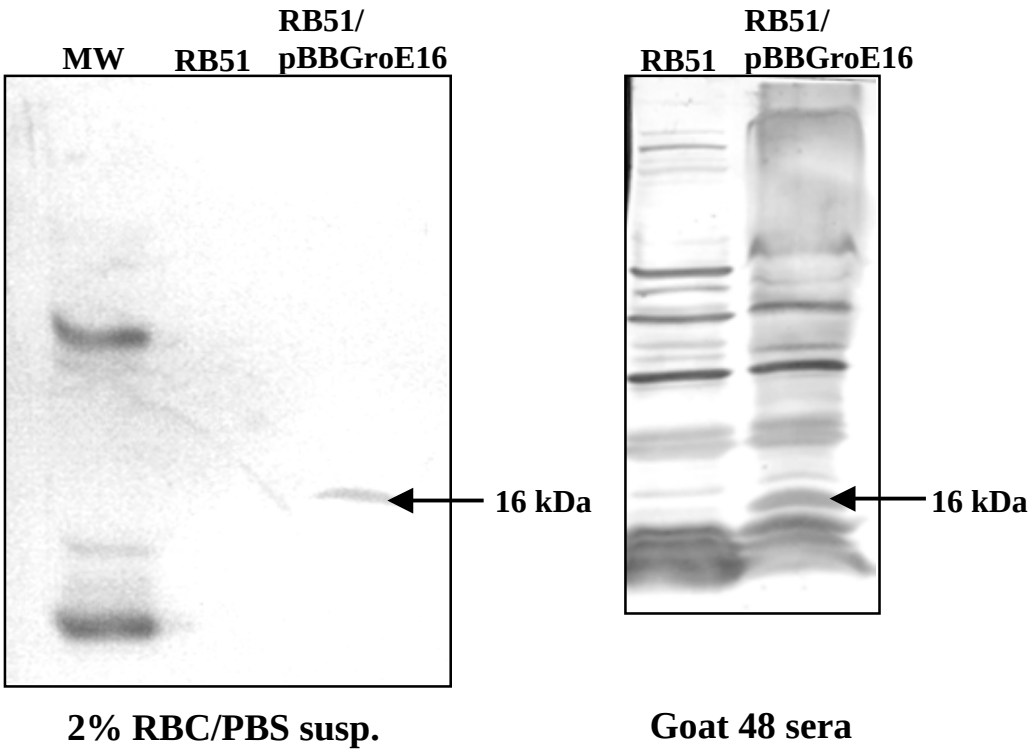

Figure 13. Preliminary evidence for the lectin-like property of the 16 kDa protein. The total antigen extracts of strain RB51 and RB51/pBBGroE16 were separated on a 15% SDS-PAGE gel, blotted onto a nitrocellulose membrane, and blocked with 2% BSA. The membrane shown on the left-side panel was then incubated with a 2% suspension of mouse erythrocytes as described in Section 3.16.1. The right-hand panel was analyzed via Western blot using Goat 48 sera as the primary antibody. In both panels, the band corresponding to the 16 kDa protein is indicated. Lane labeled MW in the left panel contains prestained protein molecular weight markers.

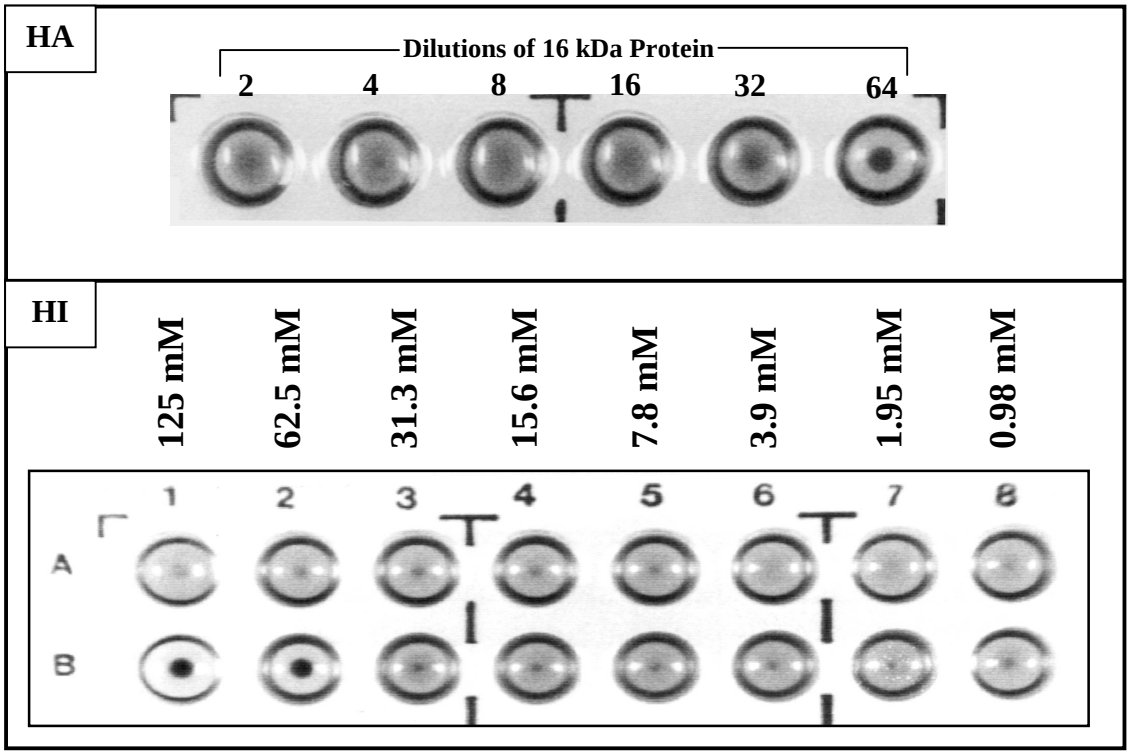

Figure 14. Hemagglutination (HA) and Hemagglutination Inhibition (HI) assays with the recombinant 16 kDa protein and mouse erythrocytes. Top Panel: Serial dilutions of the 16 kDa protein were incubated at room temperature with a 2% suspension of mouse red blood cells (RBC). Note the hemagglutination caused by the 16 kDa protein, as demonstrated by the lattice formation, up to a dilution of 1 in 32 (3.50 μg/mL final protein conc.). **Bottom Panel:** The 16 kDa protein at a concentration of 14 μg/mL, was preincubated with serial dilutions of either glucose (lane A) or mannose (lane B) and then 2% mouse RBC was added. Note the inhibition of hemagglutination, as demonstrated by button formation, by mannose – up to a concentration of 62.5 mM.

Animal Species	RBCs Binding to the 16 kDa Protein
Human	NO
Pig	NO
Goat	NO
Sheep	NO
Cow	NO
Horse	NO
Chicken	NO
Rabbit	NO
Gerbil	**YES**
Mouse (BALB/c)	**YES**

Table 5. The ability of the 16 kDa protein to bind erythrocytes from different animal species. RBC blots were performed identical to the blot shown in Figure 11. The ability of erythrocytes to bind to the 16 kDa protein was visually observed and noted by **YES** in **bold**; failure to visually confirm RBC binding is denoted by NO.

Further examination of the 16 kDa protein's ability to bind other sugars/carbohydrates could not be assessed due to problems with the purification of the 16 kDa protein under non-denaturing conditions (see Section 4.5 and Figure 8).

4.7. Protective Role of the 16 kDa Protein.

Two different studies in mice were performed to assess the role of the 16 kDa protein in protection against brucellosis. The first study evaluated if homologous overexpression of the 16 kDa protein in RB51 would result in enhanced protection against challenge infection, and the second study determined if a DNA vaccine prepared using the gene for the 16 kDa can induce protective immune response.

Two weeks after challenge, no significant difference was observed in the number of *B. abortus* 2308 CFUs in the spleens of mice that were vaccinated with either strains RB51, RB51/pBBR1MCS, or RB51pBB*groE*16 (Table 6). As expected, significant difference was observed in the splenic bacterial counts between the vaccinated and non-vaccinated (saline inoculated) mice; the non-vaccinated mice contained approximately 1.4-2.1 Log_{10} more CFUs in their spleens than the vaccinated ones. These results indicate that overexpression of the 16 kDa protein in strain RB51 did not enhance its protective ability.

Vaccination of mice with DNA of pSecTag16 did not induce any protection against infection with *B. abortus* 2308; the splenic bacterial counts in these mice were similar to those of pSecTag85 or saline inoculated ones (Table 7).

Mouse Groups	**Number mice/group (n)**	**Vaccine Dose Given (CFU/mouse)**	***Brucella* Recovered After Challenge - Mean CFU/Spleen (Log_{10})***
Saline (Control)	4	---[@]	6.236 ± 0.07584 *a***
RB51	5	3.5×10^8	4.091 ± 0.5173 *b*
RB51/pBBGroE16	5	4.5×10^8	4.842 ± 0.1356 *b*

[@] Inoculated with 0.5 mL saline only.

***** Each value is the mean of **n** observations ± standard error (S.E.)

** Means followed by the same letter are not significantly different at α=0.05 according to Tukey's HSD.

Table 6. Effect of overexpression of the 16 kDa protein in RB51 and protection against challenge. Two groups of BALB/c mice were inoculated with either RB51 or RB51 overexpressing the 16 kDa protein; a third group was inoculated with saline as a control. Mice were challenged 6 weeks postinoculation. Mice were euthanized at 2 weeks postchallenge and the CFU/spleen of challenge strain 2308 quantified. There was no significant difference in splenic counts of between mice inoculated with RB51/pBBGroE16 or RB51. Both groups (RB51 and RB51/pBBGroE16) had significantly lower CFU/spleen of challenge strain recovered than the saline control.

Mouse Groups	Number mice/group (n)	DNA Dose Given	*Brucella* Recovered After Challenge - Mean CFU/Spleen (Log_{10})*
Saline (Control)	4	---[@]	5.233 ± 0.06749 *a***
pSecTag16	5	100 μg X 2	5.459 ± 0.1068 *a*
PSecTag85	5	100 μg X 2	5.490 ± 0.1430 *a*

[@] Inoculated with 0.5 mL saline only.

***** Each value is the mean of **n** observations ± standard error (S.E.)

** Means followed by the same letter are not significantly different at α=0.05 according to Tukey's HSD.

Table 7. Inability of the DNA vaccine encoding the 16 kDa protein to protect against challenge. Two groups of mice were inoculated with either pSecTag16 (experimental group) or pSecTag85 (DNA/vector control group); a third group was inoculated with saline (inoculation control). Mice were challenged 6 weeks postinoculation. Mice were euthanized at 2 weeks postchallenge and the CFU/spleen of challenge strain 2308 quantified. There was no significant difference in splenic counts of between any of the three groups.

4.8. Construction and analysis of the 16 kDa gene disruption mutant of *B. abortus* strain 2308.

4.8.1. Construction of the 16 kDa gene disruption mutant.

Electroporation of *B. abortus* 2308 competent cells with the suicide vector pTAmp16::Cm^r resulted in the generation of several hundreds of chloramphenicol resistant colonies. Of these, 20 colonies were subsequently screened on TSA plates supplemented with ampicillin to insure that the mutants were obtained as a result of a double crossover event. Seven colonies were found to be both chloramphenicol resistant and ampicillin susceptible. Of these seven, 2 colonies (#13 and #14) were chosen for further screening. To insure that these 2 colonies represented true disruption mutants of the 16 kDa protein's gene, Southern blot analysis was performed on *Hind* III digested genomic DNA extracted from each of the colonies (Figure 15). The digoxinin-labeled probes prepared with the genes for either the 16 kDa protein or the chloramphenicol acetyl transferase were used independently to hybridize with the DNA fragments resulted from the restriction digestion. The probe specific to the gene for the 16 kDa protein hybridized with a ~4.5 kb fragment of *B. abortus* strain 2308 and a ~5.6 kb fragment of the two disruption mutants (2308Δ16) (Figure 15). The increase in size of the hybridized fragment of the disruption mutants is in accordance with the predicted size indicative of the insertion of the chloramphenicol resistance cassette within the 16 kDa gene. This was further confirmed by the hybridization of the chloramphenicol resistance gene specific probe with the 5.6 kb fragments of the disruption mutants only, but not with any fragments of the strain 2308.

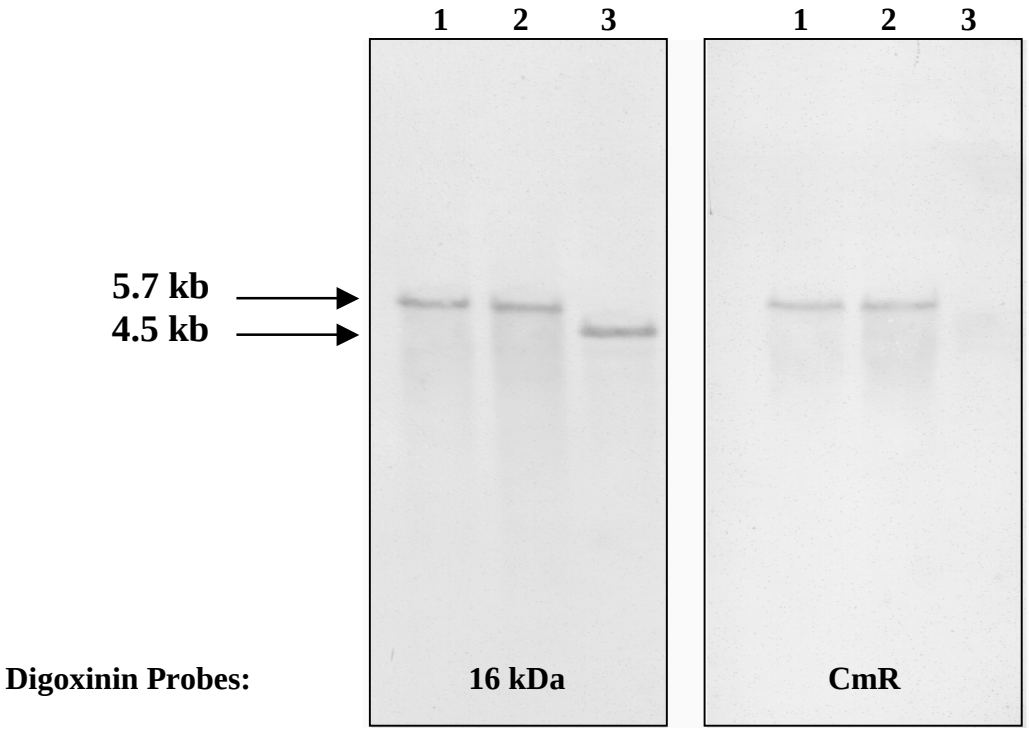

Figure 15. Confirmation of the creation of a disruption mutant in *B. abortus* strain 2308 by Southern blot analysis. Genomic DNA from parental strain 2308 (lane 3) and 2 clones (#13 and 14; lanes 1 and 2) of potential disruption mutants was digested with *Hind* III restriction endonuclease enzyme. The genomic digests were blotted and hybridized with either of 2 digoxinin-labeled probes: the 16 kDa gene (left panel) or the chloramphenicol resistance (CmR) gene (right panel). The approximate sizes of the DNA fragments hybridized with the probes are indicated at the left.

4.8.2. Phenotypic characterization of the disruption mutants.

Initial characterization of the disruption mutants consisted of examining their phenotypic characteristics. Gross visualization of crystal violet stained colonies indicated a rough-like phenotype. When the bacterial suspensions of the mutant strains were mixed with 0.1% acriflavin, autoagglutination, which is an indicator of a rough phenotype, was observed. However, the degree of autoagglutination appeared to be less than that of strain RB51, a known rough strain.

To further characterize the disruption mutants, total antigen extract of the mutants were analyzed by Western blotting with Bru38, a rat monoclonal antibody specific to *Brucella* O-antigen (Figure 16). Western blotting revealed that the disruption mutants contained far less O-antigen than their parent strain 2308. In addition, the O-antigen content of the mutants was relegated to the higher molecular weight region of the blot as compared to that of the strain 2308.

4.8.3. Persistence of the disruption mutant in the mouse model.

To aid in determining the 16 kDa protein's role in the virulence of *B. abortus*, the disruption mutants (2308Δ16) were also examined for their ability to persist in the spleens of BALB/c mice as compared to the parental strain 2308. In comparison with the mice inoculated with 2308, mice inoculated with the disruption mutant contained significantly lower numbers of deletion mutants in their spleens (CFU/spleen) at Day 1, 7, and 14 (Table 8). All bacterial colonies isolated from the mice inoculated with the disruption mutant were tested for their resistance to chloramphenicol by plating on TSA plates supplemented with 30 μg/mL Cm; all colonies were found to be chloramphenicol resistant (Cm^r). To verify these Cm^r colonies

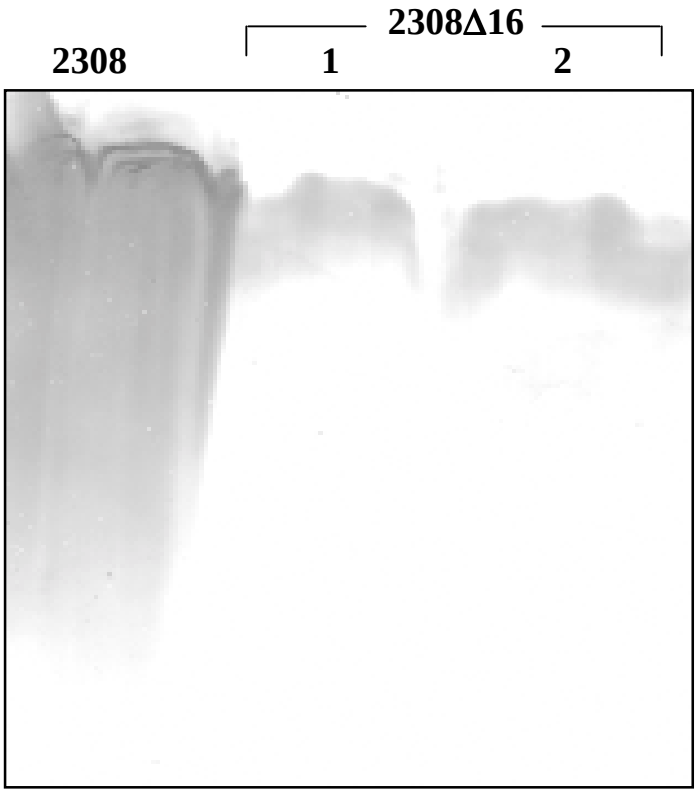

Figure 16. Western blot analysis of the *B. abortus* 2308Δ16 disruption mutant using *Brucella* O-antigen specific monoclonal antibody. Total antigen extract was obtained from *B. abortus* strain 2308 (lane 2308) as well as two clones of the disruption mutant (lanes 2308Δ16: 1 and 2). The antigen extract was separated on a 15% SDS-PAGE and Western blotting was performed. Amount of antigen extract loaded onto gel was standardized by adjusting to a transmittance of 10% (525 nm). Bru38, a rat monoclonal antibody (Mab) against *Brucella* O-antigen was used as the primary antibody. Note: the bacterial suspensions were first adjusted to 10% transmittance at 525 nm, and identical volumes were then used to prepare the antigen extracts.

				***P*-value: 2-tailed T-test**
Strain Used		**2308**	**2308Δ16**	N/A
Dose (Log_{10} CFU)		4.602	4.544	N/A
***Brucella* recovered Mean CFU/spleen (Log_{10})**	**1 day postinoculation (pi)**	4.074	2.138	3.788×10^{-4}*
	7 days pi	5.934	2.564	3.005×10^{-4}*
	14 days pi	5.750	3.478	2.164×10^{-6}*

Table 8. Persistence of the disruption mutant, 2308Δ16, in mice. Two groups of 15 BALB/c mice each were inoculated IP with either wild-type 2308 or the disruption mutant (2308Δ16) at the dosages shown above. On day 1, 7, and 14, five mice per group were euthanized and the *Brucella* CFU/spleen (Log_{10}) were quantified. Mice inoculated with 2308Δ16 had lower CFU/spleen of *Brucella* recovered than mice inoculated with 2308 on days 1, 7, and 14. Statistical analysis used was the Student's t-test. *P*-values comparing the two groups for each of the days examined are given in the far right column (shaded). Significant *P*-values are denoted by an asterisk (*).

represented the disruption mutant, genomic DNA from 10 colonies selected at random (Day 1 and 7 only) was extracted and Southern blot analysis was performed using the digoxinin labeled probe of the 16 kDa gene. In all 10 samples, the probe hybridized with a 5.6 kb fragment indicating the presence of the disrupted 16 kDa gene. In addition, all of the isolated disruption mutant colonies recovered from the mouse spleens were found to exhibit a rough-like phenotype according to autoagglutination results using 0.1% acriflavin. (See also Section 4.4.: Overexpression of the 16 kDa gene within B. abortus strain 2308 and its Persistence using the mouse model).

5. DISCUSSION

In spite of the veterinary and public health importance of *Brucella* infections, our knowledge of the virulence mechanisms employed by this bacterium is limited. Currently, only two *Brucella abortus* proteins are recognized as being protective: superoxide dismutase (SOD) and ribosomal protein L7/L12. The significance of host immune responses to specific *Brucella* antigens in protection against challenge or reinfection is poorly understood. One way of gaining insight in these areas is by identifying and characterizing the functional and immunological properties of crucial proteins of the bacterium. Utilizing recombinant DNA technology and molecular technology, studies presented in this thesis examined the 16 kDa protein's role in *B. abortus* virulence and host's immune responses.

Initially, interest in this *B. abortus* protein stemmed from the work performed by Chirhart-Gilleland (43). Chirhart-Gilleland and colleagues characterized a 14 kDa-LacZ fusion protein and identified that the protein was reactive with T lymphocytes and antibodies of both experimentally and naturally infected hosts. Nucleotide sequence analysis performed by Chirhart-Gilleland (1998) revealed that the isolated clone contained only the 3'-(carboxy) end of a *B. abortus* gene. In order to further characterize this protein, first, the missing 5'- end of the gene was cloned using a PCR-based strategy. The nucleotide sequence of this region was identical to the one concurrently reported by Chirhart-Gilleland et.al. (43), who independently cloned this region by a conventional method of screening a plasmid library containing the *B. abortus* genomic DNA fragments of specific size. Several features identified in the nucleotide and deduced amino acid sequences such as the ribosomal binding site (RBS), start codon, length of the open reading frame, amino terminal signal sequence, and the hydropathic profile were also identical to the ones concurrently reported by Chirhart-Gilleland et. al. (43). However, Chirhart-

Gilleland et. al. (1998) proposed that this gene could be a part of an operon since their computer analysis did not identify any potential promoter elements in the nucleotide sequence 5' to the RBS. In contrast, the algorithm used in this study identified a potential prokaryotic promoter sequence in the upstream region (Figure 5). Overexpression of the 16 kDa protein in RB51 harboring pBBup16, but not pBBRBS16 (Figure 1), suggests that the identified promoter sequence is functional in *B. abortus*. Nonetheless, additional studies need to be performed to further confirm the functionality of the promoter. Such studies could include the creation of a transcriptional fusion of the promoter to a reporter protein's gene (e.g. β-galactosidase). Thus the effect of specific mutations in the promoter sequence on the expression level of the reporter protein could be examined, and primer extension analysis used to identify the 5'- start of the mRNA.

Southern blot and PCR analyses (Section 4.2; Figure 7) indicate that the gene for the 16 kDa protein is present in all the *Brucella* species tested. The conserved nature of this gene suggests that the 16 kDa protein may play an important role in the biology of *Brucella*. However, the mere presence of the gene sequence does not confirm the expression of the 16 kDa protein in those *Brucella* species. I did not attempt to demonstrate the expression of the 16 kDa protein in all the *Brucella* species. As discussed in the following paragraph, the expression of this protein in strains RB51 and 2308 appeared to be very low using Western blot analysis.

In addition to the 16 kDa promoter, the *groE* and *sodC*, promoters of *Brucella* were also used to achieve overexpression of the 16 kDa protein in strain RB51. Of these three promoters, the *groE* promoter appeared to cause the highest level of expression of the 16 kDa protein. This observation corroborates a previous finding that the *groE* promoter activity drives expression of β-galactosidase in strain RB51 is higher than the *sodC* promoter (243). Comparison of the

Western blot profiles of strain RB51 and its overexpressing recombinant strains (Figure 8) indicates that the level of expression of the 16 kDa protein in strain RB51 is low. A similar observation was made with strain 2308; overexpression was needed to demonstrate clearly the presence of the 16 kDa protein. The low expression of the 16 kDa protein could not be solely because of the single copy of the gene present on the chromosomal DNA, since higher levels of expression of other proteins from single copy genes have been observed in *Brucella* (30, 139-141, 193, 223, 228, 233, 234). It is possible that the 16 kDa protein's gene promoter is inherently weak leading to less expression of the protein. It is also possible that the expression of the 16 kDa protein in *Brucella* is regulated so that only minimal expression is seen when the bacteria are grown under *in vitro* conditions. Despite the observed low expression, the 16 kDa protein has been demonstrated to evoke both antibody and CMI responses in vaccinated and infected hosts (43). Similar observation was also reported for the YajC protein of *Brucella*; while undetectable in strain RB51, vaccinated mice developed specific immune responses to the protein (244).

The role of the 16 kDa protein in eliciting a protective immune response against challenge with *B. abortus* 2308 was examined in mice by DNA immunization. A major advantage of DNA immunization is the ability to induce a strong CMI response (67), which is crucial for protection against intracellular pathogens including *Brucella*. Kurar and Splitter (1997) demonstrated that immunization of mice with DNA encoding the L7/L12 protein of *B. abortus*, a known protective antigen, resulted in the elicitation of protective immunity against challenge. This provides a strong justification for using genetic immunization strategy to test the protective role of a *Brucella* protein. However, injection of mice with the plasmid DNA encoding the 16 kDa protein did not induce any level of protection against the challenge with

strain 2308 (Table 7). In addition, serum was taken from the vaccinated mice in order to assess the humoral immune response. Given the ability of the 16 kDa protein to bind immunoglobulins this aspect of the immune response could not be investigated. It will be interesting to know if this protein induces a significant humoral and/or CMI immune response. CMI response to the 16 kDa protein was not considered under the scope of this thesis, however, CMI studies will be pursued in subsequent studies.

The lack of protection via DNA immunization could be a result of several factors, including insufficient protein expression leading to inadequate immune response, or inappropriate post-translational modifications leading to an altered antigenicity of the protein. There is evidence that antigens known to be protective when administered in the form of a conventional live, attenuated vaccine fail to provide protection when administered via DNA immunization. For example, DNA immunization of mice with the gene encoding the Cu-Zn SOD protein of *B. abortus* failed to provide protection against challenge with *B. abortus* strain 2308 (unpublished data). Although no attempts were made to examine these possibilities, the protective role of the 16 kDa protein was tested further using another strategy: inoculation with a recombinant strain RB51 overexpressing the 16 kDa protein.

Vemulapalli et al. (1998) demonstrated that overexpression of SOD, a protective antigen, in strain RB51 enhances its vaccine efficacy against the challenge with strain 2308 (243). Other investigators have noted similar observations. For example, increased expression of the Protective Antigen (PA) in the attenuated strains of *Bacillus anthracis* enhanced their ability to evoke protective immune responses in the vaccinated animals against anthrax (21). These studies suggest that overexpression of a protective antigen in a vaccine strain could enhance its vaccine efficacy. Based on this rationale, the protective efficacy of strain RB51 overexpressing

the 16 kDa protein should be enhanced if this protein were a protective antigen. Mice vaccinated with the overexpressing strain RB51 were protected to the same extent as that of the strain RB51 vaccinated ones (Table 6), indicating that the overexpression of the 16 kDa protein did not enhance the vaccine efficacy of strain RB51. Immune responses (both CMI and humoral) to the 16 kDa protein were not evaluated for the reasons given previously (see discussion on DNA vaccine, above).

Taken together, it appears that the 16 kDa protein plays a minimal (if any) role in protective immunity against *B.abortus* infections. Observations documented in the literature indicate that some *Brucella* proteins to which infected or vaccinated animals develop humoral and CMI responses do not appear to play an important role in the protective immunity (100, 140, 242). However, with regard to the 16 kDa protein, it should be emphasized that further studies are needed to completely rule out the protective potential of this protein, because it is possible that simply overproducing the protein without regard for the level of expression may not lead to enhanced protection. In the protection study, the recombinant RB51strain expressing the 16 kDa protein expressed under the strong *groE* promoter was used for the vaccination. However, it may be necessary to adjust the level and the time of protein production by strain RB51 through the use of a weaker or inducible promoter or by changing the vaccine dose. Further study of this protein's role in protective was not pursued because of the discovery of the immunoglobulin-binding and lectin-like properties of the protein.

In pursuing the study of the immunoglobulin-binding and lectin-like properties of the 16 kDa protein, it was necessary to obtained purified protein in native buffer. The 16 kDa protein expressed in *E. coli* with a His-tag fusion could only be solubilized in buffers containing denaturing agents such as urea. Removal of urea from the fractions containing the affinity

purified fusion protein by dialyzing against PBS resulted in the precipitation of the protein. Several conditions such as gradual removal of urea, addition of non-ionic detergents or glycerol to the exchanging buffer did not prevent the formation of insoluble aggregates. Similar problems have been reported for other proteins that were over produced as His-tag fusions in *E. coli* (52, 109). However, partial purification by ion-exchange chromatography yielded sufficient protein in PBS for use in the HA and HI assays. No further attempts were made to obtain more of the fusion protein in native buffers such as PBS.

Western blot analysis with serum or immunoglobulins from naïve animals indicated that the 16 kDa protein could bind with non-specific IgG of several animal species (Figure 12). This can be interpreted as an immunoglobulin binding property of this protein. However, the ability of the 16 kDa protein to interact with and agglutinate erythrocytes in RBC blots and HA tests, respectively, suggests that this protein actually could be a lectin-like protein. Moreover, its interaction with IgG could be through its affinity towards the carbohydrate moieties present toward the Fc region of the antibody molecules; although it is possible that the 16 kDa could be interacting with IgG molecule in a non-lectin like fashion. In such a case, the 16 kDa protein could be having both lectin-like and immunoglobulin binding properties.

Of the 7 sugars tested in HI experiments, only mannose inhibited the agglutination reaction, suggesting the 16 kDa protein can bind with this sugar *in vivo* (Figure 14). However, further studies with other monosaccharides, disaccharides, and other carbohydrate moieties have to be performed to determine which sugar molecules exhibit high affinity towards the 16 kDa protein. Although the 16 kDa protein is the first *Brucella* protein to be identified to contain immunoglobulin binding and lectin-like properties, proteins with such properties have been well documented in other bacteria. Immunoglobulin-binding proteins (IBP) are associated with the

cell surface of many different microorganisms including both Gram negative and Gram positive organisms. Examples include staphylococci (87), streptococci (187), peptostreptococci (125), and *Pseudomonas* species (99). IBPs most often bind to the Fc-portion of the immunoglobulin molecule (4, 78, 97, 99, 160, 188, 189, 217), although they can also bind to the Fab fragments as well (72, 73, 78, 80-82, 112, 202). The most highly studied IBPs include: Staphylococcal protein A, Streptococcal protein G, protein H (*Streptococcus pyogenes*), and protein L (*Peptostreptococcus magnus*) (162). The binding of immunoglobulins via their Fc region may help these pathogenic bacteria survive inside the host by two methods. First, binding of the IgG Fc region by IBPs may allow for coating of the bacterial surface, thereby leaving no Fc facing outward to be recognized by Fc receptors of host leukocytes (87). Second, this same coating may also mask binding sites available for opsonins that may be present in normal serum (87). In this way, IBPs act as a cloaking device with respect to the host immune system in pathogenic bacteria that possess them.

The interaction of a lectin may be with a single sugar or chains of sugars, or they may be associated with sugars found in the context of glycolipids and glycoproteins. Lectin-like proteins have been associated with several bacterial species. These proteins are often referred to as "bacterial lectins" in order that they may be distinguished from those associated with plants. Bacterial lectins are often associated with host-bacterial cell-cell interactions. This has important implications for both pathogenic and non-pathogenic bacteria and their interactions with the host. A number of bacterial lectins have been described in association with pathogenic bacteria and some of their functions as relates to pathogenesis have been elucidated (106, 156, 195, 227, 240). Most of the research thus far has uncovered the possible role of bacterial lectins in the adhesion of various pathogenic species to their respective hosts. In these various studies, it was found that

a number of these bacterial lectins were associated with bacterial pili (106, 195). These specific pili-associated bacterial lectins have been found to be responsible for receptor recognition and therefore embody a vital role in pili attachment and colonization (195). As proof of this, the isolation of bacterial lectin-deficient mutants have been shown to produce fimbriae that lack their characteristic binding properties (195). In one instance, it has been demonstrated that a bacterial lectin (Gaf D) is not only necessary for fimbrial receptor recognition, but also for fimbrial biogenesis itself (195). Because of their role in adhesion, bacterial lectins have been suggested as viable targets to be exploited in the development of vaccines (195). Antibodies generated and directed against the fimbrial adhesin by such a vaccine would have a direct effect in blocking bacterial adherence and colonization of the host. It is possible that the 16 kDa protein could play a role in *Brucella* adhesion and pathogenesis.

Overexpression of the 16 kDa protein in strain 2308 did not alter its virulence in the mouse model (Table 4). However, disruption of the protein's gene in strain 2308 reduced its ability to colonize the mouse spleens at all time points tested (Table 8). This effect of the gene disruption can be directly attributed to the rough-like phenotypic characteristic of the mutant strain 2308Δ16 (Section 4.8.2; Figure 16). In *B. abortus*, it is a well-known fact that the rough mutants are less virulent than their parent smooth strains (5, 155, 205, 236, 252). Based on the Western blot analysis, it appears that the smooth LPS of strain 2308Δ16 is aberrant in its O antigen profile. From the studies presented in this thesis, it is difficult to predict the actual role of the 16 kDa protein in the biogenesis of smooth LPS of *B.abortus*. However, mannose is known to be the major constituent of O-antigen in *Brucella*. If the 16 kDa protein can bind to O-antigen, it suggests that the protein may be involved in the assembly, transport, or attachment of O-antigen to *Brucella* LPS.

The apparent attenuation seen *in vivo* may be a result of the rough phenotype or a direct effect of the 16 kDa protein. In order to differentiate between the two, a disruption mutant of RB51 needs to be obtained. Several attempts have been made to obtain such a mutant, but have been unsuccessful. Although an extensive effort at the creation of a mutant has not been performed, it is possible that deleting/disrupting this gene within RB51 would result in a lethal mutation. In addition, complementation of the mutant strain with a functional gene of the 16 kDa protein should be performed to determine if the phenotypic and attenuation characteristics observed in the mutant strain are in fact because of the lack of the 16 kDa protein, and not due to a polar effect on the downstream gene(s). Without further studies, any attempt to assign a function to this protein is highly speculative at this point.

In conclusion, studies presented in this thesis indicated for the first time that the 16 kDa protein of *B. abortus* contains lectin-like and/or immunoglobulin binding properties. Also, preliminary evidence was obtained for the presence of a promoter element for the gene of the 16 kDa protein. Although the 16 kDa protein appears not to be involved in stimulating a protective immune response, it seems to be important for the virulence of *B. abortus*, at least in a mouse model. Further studies to understand in detail the functional role of the 16 kDa protein in *Brucella* pathogenesis will better our understanding of the strategies used by these important bacteria to inflict chronic diseases in animals including humans. Such knowledge will provide us with important clues to develop efficient vaccines and therapeutics against brucellosis.

APPENDIX 1: Media Used in Growth of Cultures

Agar Plates: Agar plates were made using any of the broth recipes below and adding an additional 15 g/L Bacto™ Agar.

Luria-Bertani Broth (LB): Per liter, to 950 mL of deionized, distilled water add: 10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl. Shake until solutes dissolve. Adjust pH to 7.0. Bring total volume of broth to 1 L. Sterilize by autoclaving.

SOC Media: Per liter, to 950 mL of deionized, distilled water add: 20 g bacto-tryptone, 5 g bacto-yeast extract, and 0.5 g NaCl. Shake until solutes dissolve. Add 10 mL of a 250 mM solution of KCl. Adjust pH to 7.0. Bring total volume of broth to 1 L. Sterilize by autoclaving. Just prior to use, add 5 mL of a sterile solution of 2M $MgCl_2$ and 20 mL of a sterile 1M solution of glucose.

Tryptic Soy Broth (TSB): Add 30 g of TSB pre-mixed powder to 800 mL of deionized, distilled water. Adjust pH to 7.0. Bring total volume of broth to 1 L. Sterilize by autoclaving.

Yeast-Tryptone Broth (2X YT): Per liter, to 900 mL of deionized, distilled water add: 16 g bacto-tryptone, 10 g bacto-yeast extract, and 5 g NaCl. Shake until solutes dissolve. Adjust pH to 7.0. Bring total volume of broth to 1 L. Sterilize by autoclaving.

APPENDIX 2: Contents of Chemical Solutions

New Wash Solution (for gel extraction of dsDNA) (5X stock): 50 mM Tris (pH 7.5), 500 mM NaCl, 5 mM EDTA. To use, take 20 mL of the 5X stock and add 50 mL ethanol (95-100%) and 30 mL of deionized, distilled water.

Phosphate-buffered Saline (PBS): To 800 mL of deionized, distilled water, add 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4. Adjust pH to 7.4 and bring the total volume to 1 L. Sterilize by autoclaving.

SDS-PAGE Electrophoresis Buffer (5X): To 900 mL of deionized, distilled water, add 15.1 g Tris Base, 94 g glycine, and 50 mL of a 10% (w/v) stock solution of electrophoresis grade SDS. Adjust the total volume to 1 L.

Silica suspension (100mg/mL; for gel extraction of dsDNA): Mix 10 g silica (Sigma; S-5631) in 100 mL PBS and allow silica to settle for 2 hours. Remove supernatant containing the fine particulate matter and repeat the procedure. Centrifuge at 2000 g X 2 min, remove supernatant, and resuspend silica in 3M NaI at 100 mg/mL. Store in the dark at 4°C.

Tris-buffered Saline (TBS): To 800 mL of deionized, distilled water, add 8 g NaCl, 0.2 g KCl, and 3 g Tris base. Adjust pH to 7.4 and bring the total volume to 1 L. Sterilize by autoclaving.

Tris-EDTA (TE): 100 mM Tris-HCl, 1 mM EDTA; pH 8.0

Western Blotting Substrate: To 5 mL of methanol (CH_30H), add 30 mg of 4-chloro-1-napthol. To the methanol mixture, add 45 mL TBS followed by 300 μL hydrogen peroxide (H_20_2). Use within a few hours.

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Vemulapalli, T. H., R. Vemulapalli, G. G. Schurig, S. M. Boyle, and N. Sriranganathan. Overexpression of an immunogenic protein of *Brucella abortus* in strain RB51. 51st Annual Brucellosis Research Conference, Chicago, IL, Nov. 1998.

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