# UTILIZATION OF THE PERSISTENT NATURE OF *BRUCELLA* IN THE DEVELOPMENT OF LIVE VACCINES

A Dissertation

by

PRISCILLA CHRISTINE HONG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Veterinary Microbiology

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# ABSTRACT

Utilization of the Persistent Nature of *Brucella* in the Development of Live Vaccines.

(August 2005)

Priscilla Christine Hong, B.S., University of San Francisco

Chair of Advisory Committee: Dr. Thomas A. Ficht

The roles of genes responsible for the survival and persistence of *Brucella* in the host and the relationship between these genes and the disease were investigated via signature-tagged transposon mutagenesis. As much as 8% of the *Brucella* genome is important for survival of this organism in the host. This is an unusually high number and may help to explain the chronic or persistent nature of *Brucella* infections. Mutants attenuated in the mouse model were divided into two groups. The early mutants failed to establish infection or colonize the host. The late mutants colonized the host but failed to maintain infection. The vaccine potential of two mutants (*virB10* and *gcvH*) that were unable to sustain infection was compared to that of a vaccine strain, S19. Survival of strain S19 *in vivo* was up to 12 weeks while *virB10* and *gcvH* mutants were cleared from spleen at 8, and 24 weeks post-inoculation, respectively. Mice were vaccinated with individual mutants and then challenged with virulent S2308 at 8, 16, and 24 weeks post-vaccination. As a result, protective immunity correlated with persistence of the mutant strain [*gcvH>virB10*].

These results suggest that survival is one of several factors that may influence protective immunity making it difficult to compare strains. For example, examination of host immune response revealed a similar pattern of host immune function ($T_H1$ over $T_H2$) in all mice except those vaccinated with *virB10* mutant. Since *gcvH* mutant provided the best immunity, experiments were designed to explore its contribution of persistence to protection. In an effort to reduce non-specific activation induced by prolonged survival of *gcvH* mutant, protection was monitored after different periods of vaccination exposure followed with doxycycline treatment. In these studies, persistence of *gcvH* mutant enhanced protection against challenge. Overall, defined mutations in genes affecting survival may render mutants as vaccine candidates capable of stimulating protective immunity equal to or better than fortuitously isolated attenuated strains. Future studies should focus on characterization of these and other genes responsible for the persistence of *Brucella* to improve the safety and efficacy of live vaccines.

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# CHAPTER I
# INTRODUCTION

## Background

***Brucella* spp.** Brucellae are small, non-motile, anaerobic, non-fermenting, Gram-negative bacteria. They are pathogenic for many mammalian species and for humans, producing chronic infections with persistent or recurrent bacteremia (121), (120). Six species are recognized within the genus: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*. This classification is mainly based on the difference in pathogenicity and in host preference (33). *Brucella* species and their different biotypes are currently distinguished by differential tests based on sero-typing, phage typing, dye sensitivity, $CO_2$ requirement, $H_2S$ production, and metabolic properties. *B. abortus*, *B. melitensis*, *B. neotomae* or *B. suis* strains may occur as either smooth or rough strains expressing smooth-lipopolysaccharide (S-LPS) or rough-lipopolysaccharide (R-LPS) as major surface antigen. *B. ovis* and *B. canis*, on the other hand, are two naturally rough species, expressing R-LPS as major surface antigen. They are responsible for ram epididymitis and canine brucellosis, respectively. *B. canis* is occasionally involved in human brucellosis. Other strains, hitherto unclassified, have been isolated from marine mammals. The main pathogenic species worldwide are *B. abortus*, responsible for bovine brucellosis; *B. melitensis*, the main etiological agent of ovine and caprine

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This dissertation follows the style and format of Infection and Immunity.

brucellosis; and *B. suis* responsible for swine brucellosis. These three *Brucella* species usually cause abortion in their natural hosts, resulting in huge economic losses.

*B. abortus* and *B. melitensis*, the causative agents of epidemic bovine abortion and Malta fever respectively, are the most extensively studied *Brucella* organisms. These bacteria commonly gain entrance via the conjunctivae, the alimentary tract, the respiratory route, and broken skin. At the site of uptake in the skin or mucous membranes, the organisms are engulfed by phagocytes, multiply and persist within membrane-bound compartments, and are carried via the lymphatic system to the regional lymph nodes. In pregnant animals, brucellae reach the gravid uterus, penetrating the trophoblasts where they proliferate extensively, resulting in abortion.

Members of the genus *Brucella* have not been described to harbor plasmids or produce capsule or exotoxins. Most of them have two chromosomes of 2.1 and 1.5 Mb, respectively; and both replicons encode essential metabolic and replicative functions and hence are chromosomes and not plasmids (97). Although infection may progress through cycles, i.e. undulant fever, the only demonstrated antigenic variation is the transition of smooth to rough phenotype (5), (111), (84).

The disease in humans is characterized by a multitude of somatic complaints, including fever, sweats, anorexia, fatigue, malaise, weight loss, and depression. Localized complications may involve the cardiovascular, gastrointestinal, genitourinary, hepatobiliary, osteoarticular, pulmonary and nervous systems. Without adequate and prompt antibiotic treatment, some patients develop a “chronic” brucellosis syndrome with many features of the “chronic fatigue” syndrome (143).

*Brucella* species are classified as facultative intracellular pathogens that can infect non-phagocytic and phagocytic cells. *B. abortus* inhibits degranulation and the oxidative burst in neutrophils (13), (85). In macrophages, *B. abortus* survives by preventing phagolysosomal fusion (18), (41), (65), (107). Inhibition of phagolysosomal maturation is also evident in HeLa cells infected with virulent *B. abortus* S2308 (108). Initially, both virulent S2308 and attenuated S19 are located transiently in autophagosomes associated with early endosomal compartments but bypass interactions with late endocytic compartments. However, at later times of the infection, the virulent S2308 is targeted to compartments of ER where massive intracellular multiplication occurs while the non-pathogenic S19 undergoes degradation after fusion of its autophagic vacuole with lysosomes. Some brucellae may be found in autophagosomes of professional phagocytic cells. However, the majority of bacteria remain in the phagosome. Eventually brucellae are found to reside in phagolysosomes and appear to be resistant to host killing mechanisms (6). To cause chronic infection, *B. abortus* relies on the intracellular survival within phagocytic cells, yet the mechanisms and virulence factors responsible for the ability of brucellae to evade antibacterial activities of the host phagocyte remain to be discovered.

Chronic infection is characterized by continued persistence within host macrophages. In the primary host, i.e., ruminants, persistence of brucellae ultimately leads to acute infection of the pregnant host resulting in abortion of the fetus. In secondary hosts such as humans, mice, or even non-pregnant ruminants including males, chronic infection alone is observed. Males do not ordinarily suffer from prolonged

illness, although orchitis may be observed which is most frequently caused by *B. ovis* infection in sheep. Since chronic infection is the key to developing clinical brucellosis, we have selected a murine model to identify *B. abortus* genes that are essential for establishing and maintaining chronic infection. The murine model has proven to be an excellent predictor of virulence in the primary host (36) (48), (67). In this animal model, we expect the attenuation of *Brucella* to serve as a predictor of virulence in both bovine and human hosts. Understanding the mechanisms of microbial persistence of *B. abortus* can also serve as a model system in studying chronic infectious diseases caused by pathogens that are genetically difficult to manipulate.

**Study I**

**Genes required for chronic persistence of *B. abortus* in mice.** The first study in this manuscript described the identification of genes in *B. abortus* necessary for the establishment and maintenance of chronic infection in the murine model. Signature-tagged mutagenesis (STM), a novel technique developed to circumvent individual and labor-intensive analysis of mutants for loss-of-function phenotype or attenuation of virulence was employed (66). This negative selection method allows a collective screening of pools of mutants simultaneously. The technique is based on the fact that DNA tags inserted within the miniTn5 system can be identified by PCR and colony blot or dot blot hybridization. The absence of tags in the recovered pools (output) compared to those present in the inoculum (input) pool is an indication of attenuation in virulence of the mutants in the host. Signature-tagged mutagenesis can therefore be used to

identify new bacterial virulence genes whose functions are required for the successful colonization of certain habitats.

To examine the ability of *B. abortus* to establish infection and persist in the host, a bank of STM mutants was constructed in which each mutant carried a unique tag as a result of a single random transposon insertion in the genomic chromosome. Observed negative phenotypes of a few single insertional mutants could be attributed to the inactivation of genes by unique tagged transposons. Having demonstrated the validity of the insertional mutagenesis, the mutant bank was screened for mutants that were attenuated in the murine model of infection. The murine model is smaller and more affordable in cost and in housing than the large animal model such as the cow, which is the primary host of *B. abortus*. More importantly, the murine model has been used extensively for detecting differences in virulence among brucellae (8), (98), (99), (100), (133). Studies have shown that vaccine strain S19 that persists longer than many attenuated strains is more rapidly cleared from mouse spleens than virulent S2308 after an infection (48), (51), (132). The ability of *Brucella* to persist within the mouse is directly attributable to intracellular survival within macrophages (1), (53), (54), (129).

The STM method provides a qualitative identification of candidates that are potentially attenuated for intracellular survival in the host. Cross feeding of mutants may occur in a complex pool of mutants, i.e., a negative phenotype in one mutant may be complemented by other mutants. Alternatively, some mutants may survive poorly in competition with 49 other mutants in the infectious dose. To address these potential problems, the mutants identified in the original STM screen were confirmed by

performing a mixed infection (1-to-1) of *B. abortus* S2308 (parental strain) with individual candidate, attenuated mutants. However, mutants that were complemented *in trans* in the presence of the other mutants might be missed using this assay. To exclude the possibility that the attenuating phenotypes of these candidate mutants are due to growth defects, individual candidate mutants along with S2308 were competitively tested for the ability to grow *in vitro* tryptic soy broth.

The ability of various strains of *B. abortus* to survive in macrophages *in vitro* has been shown to correlate with their virulence *in vivo* (1), (26), (34), (46). During the course of infection in mice, the initial growth of *Brucella* in the spleen is temporarily halted by the onset of heightened macrophage bactericidal activity (24), (87), (93). Macrophage activation occurs very early during *Brucella* infection, and persists as late as 60 days post-infection, well into the latent phase of the infection (8), (14). Therefore, to persist in a host *Brucella* must be able to survive and replicate within the environment of the macrophage. *In vitro* studies involving cytokine activation of macrophages have shown that IFN-gamma has the most consistent and dramatic enhancing effect on the ability of murine macrophages to control the growth of *Brucella* (72), (71), (74). However, the efficacy of IFN-gamma activation has no effect on the clearance of bacteria. Therefore, the ability of *Brucella* to establish its niche in the host macrophage was examined by evaluating individual STM attenuated mutants for survival in the macrophage-like cell line J774A.1.

Although housekeeping functions were of little interest in identifying novel virulence mechanisms, they would serve as targets for development of improved vaccine

strains or for therapeutic treatment. *Brucella* spp. are able to grow in what is suspected to be a nutrient-poor environment within macrophage vacuoles, suggesting that they have the ability to synthesize all the metabolites necessary for survival. Thus, a mutation in any gene encoding key enzymes in metabolic pathways that cannot be acquired from the host intracellular environment, results in reduced survival in macrophages. For example, certain *Brucella* auxotrophs including amino acid biosynthesis (*car*AB, *leu*A), and glutamine metabolism (*gln*D) are attenuated in macrophages (50), (53). Purine auxotrophs of *B. melitensis* and of several pathogens are also attenuated in macrophages and in mice (34), (42). Thus, to elucidate the metabolic pathways required by *Brucella* to survive intracellularly in host macrophages, the attenuated STM mutants were screened for auxotrophs. The screening for auxotrophs helps to identify nutrient compounds that may not be readily available to *Brucella* living inside a phagosome. In addition, when the attenuating phenotype was not due to metabolic defects, the screen provides a mean to demonstrate that the observed attenuation was caused by the inactivation of novel virulence factors that are necessary for the survival of *Brucella* within the macrophage.

**Study II**

***B. abortus* mutants defective in persistence in mice confer protection against virulent challenge.** The second study described the comparison of *B. abortus* STM mutants defective in persistence in the mouse model for their ability to stimulate protective immunity. In contrast to mutants defective for resistance to stress and other

environmental hazards that poorly colonize the host, mutants defective for maintaining chronic infection colonized the host normally, but failed to persist or thrive. Mutants with inactivated genes that were required for chronic infection became ideal for live vaccine development. To be effective, live attenuated vaccines against brucellosis must persist long enough to elicit protective immunity, but should be cleared as quickly as possible to avoid unnecessary side effects. In order to determine whether greater persistence correlates with greater protection, the ability of selected mutants to protect vaccinated mice against a challenge infection of wild type *B. abortus* was examined at different time points following vaccination. Protective immunity against *Brucella* infection has been studied in several mouse models (BALB/c, CD-1, CBA, C57Bl/10 mouse strains) (16), (25), (28), (70), (138), (152), (153). The criterion used for measuring protection in immunized mice is the reduction, at a specified time, after a virulent challenge, of the number of colony-form units (CFU) of *Brucella* recoverable from the spleen or liver or both (110). The level of protective immunity was also analyzed by spleen weights from groups of mice vaccinated with different mutants, since splenomegaly is indicative of severity of the infection. T cells play a major role in the acquired specific resistance to intracellular bacteria determining the resolution of infection. Thus, cytokine profiles induced by different mutants were analyzed and compared to that induced by the wild type S2308. The $IgG_{2a}$ and $IgG_1$ patterns analyzed from each group of vaccinated animals was another measure of the cell-mediated immunity. In addition, the adaptive or acquired immunity were characterized by lymphocyte proliferative response and antibody titers. The comparison of T cell-

mediated responses between different mutant strains helps to identify predictive correlates of adaptive immunity and to establish whether optimal immunity is a function of the survival and/or the intracellular trafficking and processing of the organism.

**Study III**

**Isolation of *gcvH* in *B. abortus.*** The last study described the isolation of the *gcvH*, an important locus shown to be required for persistence within the host. The ability of *B. abortus* to successfully establish and maintain chronic infection can be attributed to the long-term survival and growth of *Brucella* within the host macrophage. In the hostile conditions encountered within the macrophage, brucellae elicit changes in gene expression resulting in the induction of stress response proteins (89), (80), (118). Stress response proteins such as DnaK, GroE, Lon, and Clp, may serve to aid in adaptation to the harsh environment of phagocytic cells (44), (88), (118), (142). These gene products may be involved in removing damaged or misfolded proteins before they can accumulate to toxic levels. Others such as antioxidants, SodC and KatE, may play a role in the intracellular oxidative defense mechanisms and hence protecting brucellae from the oxidative burst in the host macrophage (141), (77), (78). Interestingly, previous studies have shown that the intracellular survival of *B. abortus* can be reduced by the disruption of the *cydBA* operon whose products are involved in the terminal step of the electron transport chain (46). The function of this protein appears to be essential to prevent oxidative stress under the conditions encountered in the host.

The intracellular niche of the phagocytic cell is the prominent feature shared by another facultative intracellular pathogen, *Mycobacterium tuberculosis*. Infection with *M. tuberculosis* can result in the active tuberculosis and/or the latent infection. The latency of the disease in humans and experimental animals is due to the persistence of the tubercle bacillus in the host (105). Wayne *et al*. (150) has reported that *M. tuberculosis* is capable of adapting to prolonged periods of dormancy in tissues and that dormant bacilli are responsible for the latency of the disease itself. The process of shiftdown of *M. tuberculosis* from active replication to dormancy, but not death, has been described through the *in vitro* system as (a) changes in tolerance to anaerobiosis, (b) production of a unique antigen and (c) a ten-fold increase in glycine dehydrogenase production (147), (149). The unique antigen, URB, is present only in dormant bacilli, and not in well-aerated replicating cultures. The enzyme glycine dehydrogenase catalyzes the reductive amination of glyoxylate, concomitantly oxidizing NADH to NAD. Under conditions of reduced oxygen availability found in inflammatory and necrotic tissues, glycine dehydrogenase can serve to regenerate NAD from its reduced form, thus providing energy for *M. tuberculosis* to shift down to a stable, non-replicating form that merely tolerates anaerobiosis. Providing NAD and thus ATP may be necessary to support the completion of a final cycle of DNA synthesis before the shutdown of replication.

Although the outcomes of the two diseases are different, persistence of bacteria in the host is the hallmark of chronic infection caused by *Brucella* and of the latent infection caused by *Mycobacteria*. In the latency of tuberculosis, the level of glycine

dehydrogenase increases in dormant tubercle bacilli, yet the *gcvP* gene encoding the enzyme has not yet identified in *M. tuberculosis*. In *B. abortus* the *gcvH* mutant shows a defect in the ability to persist in the host, indicating that *Brucella* and *Mycobacteria* may depend on a similar metabolic pathway for chronic persisence in the host. The next step in characterizing the *gcvH* locus was to restore or rescue the attenuated phenotype of the *gcvH* mutant. First, all three genes of the *gcv* operon must be fully expressed in order to have a functional glycine cleavage system. The introduction of wild-type copy of *gcvH* and *gcvP* into the mutant was accomplished via the use of a low-copy, broad host range plasmid. Then the complemented mutant strains were evaluated for their ability to survive in mice as well as in the macrophage. The outcome of the *in vivo* and *in vitro* survival of the complemented mutant strains would be invaluable for future studies on the regulation and expression of the *gcv* operon in *B. abortus*.

# CHAPTER II
# IDENTIFICATION OF GENES REQUIRED FOR CHRONIC PERSISTENCE OF *BRUCELLA ABORTUS* IN MICE *

## Overview

The genetic basis for chronic persistence of *Brucella abortus* in lymphoid organs of mice, cows, and humans is currently unknown. We identified *B. abortus* genes involved in chronic infection, by assessing the ability of 178 signature-tagged mutants to establish and maintain persistent infection in mice. Each mutant was screened for its ability to colonize the spleens of mice at 2 and 8 weeks after inoculation. Comparison of the results from both time points identified two groups of mutants attenuated for chronic infection in mice. The first group was not recovered at either 2 or 8 weeks post-infection and was therefore defective in establishing infection. Mutants in this group carried transposon insertions in genes involved in lipopolysaccharide biosynthesis (*wbkA*), in aromatic amino acid biosynthesis, and in type IV secretion (*virB1* and *virB10*). The second group, which was recovered at wild-type levels 2 weeks post-infection but not 8 weeks post-infection, was able to establish infection but was unable to maintain chronic infection. One mutant in this group carried a transposon insertion in a gene with homology to *gcvB* of *Mycobacterium tuberculosis*, encoding glycine dehydrogenase, an

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enzyme whose activity is increased during the state of nonreplicating persistence. These results suggest that some mechanisms for long-term persistence may be shared among chronic intracellular pathogens. Furthermore, identification of two groups of genes, those required for initiating infection and those required only for long-term persistence, suggests that *B. abortus* uses distinct sets of virulence determinants to establish and maintain chronic infection in mice.

**Introduction**

Bacteria causing chronic infections, such as *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, and *Brucella abortus* are able to evade the host's immune system throughout the infection by colonizing an intracellular niche. This lifestyle may require adaptations other than the brief survival in phagocytic cells observed for well-characterized intracellular pathogens such as *Salmonella* serotypes which cause an acute infection. While *M. tuberculosis* and *C. trachomatis* are difficult to manipulate genetically, the genetic manipulation of the *Brucella* genome can be performed routinely, using tools such as plasmid vectors and systems for Tn*5* mutagenesis (75), (83), (86). Thus, identification of the genes required by *B. abortus* to cause infection may reveal virulence mechanisms of chronic disease caused by other intracellular pathogens. A recent improvement in Tn*5* mutagenesis, known as signature-tagged transposon mutagenesis (STM), has been developed for the in vivo selection of Tn*5* mutants that are defective in colonization (66). This method uses experimentally infected animals to identify mutants that are attenuated *in vivo* from a large, mixed pool

of mutants (126). Since Tn*5* can be used in *B. abortus*, STM can be used to identify genes that are necessary for chronic intracellular infection.

Brucellosis is endemic in Mediterranean countries and Central and South America and is manifested as an undulant fever in humans that, if untreated, can develop into a chronic infection with symptoms persisting for several months (154). Chronic infections may result in infection of secondary tissues, including heart and brain, if the infection is left untreated. Symptoms may also recur years after the original infection. *B. abortus* infection is acquired by humans through contact with infected livestock and consumption of unpasteurized dairy products. Bacteria cause a systemic infection and localize preferentially to organs that are rich in elements of the reticuloendothelial system, such as liver, spleen, and lymph nodes, where they survive and multiply within host macrophages. *B. abortus* has been found to inhibit the bactericidal functions of phagocytes, including phagolysosomal fusion, neutrophil degranulation, and the oxidative burst (8); however, as with other intracellular pathogens which cause chronic infection, the genetic basis for the interaction of *Brucella* with phagocytic cells is still poorly understood. *B. abortus* virulence is conveniently studied in a mouse model that mimics the chronic infection observed in humans. Here bacteria are found intracellularly, within macrophages of infected organs (96). Experimental infection of BALB/c mice has shown that the infection has two phases: during the first 2 weeks, bacteria multiply rapidly. In the second phase, bacterial numbers stabilize over the next 5 to 6 weeks and then decrease slowly. Bacteria have been recovered from spleens of infected mice as late as 24 weeks post-infection (98), (112). The different phases of

*B. abortus* infection in mice raise the question whether this pathogen uses different sets of virulence genes during the early and late stages of this disease.

To address this question, we have performed a random screen of the genome of *B. abortus* to identify genes required for infection at an early (2 weeks post-infection) and a late (8 weeks post-infection) time point postinoculation. Comparative analysis of these results provided new insight into the genetic basis for chronic intracellular infection and *B. abortus* pathogenesis. Furthermore, our results suggest that a better understanding of the mechanisms by which *B. abortus* is able to cause chronic intracellular infection may ultimately reveal strategies that are shared by other, less tractable pathogens.

## Materials and Methods

**Construction of mutants and growth conditions.** *B. abortus* strain 2308 (obtained from B. L. Deyoe, National Animal Disease Center, Ames, Iowa) was used as a host for STM. A bank comprising 240 signature-tagged *B. abortus* mutants (ST mutants) was constructed using a pool containing 10,000 uniquely tagged mini-Tn*5*Km2 derivatives carried on suicide plasmids, which was obtained from David Holden (66). The transposons were introduced into *B. abortus* S2308 by electroporation and selected on tryptic soy agar (TSA; Difco) containing 100 mg of kanamycin (Km) per liter (1). Approximately 20 mutants were taken from each of 10 individual electroporations in order to minimize the isolation of siblings. Mutants resistant to ampicillin were eliminated from the pool, since they carry the suicide vector inserted into the

chromosome. For infection of mice, *B. abortus* strains were grown on potato infusion agar (PIA; Difco) for 48 h and resuspended at the appropriate concentration in phosphate-buffered saline (PBS) (3). For determination of auxotrophy, the chemically defined solid medium formulated by Gerhardt was used, which contains glycerol, glutamate, lactate, thiamine, nicotinic acid,biotin, calcium pantothenate, and inorganic salts (58). This basal medium was supplemented with 10 groups of amino acids to perform auxanography for attenuated mutants. All work with live *B. abortus* was performed in a biosafety level 3 containment facility following Centers for Disease Control-National Institutes of Health guidelines.

**Infection of mice.** For the mutant screen, pools of 46 mutants were used to infect groups of six 6- to 8-week-old BALB/c mice intraperitoneally (i.p.) at a total dose of approximately $10^6$ CFU. Groups of three mice were sacrificed at 2 and at 8 weeks post-infection, and bacteria were recovered from the spleens. Spleens were homogenized in 3 ml of PBS, and serial 10-fold dilutions were plated on TSA-Km.

**Signature-tagged screen and hybridization analysis.** Bacteria recovered from murine spleens at 2 and 8 weeks post-infection were pooled to obtain one output pool from each mouse. Total chromosomal DNA was prepared from each output pool to serve as a template for generating probes containing labeled tags, as described before (66). Labeled tags used as probes were prepared by incorporation of [-32P]dATP during PCR amplification of tags from total chromosomal DNA. Blots containing chromosomal

DNA from individual mutants in each pool were prepared by transferring pools of *B. abortus* ST mutants from 96-well plates onto nylon membranes laid on top of TSA plates with a 48-prong replicator. Hybridization and washes were carried out under stringent conditions (7). To identify potentially attenuated mutants, hybridization signals on a blot probed with labeled tags prepared from the input pool (inoculum) were compared with three identical blots hybridized with tags prepared from the output pools recovered from the three mouse spleens at each time point. Mutants giving a hybridization signal with the input pool but no signal in the output pool of at least two mice were selected for further study. To confirm that each mutant carried a single insertion of mini-Tn*5* in the chromosome, mutants were analyzed by Southern blot (7). Chromosomal DNA was prepared (7) and digested with *Eco*RI, which cuts once within the transposon, outside the kanamycin resistance gene. After agarose gel electrophoresis and transfer to nylon membranes, chromosomal DNA was analyzed by hybridization with a probe containing a 1.3-kb fragment of the Tn*903* kanamycin resistance gene from plasmid pUC4KSAC (Pharmacia). Mutants whose chromosomal DNA gave a hybridization signal with a single band on the Southern blot were determined to have only one copy of the transposon inserted into the chromosome.

**Competitive infection assay.** For competitive infection experiments, groups of four mice were inoculated i.p. with an approximately 1:1 mixture of mutant and wild-type *B. abortus*, at a total dose of approximately $10^5$ CFU. Mice were sacrificed at 2 or 8 weeks post-infection, and bacteria were recovered from infected spleens. The CFU of

mutant and wild-type *B. abortus* recovered from infected spleens were enumerated by serial dilution in PBS and plating in parallel on TSA and TSA-Km. Numbers of wild-type and mutant bacteria were calculated by subtracting the CFU recovered on TSA-Km, on which only the Tn*5* mutants are able to grow, from the CFU recovered on TSA plates, representing the total number of bacteria recovered. Data were normalized by dividing the output ratio of (CFU [wild type]/CFU [mutant]) by the input ratio of (CFU [wild type]/CFU [mutant]). All data were then converted logarithmically for statistical analysis. A Student's *t* test was used to determine whether the wild-type/mutant ratio recovered from infected spleens was significantly different from the wild-type/mutant ratio present in the challenge inoculum.

**Cloning and sequence analysis.** Transposon-flanking DNA was cloned by inverse PCR as described previously (9) using *Rsa*I for digestion of chromosomal DNA and the primer pair SIGN-10 (5'-GCCGAACTTGTGTATAAGAGTCAG-3') and SIGN-11 (5'-AAAGGTAGCGTTGCCAATG-3'). The PCR products were ligated into cloning vector pCR II (Invitrogen), and plasmid DNA for sequencing was isolated from *Escherichia coli* strain DH5 (62) using ion-exchange columns from Qiagen. In addition, larger DNA fragments flanking the transposon insertions in several of the mutants were cloned by ligating *Pst*I- or *Eco*RI-restricted genomic DNA into cloning vector pBluescript SK+ restricted with the appropriate enzyme and selection for the kanamycin resistance marker of mini-Tn*5*Km2 (39). Nucleotide sequences were analyzed using the MacVector 6.5 software package (Oxford Molecular Group). Sequence homology was determined

using the BLAST2 search algorithm at the National Center for Biotechnology Information (NCBI) (4).

## Results

**Generation of signature-tagged mutants of *B. abortus*.** *B. abortus* 2308 was mutagenized with a pool of signature-tagged mini-Tn*5*Km2 derivatives carried on plasmid pUT by electroporation, and mutants were selected on TSA-Km. These mutants were screened for susceptibility to ampicillin to eliminate strains (fewer than 2% of mutants) carrying cointegrates of the suicide vector pUT inserted in the chromosome. To confirm that mutants obtained from the same electroporation are not siblings but arise from independent transposition events, Southern hybridization was performed using the 1.3-kb *Eco*RI fragment of pUC4KSAC, which contains the Tn*903* Kmr gene (39). Using this probe, the hybridization profiles of *Eco*RI-digested chromosomal DNA from 10 randomly chosen mutants from a single electroporation were compared. Since *Eco*RI cuts only once within miniTn*5*Km2, a mutant with a single transposon insertion should have only one band hybridizing with the probe. All of the mutants examined had a single band of unique size hybridizing with the probe, indicating that each mutant contained a single, unique insertion of the transposon (data not shown).

**Signature-tagged screen of *B. abortus* mutants in mice.** To identify mutants defective in establishing chronic infection, we used the BALB/c mouse model of chronic brucellosis. Mice infected i.p. with *B. abortus* have been shown to harbor bacteria in the

spleen for up to 24 weeks post-infection (8), (14). The persistence of *B. abortus* in mice is similar to chronic infection observed in other host species, including humans. It has not been shown whether attenuated *B. abortus* mutants exhibit competitive infection defects in mice when coinoculated with a virulent strain. We therefore performed a preliminary competitive infection experiment using *B. abortus* 2308 and CA180, a Tn*5* mutant defective in the synthesis of the O antigen of lipopolysaccharide (LPS), which was characterized previously (1). Three mice were infected i.p. with a mixture containing 6.4 Å~ $10^4$ CFU of *B. abortus* 2308 and 4.1 Å~ $10^4$ CFU of CA180. At 1 week postinfection, no mutant *B. abortus* could be recovered from the spleens of any infected mouse, while the number of wild-type *B. abortus* ranged from 6 Å~ $10^3$ to 2 Å~ $10^6$ CFU/spleen. This result showed that a mutant with a defect in a known *B. abortus* virulence factor, LPS, exhibits a competitive infection defect in the BALB/c mouse model of brucellosis. Furthermore, it suggested that STM, which utilizes competitive infection as the basis for screening pools of mutants, could be used to screen for mutants defective in chronic infection.

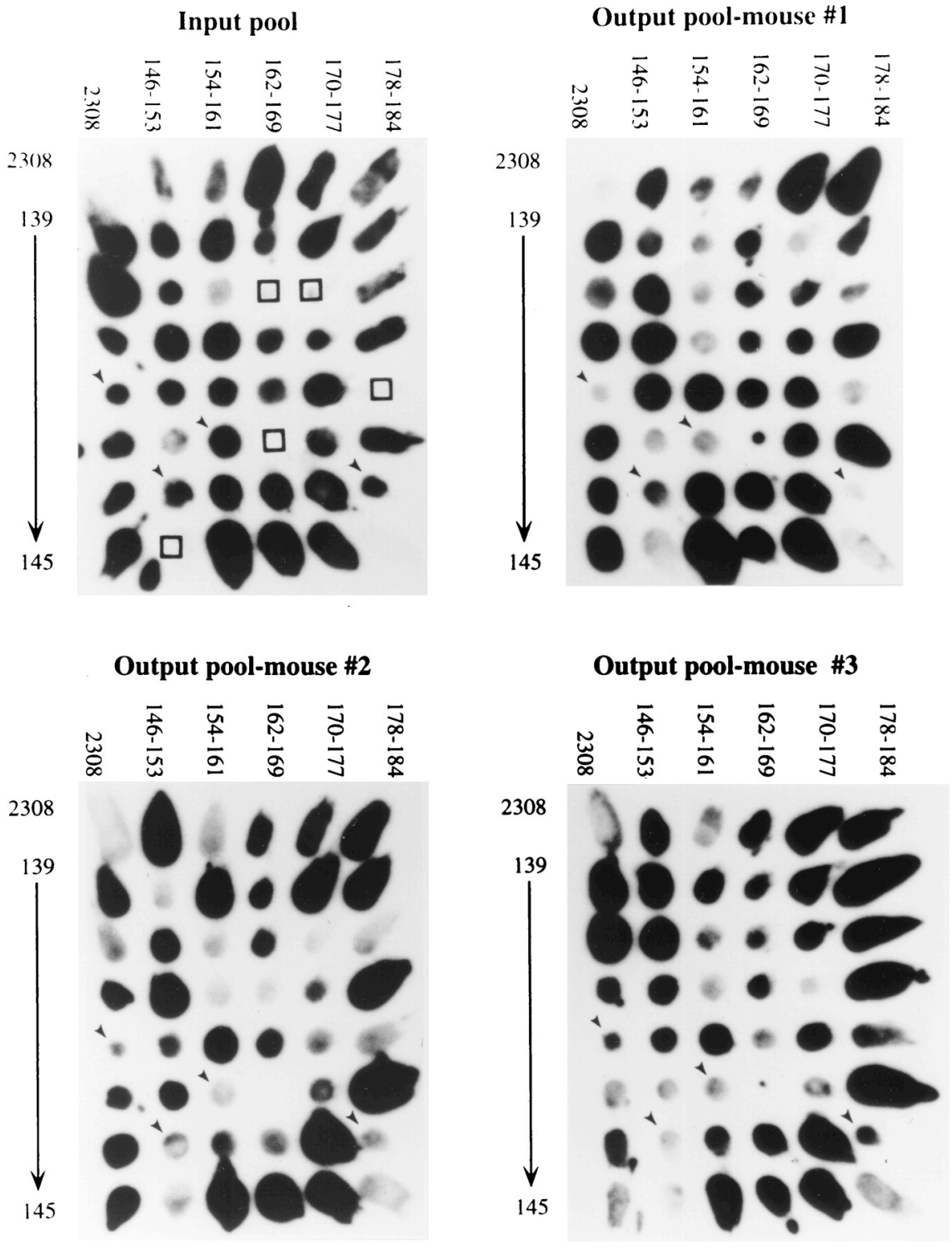
For the screen in mice, pools of 46 mutants were grown individually in tryptic soy broth (TSB) in 96-well plates for 48 h and then stamped with a 48-prong replicator onto PIA plates and grown for 48 h. Bacteria were resuspended from PIA plates, and the concentration was adjusted to approximately $10^7$ CFU/ml with PBS. Of this suspension, 0.1 ml was injected i.p. into each of six BALB/c mice. In the pooled inoculum, the dose of each individual mutant was therefore approximately $10^4$ CFU. At 2 and 8 weeks post-infection, groups of three mice were sacrificed. At necropsy, the only sign of disease

evident was the enlargement of the spleen. To recover bacteria, spleens were homogenized in PBS, and serial 10-fold dilutions were plated on TSA-Km. After incubation for 4 days, bacteria were resuspended from plates containing between 1,000 and 5,000 colonies, and chromosomal DNA was prepared from the recovered pool of mutants. Probes of input and output pools were generated by PCR amplification of tags from chromosomal DNA of pooled STM mutants as described before (66) and used for hybridization with dot blots containing individual mutants in the corresponding pool. For each time point, a fresh input pool probe was prepared and a blot was hybridized for comparison with the output pools recovered from the mice. We found that 39 mutants failed to give a reproducible hybridization signal when the input pool probe was prepared more than once from a chromosomal DNA preparation. These mutants were eliminated from the screen. Thus, a total of 178 mutants with consistently hybridizing tags were screened.

Mutants which gave weak or no hybridization signals on output pool blots from at least two of the three mice at each time point were identified as putatively attenuated by comparison with input pool blots (Fig. 1). At 2 weeks post-infection, 28 mutants exhibited a reduction in the hybridization signal, suggesting reduced recovery from the mouse spleens. Of these 28 mutants, 11 exhibited a reduced hybridization signal in output pools recovered at 8 weeks post-infection.

In addition to the 11 mutants reduced in colonization at both 2 and 8 weeks, we identified 16 mutants that were only defective for colonization at 8 weeks. While mutants identified from both the 2- and 8-week output pools may be unable to establish

**Fig. 1.** Dot blots. Colony blots showing hybridization of *B. abortus* ST mutants with probes containing tags amplified from the input pool and from output pools recovered from spleens of three mice at 8 weeks postinfection. At the upper left and lower right corner of each blot is the parent stain, *B. abortus* 2308. Arrows indicate mutants (BA142, BA152, BA159, and BA184 [left to right]) which gave weak signals in the output pools of at least two of the three mice inoculated and whose competitive infection defect was confirmed subsequently. Open squares indicate mutants that gave inconsistent hybridization results and were therefore eliminated from the screen.


Input pool
2308
146-153
154-161
162-169
170-177
178-184
2308
139
145
Output pool-mouse #1
2308
146-153
154-161
162-169
170-177
178-184
2308
139
145
Output pool-mouse #2
2308
146-153
154-161
162-169
170-177
178-184
2308
139
145
Output pool-mouse #3
2308
146-153
154-161
162-169
170-177
178-184
2308
139
145

infection, those identified only from the 8-week output pools may be defective in sustaining chronic infection. The 17 mutants identified only from the 2-week output pools may represent mutants, which are slow to colonize the spleen, but are still able to persist. However, infection defects in these mutants were not characterized further, since these mutants were able to sustain chronic infection. Thus, two groups of mutants, those identified at both 2 and 8 weeks and those identified only at 8 weeks, were considered putatively attenuated for chronic infection and were chosen for further study (Table 1).

**Competitive infection of mice with mutants identified by STM.** Following our identification of mutants putatively attenuated for chronic infection, we performed a quantitative assay to confirm their colonization defect. To this end, each of the 27 mutants identified by the STM screen was inoculated i.p. at a 1:1 ratio with wild-type *B. abortus* at a total dose of approximately $10^5$ CFU to groups of four mice (Table 1). As a control, mutant BA53, which was recovered at both 2 and 8 weeks post-infection from mice, was selected at random from the STM pool as a negative control and administered to mice in a competitive infection experiment as described above. During mixed-infection experiments, 15 mutants identified in the STM screen were found to have a significant defect in persistence in mouse spleens. One of these mutants, BA11, had a significant colonization defect only at 2 weeks and therefore was not characterized further. Thus, for 14 of 27 mutants identified in the preliminary STM screen, the putative chronic infection defect could be confirmed by a significant reduction in

**Table 1:** Confirmation of competitive defects of STM mutants by competitive infection of mice with *B. abortus* S2308

| Mutant | competitive index[a] *in vivo* | | competitive index *in vitro* | Mutant | competitive index *in vivo* |
|---|---|---|---|---|---|
| | 2 weeks | 8 weeks | | | 8 weeks |
| BA11 | 2.29 | 1.86 | | BA18 | 0.064 |
| BA41 | 4.74* | >29512*** | 0.97 | BA27 | 2.24 |
| BA73 | 3.36 | 147.9* | 2.74 | BA31 | 3.63* |
| BA87 | 1.08 | 10.47** | 0.79 | BA38 | 7.76* |
| BA100 | 20.89*** | >758.6*** | 4.70 | BA63 | 1.86* |
| BA102 | 1.47 | 93.3*** | 1.80 | BA70 | 3.39 |
| BA109 | 0.92 | 3.21 | | BA99 | 1.02 |
| BA114 | 133.07** | >1047.1*** | 0.91 | BA105 | 2.00 |
| BA157 | 0.74 | 1.58 | | BA119 | 6.31 |
| BA159 | 1.50 | 23.44* | 2.20 | BA116 | 2.51 |
| BA184 | 1306.8*** | >2168.2*** | 0.57 | BA122 | 95.50* |
| | | | | BA130 | 3.02 |
| | | | | BA138 | 3.72 |
| | | | | BA140 | 1.20 |
| | | | | BA142 | 70.79*** |
| | | | | BA152 | 72.44*** |

[a] competitive index was calculated as $(CFU_{S2308}/CFU_{mutant})_{recovered}/(CFU_{S2308}/CFU_{mutant})_{inoculated}$.
Significance of differences between competitive indices of STM mutants and a control, non-attenuated mutant were compared using the Student's *t* test.
*, $p<0.05$; **, $p<0.01$, ***, $p<0.005$.

colonization during competitive-infection experiments.

Mutants which did not have significant colonization defects in the competitive-infection assay either may have been false-positives in the STM screen or may only display competitive colonization defects when inoculated at a wild type-to-mutant ratio of greater than 1. However, these possibilities were not investigated further. Mutants with significant colonization defects during mixed infections fell into two groups: those reduced in colonization at both 2 and 8 weeks, and those reduced at only 8 weeks (Fig. 2). Subsequent mouse infection experiments showed that mutants with competitive colonization defects are also attenuated when administered alone to mice (data not shown). To determine whether colonization of the spleen was reduced due to a general growth defect, mutants displaying significant defects at both 2 and 8 weeks post-inoculation were assayed for *in vitro* growth defects by inoculating TSB with a mixture of wild-type and ST mutant strains (Table 1). With the exception of BA100, which was outgrown almost fivefold by the parent strain, none of the mutants displayed a strong growth deficiency in laboratory medium.

**Identification of inactivated genes.** Genes inactivated by mini-Tn*5*Km2 insertions were identified by cloning transposon-flanking DNA, sequence determination, and comparison with the GenBank database using the BLAST2 search program at NCBI. Transposon-flanking DNA was cloned from eight mutants.

Mutants with defects in establishing chronic infection included BA184, which carried a transposon insertion in a *B. abortus* homologue of *Brucella melitensis wbkA*,

| 2 and 8 weeks: 4 mutants | 8 weeks: 10 mutants | |
|---|---|---|
| BA41 (*virB1*) | BA31 | BA102 (*gcvP*) |
| BA114 (*virB10*) | BA38 | BA122 |
| BA100 (*aro*) | BA63 | BA142 |
| BA184 (*wbkA*) | BA73 (n.h.) | BA152 (*gltD*) |
| | BA87(HP1225) | BA159 (*gluP*) |

**Fig. 2.** Attenuated mutants. Mutants with statistically significant colonization defects, as confirmed by individual competitive infections and statistical analysis. Homologues of disrupted genes are given in parentheses. nh, no homology.

encoding a mannosyltransferase that functions in the biosynthesis of O antigen (61). LPS biosynthesis is required for virulence in *Brucella* species, and it has recently been shown that strains of *B. abortus* and *B. melitensis* carrying defined mutations in genes required for LPS biosynthesis are unable to establish infection in mice (1), (61), (95). Identification of this known virulence factor thus validated the STM screen.

Two additional mutants with defects in establishing persistent infection, BA41 and BA114, carried transposon insertions in homologues(*virB1* and *virB10*, respectively) of the *Brucella suis virB* locus (103). *B. suis* mutants carrying disruptions at this locus show a reduced ability to multiply *in vitro* within macrophages and HeLa cells. Our data suggest that this locus is required for establishing chronic infection in organs of the mouse.

We could obtain no clone from DNA flanking the transposon insertion of the fourth mutant (BA100) defective in establishing chronic infection. However, BA100 was unable to grow on *Brucella* minimal medium unless supplemented with a combination of aromatic amino acids. These auxanography data indicated that this mutant was defective in an early step of the aromatic amino acid biosynthesis pathway.

Sequences obtained from the transposon insertion site in three mutants defective in sustaining chronic infection showed homology to metabolic genes. BA159 was interrupted at the *gluP* locus, encoding a putative transporter for glucose and galactose (49). Mutant BA152 carried a transposon insertion in a homologue of *Rhizobium etli gltD*, encoding the small subunit of glutamate synthase (21). The identification of these two mutants in the STM screen suggests that glucose, galactose, or glutamate may serve

as carbon and/or nitrogen sources during growth of *B. abortus* in the host. BA102 carried an insertion in a putative glycine dehydrogenase or glycine cleavage system. Since BA102 did not have a competitive growth defect in TSA and was able to grow on minimal medium without glycine, the virulence defect of BA102 is likely not due to auxotrophy. Interestingly, the activity of glycine dehydrogenase has been shown to increase 10-fold upon entry of *M. tuberculosis* into a state of nonreplicating persistence (148), (150). Since *B. abortus*, like *M. tuberculosis*, is able to persist chronically in infected hosts, glycine dehydrogenase may play a similar role in the entry of these pathogens into a latent state in the host.

Finally, DNA flanking two insertions in mutants defective for maintaining chronic infection showed either no homology to genes in the database (BA73) or homology to an open reading frame of unknown function (BA87) (Table 2).

**Discussion**

The goal of the STM screen was to identify and compare *B. abortus* genes required for establishment and maintenance of chronic persistence in the mouse. Since fewer than 200 genes have been sequenced in *Brucella* species, we reasoned that screening a small number of mutants would be sufficient to identify new *B. abortus* genes required for chronic infection. The STM screen identified 27 mutants putatively attenuated for chronic infection. A statistically significant competitive-infection defect could be detected in 14 of these mutants. Thus, statistically significant evidence for attenuation was obtained for 8% (14 of 178) of the mutants screened. Assuming that our

**Table 2:** Characterization of *B. abortus* genes identified by STM

| Mutant | Closest homolog (organism) | GenBank Accession Number (reference) | inferred function |
|---|---|---|---|
| BA41 | *virB1* (*Brucella suis*) | AF141604 | Type IV secretion |
| BA114 | *virB10* (*Brucella suis*) | AF141604 | Type IV secretion |
| BA184 | *wbkA* (*Brucella melitensis*) | AF047478 | O-antigen biosynthesis |
| BA159 | *gluP* (*Brucella abortus*) | U43785 | uptake of glucose and galactose |
| BA102 | P-protein (*Synechocystis spp.*)<br>*gcvB* (*M. tuberculosis*) | D90914<br>Q50601 | glycine dehydrogenase |
| BA152 | *gltD* (*Rhizobium etli*) | AF107264 | glutamate synthase |
| BA87 | HP1225 (*Helicobacter pylori)* | AE000628 | conserved inner membrane protein |
| BA73 | no homology | | ? |

mutagenesis was random and that the coding density of the 3,200-kb *B. abortus* genome is similar to that of the *E. coli* genome, our data suggest that an estimated 257 genes may be required for establishing and maintaining chronic persistence in mice after i.p. infection. In contrast, i.p. infection of mice with an STM bank of *Salmonella typhimurium* revealed that only 3% of its 4,400-kb genome, or an estimated 153 genes, is required for the acute infection caused by this intracellular pathogen (66). The greater number of virulence genes required for chronic infection versus acute disease may reflect the requirement for additional adaptations to ensure long-term persistence, such as those which prevent clearance of *B. abortus* by the host immune system.

The working hypothesis of this study was that different sets of genes may be required for the initial steps or the establishment of chronic infection, which is characterized by rapid bacterial growth, than for maintenance of chronic infection, in which little or no growth is observed (8), (14). Indeed, the 14 attenuated mutants identified in this study fell into two classes (Fig. 2). Four mutants were unable to establish infection by either 2 or 8 weeks post-infection. In contrast, the remaining 10 mutants were able to establish infection at 2 weeks post-infection but displayed a defect in chronic persistence at 8 weeks post-infection. The first class included mutants with transposon insertions in genes required for O-antigen biosynthesis (BA184), type IV secretion (BA41 and BA114), and biosynthesis of aromatic amino acids (BA100). The genes inactivated in these mutants are predicted to play a role early during infection. For example, BA184, a *wbkA* mutant, was the most highly attenuated and was outcompeted by the wild type by 1,000-fold at 2 weeks postinfection, suggesting that it

was eliminated early in the infection process. Since rough mutants are sensitive to the bactericidal action of complement, it is possible that BA184 is cleared by complement-mediated lysis and may reach the spleen only in small numbers (1), (30), (43). The transposon insertion in BA100 rendered this mutant defective in the biosynthesis of aromatic amino acids, as determined by auxanography. This biosynthesis pathway is also required for the biosynthesis of 2,3-dihydroxybenzoic acid, the only siderophore known to be produced by *B. abortus* (90). However, since this siderophore has been shown to be dispensable for growth in mice (11), it is more likely that attenuation of this mutant is the result of its inability to acquire aromatic amino acids in the host. *Salmonella aro* mutants are unable to survive and replicate within macrophages and are attenuated for virulence (68). Thus, some of the virulence genes required in an early phase during chronic *B. abortus* infection may be similar to those used by intracellular pathogens, such as *S. enterica* serovar *Typhimurium*, which cause an acute infection.

Two mutants (BA114 and BA41) defective for initiation of chronic infection carried insertions in a putative type IV secretion system of *B. abortus*, encoded by the *virB* genes (103). Mutant BA114 (*virB10*) displayed a greater competitive colonization defect in murine spleens at 2 weeks post-infection than BA41 (*virB1*) (Table 1). A similar effect has been described for the homologues of the *B. abortus virB10* and *virB1* genes present in the genome of the plant pathogen *Agrobacterium tumefaciens*. Inactivation of *virB1* in *A. tumefaciens* causes a lower degree of attenuation than a mutation in *virB10* (12). Mutations in the *virB* locus render *B. suis* unable to multiply in HeLa cells or macrophage cell lines *in vitro* (103). These data, together with our

findings that *B. abortus virB1* and *virB10* mutants are unable to persist in mouse spleens after i.p. inoculation, suggest that attenuation in the animal model is due to an inability of these strains to grow intracellularly.

The second class of mutants, which were unable to maintain chronic infection, included strains defective in production of glutamate synthase (BA152), glycine cleavage (BA102), nutrient uptake (BA159), and several unknown functions (BA31, BA38, BA73, BA87, BA63, BA122, and BA142). While the inactivated genes in these mutants were not required for initiation of infection, they were required for chronic persistence. Mutant BA102 carried a transposon insertion in a gene with homology to *gcvB*, encoding glycine dehydrogenase, from *M. tuberculosis*. The activity of this enzyme has been found to increase 10-fold upon entry of *M. tuberculosis* into a state of nonreplicating persistence *in vitro* (150). The finding that glycine dehydrogenase is required for persistence of *B. abortus* in the mouse spleen suggests that *M. tuberculosis* and *B. abortus* may depend on similar metabolic pathways for chronic persistence in the host. This result underscores the potential for research on host pathogen interactions of *B. abortus* to elucidate mechanisms of intracellular persistence which are shared by other chronic intracellular pathogens.

# CHAPTER III
# *BRUCELLA ABORTUS* MUTANTS DEFECTIVE FOR MAINTAINING CHRONIC INFECTION CONFER PROTECTION IN BALB/C MICE

## Overview

Protection against brucellosis requires a $T_H1$ response including the enhanced activation of professional phagocytes. Immunity to *Brucella* infection relies on the use of fortuitously attenuated vaccine strains. Research in the development of live vaccines has been focusing on rough mutants lacking the major antigenic determinant, the O-antigen. However, the loss of O-antigen severely attenuates the survival of the organism in the host and limits protection. The most effective vaccine strains available such as *B. abortus* S19 and *B. melitensis* Rev-1 have several limitations, not the least of which is virulence in humans. In an effort to identify vaccine candidates with enhanced efficacy and improved safety, we have explored protection induced by attenuated mutants with different survival characteristics, the rapidly clearing *virB10* (BA114) and the longer-persisting *gcvH* (BA102) mutants. The results in this study suggest that the persistence of the vaccine candidate in the mouse enhances long-term protection. The *gcvH* mutant induced significant levels of protection at 8, 16 and 24 weeks post-vaccination. Even though the protection against challenge began to decline from 16 to 24 weeks post-vaccination for both candidates, BA102 still induced a significant level of protection. Both vaccine candidates appeared to elicit a $T_H1$ memory response in the BALB/c mouse model as judged by the levels of $IgG_{2a}$ and $IgG_1$ in the sera of vaccinated mice and the induction of IFN-gamma and IL-2 in splenocytes obtained from vaccinated mice

in response to heat-killed *Brucella* or cell lysates. Surprisingly, the production of IL-10, a $T_H2$ cytokine, was also enhanced.

**Introduction**

The vaccination against bovine brucellosis with live attenuated smooth *B. abortus* strain S19 is used in most of the world because it has been useful for the control and eradication of this disease (101), (102). This vaccine is effective in conferring protection against virulent strains of *B. abortus*. However, there is a number of disadvantages. Strain S19 can cause abortion in pregnant animals and is still fully virulent for humans. In addition, it is difficult to differentiate between vaccinated and naturally infected animals, because S19 induces a humoral response that is similar to the response after natural infection, thus hampering procedures for the detection of infected animals by serological testing. In order to avoid these drawbacks, a stable, lipopolysaccharide O-antigen-deficient mutant of virulent *B. abortus* S2308 was developed (124). This rough, rifampin-resistant, attenuated variant is designated as RB51 and is currently being used in many countries as an alternative vaccine to *B. abortus* S19 because this strain does not induce antibodies to *Brucella* lipopolysaccharide O-antigen. Thus, it is possible to discriminate between infected and vaccinated animals. *B. abortus* RB51 retains the ability to confer protection against infection with pathogenic strains in both mice and cattle (45), (73), (91), (104), (135), (134), (131). However, the duration of protective efficacy is short-lived because RB51 can only persist for a short period in the spleens of mice vaccinated by intraperitoneal

injection. Since protection against *Brucella* infection requires a long-lived cellular immune response, the survival of the vaccine strain is therefore crucial for the development of a protective cellular immune response against *B. abortus* in cattle.

Two mutant strains (*gcvH* and *virB10*) among 14 attenuated *B. abortus* mutants isolated previously by signature-tagged mutagenesis (STM) were further analyzed for possible use as live attenuated vaccines. Selection of these two mutants was based on contrasting differences in the survival phenotype. *B. abortus* BA102 harbors a mini-Tn5 kanamycin cassette insertion in *gcvH* that exhibits a defect in chronic persistence in the mouse model (69), but no severe reduction on the intracellular survival within macrophages *in vitro*. The insertional mutation in BA102 is predicted to cause premature transcriptional termination of *gcvH* and abolishment of *gcvP* expression, and thus, resulting in the absence of a functional enzyme that may be essential for the survival of intracellular pathogens at reduced oxygen concentration (147), (149), (151). Consistent with an anaerobic state for *Brucella* is the observed requirement for cytochrome bd oxidase during intracellular growth (46), (81), (82).

Defective persistence in mice was also observed for BA114, the *virB10* mutant of *B. abortus* carrying a mini-Tn5 insertion in the *virB10* gene of the type IV secretion system (69). Type IV secretion systems (T4SS) are found in several mammalian and plant pathogens including *Bordetella pertussis*, *Helicobacter pylori* and *Agrobacterium tumefaciens*, (27). In the BALB/c mouse model, the defective *virB10* mutant (BA114) exhibited a severe reduction in numbers as early as two weeks post-infection (69). In murine bone marrow-derived macrophages and J774A.1, *B. abortus* and *B. melitensis*

mutants defective in T4SS were killed in the phagolysosomal compartment, suggesting that unlike the wild type these mutants were unable to inhibit phagosome maturation (22), (40), (146).

Both mutants of *B. abortus*, BA102 (*gcvH*) and BA114 (*virB10*), exhibit no growth defects when grown in liquid or on solid media (69). Therefore, attenuation in the mouse model was not simply the result of defects in growth or replication. Furthermore, both mutants did not require supplements when grown on *Brucella* minimal media, suggesting that the transposon insertions in *gcvH* and *virB10* do not render them auxotrophic.

The present study focuses on an organism such as the *gcvH* mutant of *B. abortus* that survives and replicates within the host for extended periods, presumably preventing phagolysosomal fusion yet is incapable of persisting at late times due to a failure to adapt to the changing environmental conditions, which are not experienced by bacteria infecting macrophages in culture. For comparison, an *in vivo* attenuated *virB* mutant of *B. abortus*, which fails to prevent fusion and hence is rapidly degraded, has been selected. The duration of survival of both mutants is expected to be sufficient to stimulate an adaptive immune response; however, differences in the replicative burst and the duration of exposure are expected to provide significantly different protective immune responses against wild type S2308.

As a result, the longer-lived *gcvH* mutant (BA102) elicited higher levels of $T_H1$-associated cytokines (IFN-gamma and IL-2) and proliferative responses of splenocytes, which persisted up to 24 weeks post-inoculation. In contrast, the shorter-lived *virB10*

mutant (BA114) only induced a significant level of IFN-gamma during the later phase of infection, and additionally, BA114 elicited a transient $T_H1$ response that was observed between 8 and 24 weeks post-vaccination. Surprisingly, neither BA102 nor BA114 mutant elicited significant splenomegaly at 8, 16, and 24 weeks post-inoculation. Taken together, the *gcvH* mutant or similar longer-surviving strains offer the best choice for long-lasting protective immunity. Side effects associated with persistence must be eliminated in order to make them safe for human use.

**Materials and Methods**

**Bacteria and bacterial products.** Wild-type *B. abortus* S2308 and vaccine strain 19 were obtained from B. L. Deyoe, National Animal Disease Center, and Ames, Iowa, respectively. Strains BA102 and BA114, attenuated mutants containing a single insertion of mini-Tn5 kanamycin cassette in *gcvH* and *virB10*, respectively, as described elsewhere (69) were derived from signature-tagged mutagenesis of *B. abortus* S2308. Strain BA582 is an attenuated mutant of *B. abortus* containing a wild-type Tn-5 insertion in *cydB* (46). All strains (Table 3) were grown on *Brucella* agar with or without kanamycin (100 □g/mL) for 3 days at 37°C. For infection of mice, bacteria were harvested from plates into PBS (pH 7.4) and ten-fold serial dilutions were made to obtain the appropriate inoculum ($10^7$ CFU/mL mutant or $10^5$ CFU/mL S2308). For *in vitro* growth and macrophage survival assays, isolated bacterial colonies grown on plates were used to inoculate 5-mL tryptic soy broth (TSB) containing the appropriate antibiotics followed by a 24-h incubation at 37°C in a rolling drum. The 24-h liquid

cultures were then used to prepare 48-h liquid cultures (a 1:50 dilution of the 24 h culture in 5 mL of fresh TSB containing the appropriate antibiotics). The bacteria from a 48-h culture were pelleted by centrifugation for 30 sec at 2500 x g, washed twice with PBS (pH 7.4) and resuspended in PBS (pH 7.4) to adjust the concentration to 5 X $10^9$ CFU/mL based on turbidity measurements using the Klett meter. For ELISA, bacterial suspensions in PBS (pH 7.4) were heat-killed overnight at 68°C. Heat-killed *B. abortus* (HKBA) was sonicated to generate a bacterial cell lysate (BACL) containing approximately 1.7 mg of protein/mL as estimated by the BioRad protein dye (California) and SoftMax 2.02 (California). All work with live *B. abortus* was performed in a Biosafety Level-3 containment facility following the Centers for Disease Control-National Institutes of Health guidelines appearing in the BMBL 4th edition (116).

**Tissue culture and macrophage survival assay.** The macrophage-like cell line J774A.1 was obtained from the ATCC and maintained as frozen stock in 40% DMEM (high glucose, 4500 mg/L) containing 1% (v/v) non-essential amino acids, 7.3 mM L-glutamine, 10% (v/v) DMSO, and 50% (v/v) fetal bovine serum at –180°C. The J774A.1 cells were grown in complete DMEM (Sigma, 4500 mg glucose/L, 25 mM HEPES, without L-glutamine) containing 10% (v/v) heat-inactivated fetal calf serum, 1% non-essential amino acids, and 7.3 mM L-glutamine in the presence of 100U penicillin and 10 mg streptomycin (Difco) for 3 days at 37°C, 5% $CO_2$ or until the plate was confluent. Cells were collected and resuspended in fresh serum-containing DMEM without penicillin-streptomycin and the concentration was adjusted to approximately 4 x $10^5$

cells/mL. Each well of the 24-well microtiter plate contained approximately 2 x $10^5$ cells. The cells were then incubated for 24 h at 37°C, 5% $CO_2$. Before the introduction of bacteria, media were replaced with serum-free DMEM without penicillin-streptomycin. Each set of triplicate wells received 2.5 x$10^7$, 5 x $10^7$, or 7.5x$10^7$ CFU of bacterial strain and cells were incubated for 20 min at 37°C, 5% $CO_2$. To synchronize infection, cells were centrifuged for 5 min at 411 x g, 10°C. Old media were replaced with fresh complete DMEM containing 15mg/mL gentamicin. For t=0 h, medium containing 15 g/mL gentamicin was removed after incubating at room temperature for 5 min, followed by washing three times with PBS (pH 7.4) to remove extracellular bacteria. Infected macrophages were lysed by the addition of 500 mL of 0.5% (v/v) Tween-20. The lysate was transferred to a clean tube and each well was rinsed with an additional 500 L of PBS (pH7.4), which was then added to the tube containing the initial lysate. Ten-fold serial dilutions were performed on the inoculum and each lysate, and dilutions were plated on TSA or TSA containing the appropriate antibiotics. Macrophage survival assay for each mutant was performed in 3-4 separate experiments.

**Infection of mice.** Groups of eight 6-to-8-week-old female BALB/c mice (Jackson laboratories, Maine) were infected intraperitoneally (i.p.) with a dose of $10^6$ CFU of the mutant per animal. Non-vaccinated, control mice received only PBS (pH 7.4) intraperitoneally. At various time points (8, 16, and 24 weeks post-vaccination), sera were collected to test for antibody before the 21-days of treatment with doxycycline (DOX) (50 mg doxycycline/ kg of body weight per 12 h, oral). In order to determine the

length of time required for doxycycline to clear from the mice, DOX-treated animals were given a challenge infection of S2308 at 2, 10, and 15 days post-DOX treatment. A control group of untreated animals also received a challenge infection of S2308. The clearance of doxycycline within the host was established by similar levels of recovered splenic S2308 observed at one week post-challenge in animals of the 15-day-post-DOX-treatment group and in those of the untreated group. In selected experiments, groups of four-to-five vaccinated and non-vaccinated mice were sacrificed two weeks after the DOX treatment, in order to obtain spleen cells for proliferation and cytokine production assays. Sera were also collected and spleens were aseptically removed and weighed. Splenocytes were harvested from one half of the spleen; the other half was homogenized in 250 μL of PBS (pH 7.4) and serial ten-fold dilutions were plated on TSA or TSA supplemented with kanamycin (100 μg/mL) plates in order to determine the number of viable brucellae remaining in the spleens. Other groups of vaccinated mice and the control group of non-vaccinated animals after two weeks of DOX treatment received a challenge infection of *B. abortus* S2308, in which each animal received a challenge dose of $10^4$ CFU S2308. At one week post-challenge, sera were collected and mice were sacrificed. Spleens were then aseptically extracted, weighed and processed for bacterial count. Those of the S2308-infected groups were also included for comparison.

**Detection of *Brucella*-specific antibodies.** Sera of vaccinated mice (5 mice per group) were collected at two weeks after doxycycline treatment. Sandwich ELISA was performed in 96-well flat-bottom polystyrene microtiter plates (Costar, Massachusetts).

Wells were coated with 10 μg of BACL and incubated for 3 h at 37°C. Excess binding sites were then blocked with 1% (w/v) casein (Fisher Scientific, Maryland) in PBS for 1 h at 37°C. Wells were washed with PBS between steps to remove unbound materials. The antigen-coated wells were incubated with serial two-fold dilutions of primary antibodies (sera from mice) for 16 h at room temperature (25°C). The plates were then incubated with phosphatase-labeled goat anti-mouse immunoglobulins, IgG(H+L), $IgG_1$(□1), or $IgG_{2a}$(□2a) for 20 h at room temperature using protocols obtained from Southern Biotech (Alabama). The values of IgG, $IgG_{2a}$ and $IgG_1$ were calculated from the cutoff values (IgG=0.737, $IgG_{2a}$=0.150, and $IgG_1$=0.151) that were generated as follows. Sera obtained from 5 (naïve) control animals were diluted 1:100 and detected by standard ELISA procedure (20). The cutoff value is the sum of the mean $OD_{450}$-readout result obtained from serum samples of 5 control animals plus 3 times the average standard deviation. For each sample, 2-fold serial dilutions were performed before adding to the ELISA plate. The titer was calculated by transforming the highest 2-fold dilution that has an $OD_{450}$-readout value above the cutoff value to the titer $\log_2$.

**Splenocyte proliferation and cytokine production.** One-half of individual spleens from vaccinated/DOX-treated mice and naïve control animals were ground lightly (7). After lysis of erythrocytes with ACK lysis buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 100 mM $Na_2EDTA$, pH 7.2-7.4), cell suspension was washed three times in RPMI-1640 medium. Then the concentration of cells was adjusted to 2 x $10^6$ cells/ml with complete RPMI-1640 containing 25 mM HEPES, 2 mM L-glutamine, 10% (v/v) heat-inactivated

(for 30 min at 56°C) fetal bovine serum (Difco), and 5.5 x $10^5$ M 2-mercaptoethanol in the presence of 100U penicillin and 10 mg streptomycin (Difco). For proliferation, 2 X$10^5$ cells were cultured with 2 μg of concanavalin A (ConA), 1 X $10^8$ heat-killed *B. abortus* S2308, or 1 μg of *Brucella* lysate in each well of a 96-well flat-bottom tissue culture treated plate (Corning, New York) for 5 days at 37°C, 5% $CO_2$. Cells in control wells received complete RPMI-1640 only (as background control). On day 5, cultures were pulsed with 1 μCi of [$^3$H]-thymidine for 18 h. Cells were then harvested onto glass fiber filters (Packard). These filters were placed into vials containing scintillation cocktail, and incorporated radioactivity was measured by liquid scintillation counting in Beckman LS6000 Series Scintillation System (California). For cytokine production by splenocytes, 4 x $10^6$ cells were cultured as described in a 24-well tissue culture treated plate (Costar, Massachusetts) for 3 days at 37°C, 5% $CO_2$. Cell suspensions were harvested and assayed for cytokine levels. Filtrates were analyzed by ELISA for IL-2, IL-4, IL-10, and IFN-gamma using monoclonal antibody pairs (eBioscience, California).

**Statistical methods.** For macrophage survival experiments, results obtained at t=24h and 48h of the macrophage survival assay were calculated in relative to t=0 and presented as CFU of *t* / CFU of t=0 ratios. The significant differences between groups were analyzed by the one-way ANOVA using Fisher's LSD pairwise comparisons while the differences in the survival of a strain between t=24 and t=48 were analyzed by the Student's *t* test of one-tailed distribution and two-sample equal variance.

For the *in vivo* experiments including the survival of mutants in the moue model

and the vaccine potential, data reported from spleens of immunized versus non-immunized animals were expressed as mean CFU $\pm$ standard error (SE); as noted, culture-negative spleens were assigned a value of 1 CFU $\pm$ 1 SE. These data were graphically presented as CFU of *Brucella* per spleen. Spleen weights of immunized and control mice were also recorded and the average spleen mass is calculated from groups of four-to-five mice and presented in grams with $\pm$standard error. Statistical analyses were performed using the Student's *t* test with one-tailed distribution and two-sample equal variance.

For the measurements of IgG, $IgG_{2a}$ and $IgG_1$, results obtained were presented graphically as titer $\log_2 \pm$ SE. Statistical analyses of titers were performed using the Student's *t* test with one-tailed distribution and two-sample equal variance.

For the proliferation experiments and the cytokine profiling experiments, data obtained from the [$^3$H]-thymidine cultured-splenocytes were expressed as mean cpm. Results of cytokine induction in (heat-killed *B. abortus*) HKBA-stimulated splenocytes were calculated along with individual cytokine standards and graphically presented as units (pg/mL) $\pm$ SE.

## Results

**Growth characteristics of attenuated STM mutants**. STM mutants BA114 (*virB10*) and BA102 (*gcvH*), were originally characterized as early and late mutants due to survival defects at two weeks and eight weeks, respectively (69). Our previous results showed that these two mutants did not differ from the wild type in growth on either

Gerhardt's minimal medium or in TSB (69). We were therefore interested in determining whether the different survival characteristics in mice correlated with the differences in the ability to survive within macrophages. The *in vitro* growth in macrophages was evaluated using the murine macrophage-like cell line J774.A1. The macrophage-killing assays revealed that BA102 and BA114, as well as all other strains tested including BA582, which is defective in cytochrome d oxidase (46), and the control strains S19 (vaccine) and S2308 (challenge), were reduced by approximately 2 logs at 24 h after infection (Table 3, Fig. 3). However, CFU of S19 and S2308 strains increased significantly between 24 and 48 h ($p<0.0212$ and 0.0210, respectively) while much less growth was apparent with BA102 and BA582 ($p<0.0465$ and 0.0804, respectively). In contrast, the bacterial load of BA114 in macrophages continued to decline (one-log reduction) until 48 h post-infection. The results show that BA102 (*gcvH*), BA582 (*cydB)* and S19 are moderately attenuated for growth in macrophages, while BA114 (*virB10*) has a more marked defect in the intracellular persistence.

**Survival of *gcvH* and *virB10* mutants in the mouse model**. Long-term persistence in the host of fortuitously isolated vaccine strains including *B. abortus* S19 and *B. melitensis* Rev-1 has been offered as one explanation for their success in stimulating immune protection. In order to compare the vaccine potential of strains with similar genetic backgrounds yet distinctly different survival characteristics, the persistence of the STM mutants, BA102 (*gcvH*) and BA114 (*virB10*), in the spleen was evaluated using

**Table 3:** *Brucella abortus* strains involved in the study

| Strain | Description, Source/Reference |
|---|---|
| S2308 | Wild-type *B. abortus* (biovar 2) that was used as the parental strain for STM mutant bank, obtained from B. L. Deyoe, National Animal Disease Center. |
| S19 | *B. abortus* vaccine strain 19 obtained from Ames, Iowa. |
| BA102 | *B. abortus* signature-tagged mutagenesis (STM) mutant carrying a single mini-Tn5 cassette insertion in the *gcvH* gene (69). |
| BA114 | *B. abortus* signature-tagged mutagenesis mutant carrying a single mini-Tn5 cassette insertion in the *virB10* gene (69). |
| BA582 | *B. abortus* cydB::Tn5 (46). |

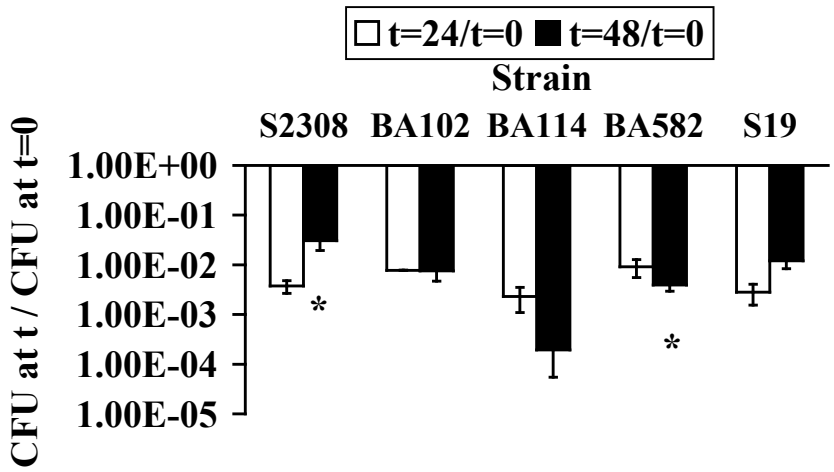



**Fig. 3.** Intracellular survival of *B. abortus* STM mutants. Survival of each *Brucella* strain (*B. abortus* S2308, BA102 (*gcvH*), BA114 (*virB10*), BA582 (*cydB*), and vaccine S19) in the murine macrophage-like cell line J774A.1 is graphically presented by the ratio of the average CFU at *t* to the average CFU at t=0. The average CFU recovered from infected macrophages at each *t* was calculated from a triplicate set and the assay was performed as 3-4 separate experiments. In each experiment, $10^7$ CFU per strain was used to infect $10^5$ macrophages, and t=0 was taken after the 5-min room temperature incubation. Statistical analyses were performed as follows. One-way ANOVA followed by the Fischer's LSD pairwise comparisons was used to compare the differences in the ability to survive intracellularly between strains (S2308 vs. BA102, BA114, BA582, and S19); the p-values $<0.05$ were considered statistically significant. The Student's *t* test with one-tailed distribution and two-sample equal variance was used to compare the differences in the survival of a strain within the macrophage between t=24 and t=48; *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

the BALB/c mouse model. In an effort to compare strains for potential vaccine use, strains exhibiting various survival characteristics were chosen. The *cydB* (BA582) and *virB10* (BA114) mutants have been shown to exhibit a large reduction in numbers of splenic brucellae between one and two weeks after inoculation; and by 8 weeks post-infection few, if any, bacteria were recoverable ((46), Fig. 4). In contrast, the *gcvH* (BA102) mutant dramatically decreased in number in the spleens of mice over the first 16 weeks post-inoculation and then cleared much more gradually over the next 8 weeks, i.e., showing a slight decrease in numbers between 16 and 24 weeks post-inoculation. Thus, the decline in CFU of BA102 distinguished it from the early-clearing BA114, which was below the level of detection by 16 weeks post-inoculation.

**Vaccine potential (induced protective immunity) of attenuated mutants.** The attenuated *in vivo* survival of STM mutants is an attractive attribute for live vaccine candidates against brucellosis. To be effective, live attenuated vaccines must persist long enough to elicit protective immunity, yet they should be cleared as quickly as possible to avoid unnecessary side effects. In order to determine whether greater persistence correlates with greater protection, the ability of selected mutants to protect mice against a wild-type *B. abortus* 2308 challenge infection examined. Since both the duration of bacterial colonization and the level (CFU) of colonization of these strains differed, (Fig. 4), it was difficult to relate the potential differences in the level of protection among these strains solely to the level of colonization or the duration of colonization by the vaccine strain that exhibit such a wide range of *in vivo* persistence

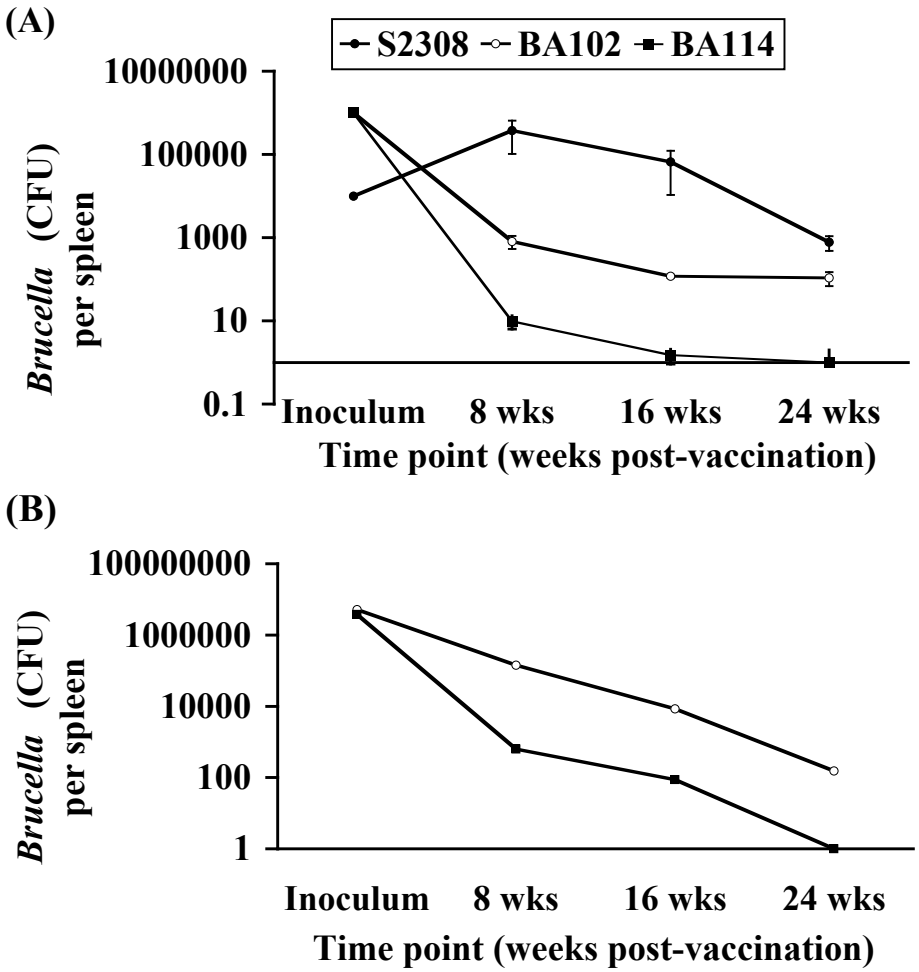



**Fig. 4.** Survival curves of individual STM mutants in BALB/c mice at 8, 16, and 24 weeks post-vaccination. (A)followed by 21 days of doxycycline treatment (50 mg DOX/kg of body weight per 12 h, oral), (B)without doxycycline treatment. Each animal was inoculated with $10^6$ CFU per mutant strain or $10^4$ CFU S2308. At 8, 16, and 24 weeks post-inoculation, animals were either treated not treated with doxycycline for 21 days. Two weeks post-treatment, all mice were sacrificed and spleens were extracted aseptically for bacterial counts. Each data point represented the average CFU recovered from spleens of 4-5 mice. Statistical analysis was performed using the Student's *t* test with one-tailed distribution and two-sample equal variance; the vertical bars show the standard errors. STM, signature-tagged mutagenesis; p.v., post-vaccination.

these two variables. In order to reduce the bacterial burden of the persisting strain prior to challenge and set a defined period of vaccine strain colonization, oral treatment with doxycycline (DOX) was administered daily for 21 days at 8, 16, or 24 weeks post-vaccination (Fig. 5). To ensure that low levels of DOX that might persist after cessation of the treatment would not affect the persistence of the challenge inoculum, a control experiment was performed, in which groups of mice that had been treated with DOX two weeks previously or untreated control animals were inoculated with $10^4$ CFU of *B. abortus* 2308. The survival of the challenge strain 2308 in animals at two weeks post-DOX treatment was not significantly different from that in the untreated animals (Fig. 6).

The level of protection conferred by vaccination with BA114 (*virB10*), BA102 (*gcvH*), BA582 (*cydB*) or Strain 19 was compared by enumeration of splenic S2308 at one week after challenge (Fig.7). The control group of unvaccinated animals showed a recovery of nearly $10^7$ CFU of S2308 per spleen one week post-challenge, which is approximately a 3-log increase in the bacterial load in comparing to the inoculum dose. In contrast, BA102- and BA114-vaccinated animals of the 8-week-post-vaccination time point exhibited significantly lower splenic loads of S2308 at one week post-challenge when compared to those of the control group (p-values: 0.0077 and 0.0079, respectively). At 16 weeks post-vaccination, both BA102- and BA114-induced protection against challenge reached a maximum with no discernable difference between these two mutants in the level of protective immunity, despite an almost 2-log difference and bacterial burden. Therefore, an experimental design was used to allow separation of

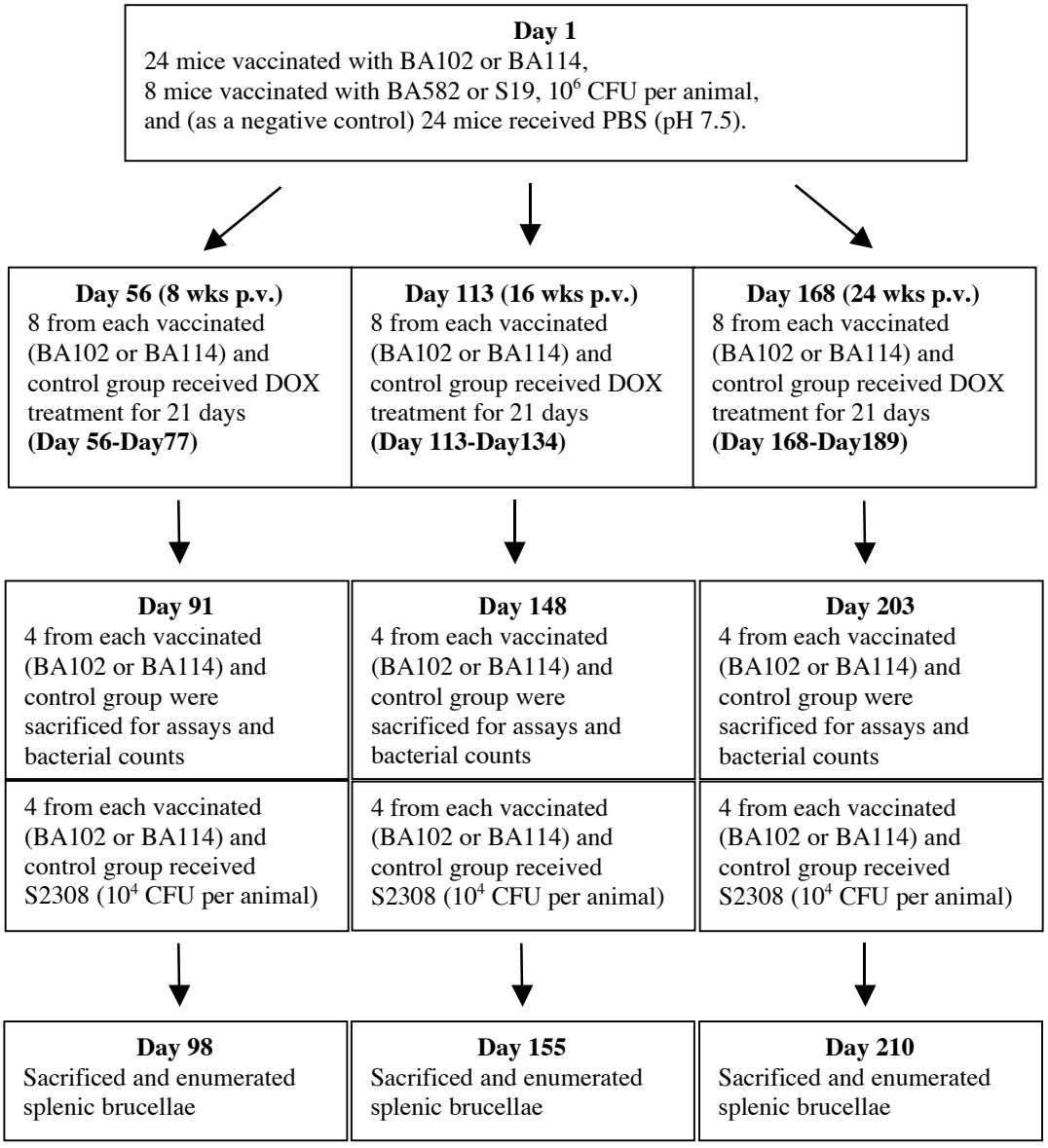
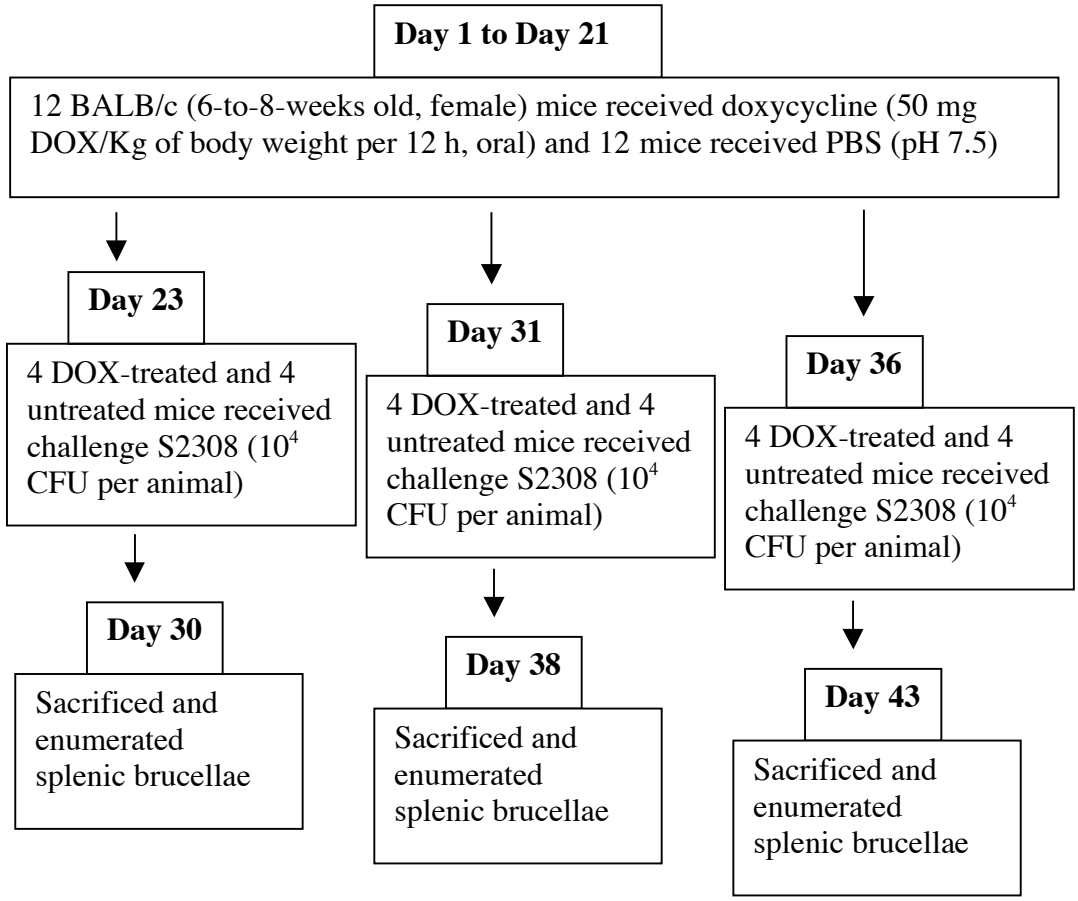


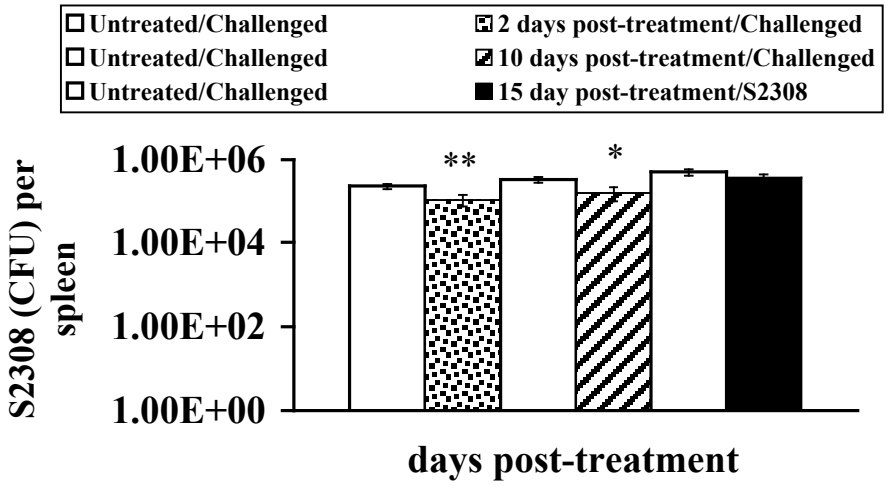

**Fig. 5.** The time line of vaccination, doxycycline treatment, and challenge infection with *B. abortus* S2308. p.v., post-vaccination; doxycycline treatment, 50 mg DOX/kg of body weight per 12h (oral).

**Fig. 6.** Doxycycline clearance. (A) Time course study to determine the clearance of doxycycline from the host, (B)The effect of doxycycline in mice at 2, 10, and 15 days after treatment. Groups of 6-to-8-week old female BALB/c mice were treated for 21 days with doxycycline (50 mg DOX/kg of body weight per 12 h, oral). At 2, 10, and 15 days after treatment, animals were inoculated with *B. abortus* S2308. As a control, untreated mice were also received challenge infection with S2308. One week post-challenge, animals were sacrificed for bacterial counts of splenic brucellae. Statistical analysis was performed using the Student's *t* test with one-tailed distribution and two-sample equal variance. The vertical bars show the standard errors. The p-values indicate the differences between the untreated group and the group that received DOX treatment 2, 10, or 15 days previously: *, $p<0.05$; **, $p<0.01$.

**A**


Day 1 to Day 21
12 BALB/c (6-to-8-weeks old, female) mice received doxycycline (50 mg DOX/Kg of body weight per 12 h, oral) and 12 mice received PBS (pH 7.5)
Day 23
4 DOX-treated and 4 untreated mice received challenge S2308 ($10^4$ CFU per animal)
Day 30
Sacrificed and enumerated splenic brucellae
Day 31
4 DOX-treated and 4 untreated mice received challenge S2308 ($10^4$ CFU per animal)
Day 38
Sacrificed and enumerated splenic brucellae
Day 36
4 DOX-treated and 4 untreated mice received challenge S2308 ($10^4$ CFU per animal)
Day 43
Sacrificed and enumerated splenic brucellae

**B**


Untreated/Challenged
Untreated/Challenged
Untreated/Challenged
2 days post-treatment/Challenged
10 days post-treatment/Challenged
15 day post-treatment/S2308
S2308 (CFU) per spleen
1.00E+06
1.00E+04
1.00E+02
1.00E+00
**
*
days post-treatment

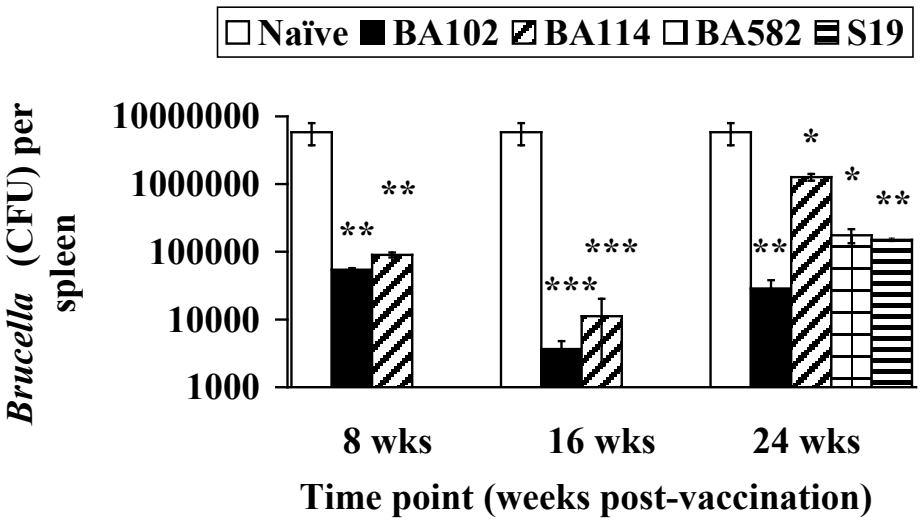



**Fig. 7.** Recovery of *B. abortus* S2308 from vaccinated animals one week after challenge. At each time point (8, 16, and 24 weeks post-vaccination), animals were treated with doxycycline (DOX) for 21 days (50 mg DOX/kg of body weight per 12 h, oral). Animals of the unvaccinated control group were also received DOX treatment. At 2 weeks post-treatment, groups of 4-5 mice received a challenge infection with *B. abortus* S2308 intraperitoneally at a dosage of $10^4$ CFU per animal. Following one week post-challenge, mice were sacrificed and bacteria were harvested from spleens. The total CFU of S2308 recovered was calculated from the difference between the total CFU on TSA plates and the total CFU on TSA containing kanamycin. Statistical analysis was performed using the Student's *t* test with one-tailed distribution and two-sample equal variance; the vertical bars show the standard errors. The p-values indicated the significant differences between the control/DOX group and the mutant-vaccinated/DOX-treated group: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

in the persisting bacterial load between these strains prior to challenge. However, by 24 weeks post-vaccination, the BA102-induced protective immunity against challenge remained significantly high at approximately 2-logs greater than the protection induced by BA114 mutant. Nevertheless, the recovered splenic S2308 load in BA114-vaccinated mice of the 24-week post-vaccination time point was reduced significantly when compared to naïve animals at one week after challenge (p-value: 0.0210). For comparison, S19-vaccinated mice exhibited levels of protection intermediate to those of BA102- and BA114-vaccinated animals.

In mice, infection with *Brucella* has been shown to induce an inflammatory response and trigger the recruitment of macrophages in the spleen, resulting in splenomegaly. Hence, spleen mass, as a result of live-strain inoculation, can serve as an indicator for the severity of the infection, which often corresponds to the bacterial burden. Thus, mice inoculated with attenuated organisms are expected to exhibit reduced splenomegaly while those inoculated with S2308 will show a pronounced level of spleen-enlargement. Overall, vaccinated mice after receiving a challenge infection exhibited a greatly reduced splenomegaly when compared to the unvaccinated animals after challenge, i.e., 25-50% and 100% increase in spleen mass, respectively. However, measuring protection by the degree of splenomegaly proved to be less sensitive than that by enumeration of brucellae; particularly, when compared the BA102-inoculated to the BA114-inoculated animals, those vaccinated with BA114 appeared to show better protection against splenomegaly than against colonization (Table 4, Fig. 7).

**Table 4:** Spleen mass of vaccinated/DOX-treated BALB/c mice

| Group | Spleen mass (g) before challenge[1] | | | Spleen mass (g) post-challenge[2] | | |
|---|---|---|---|---|---|---|
| | 8 wks p.v. | 16 wks p.v. | 24 wks p.v. | 8 wks p.v. | 16 wks p.v. | 24 wks p.v. |
| Control | 0.143 ±0.022 | | | 0.324 ±0.051 | | |
| BA102 | 0.163 ±0.007 | 0.133 ±0.030 | 0.188 ±0.025 | 0.186 ±0.018 | 0.207 ±0.008 | 0.266 ±0.023 |
| BA114 | 0.0860 ±0.010 | 0.115 ±0.014 | 0.116 ±0.010 | 0.162 ±0.020 | 0.130 ±0.011 | 0.136 ±0.028 |
| S2308 | 0.220 ±0.036 | 0.190 ±0.017 | 0.178 ±0.018 | N/A | | |
| BA582 | N/A | N/A | 0.147 ±0.029 | N/A | N/A | 0.203 ±0.017 |
| S19 | N/A | N/A | 0.145 ±0.015 | N/A | N/A | 0.192 ±0.028 |

[1]8, 16, or 24 weeks post-vaccination plus 21 days of doxycycline treatment and two additional weeks post-doxycycline treatment.
[2]One week post-challenge.
p.v., post-vaccination.
N/A, no animals were tested.

**Antibody subclass as a measure of T-helper cell driven response.** We have demonstrated that two STM mutants BA102 (*gcvH*) and BA114 (*virB10*), exhibiting different survival characteristics in the mouse model and the murine macrophage-like cell line J774.A1, stimulated protection against wild-type *B. abortus* S2308. However, BA102 (*gcvH*), the longer-persisting mutant, appears to provide a longer-lasting protective immunity. Therefore, in order to characterize factors contributing to this increased protection, the humoral response IgG isotypes and the cellular responses including lymphocyte proliferation and cytokine profiles of T cell-mediated immune responses ($T_H1/T_H2$) were evaluated in vaccinated mice after the DOX treatment and after challenge. *Brucella*-specific IgG was present at similar levels in all vaccinated groups before and after doxycycline treatment (Fig. 8). The levels of $IgG_{2a}$ isotype in vaccinated animals showed no significant changes throughout the experiment (Table 5). However, the $IgG_1$ levels were significantly different between animals vaccinated with BA114 and those vaccinated with BA102. At 24 weeks post-vaccination and followed by 21 days of DOX treatment, BA114-vaccinated mice exhibited a significant increase of $IgG_1$ while BA102-vaccinated animals showed a reduction in $IgG_1$. In general, when $IgG_{2a} \geq IgG_1$, the response will favorably shift toward $T_H1$ while a $T_H2$-driven response is ensured when $IgG_{2a} < IgG_1$ (119). Taken together, unlike BA102, the rapidly clearing mutant BA114 appeared to elicit an immune response that has characteristics of $T_H2$ at 24 weeks post-vaccination, which may reflect the loss of memory response over time and hence leading to the decline in protective immunity observed in the BA114-vaccinated groups.

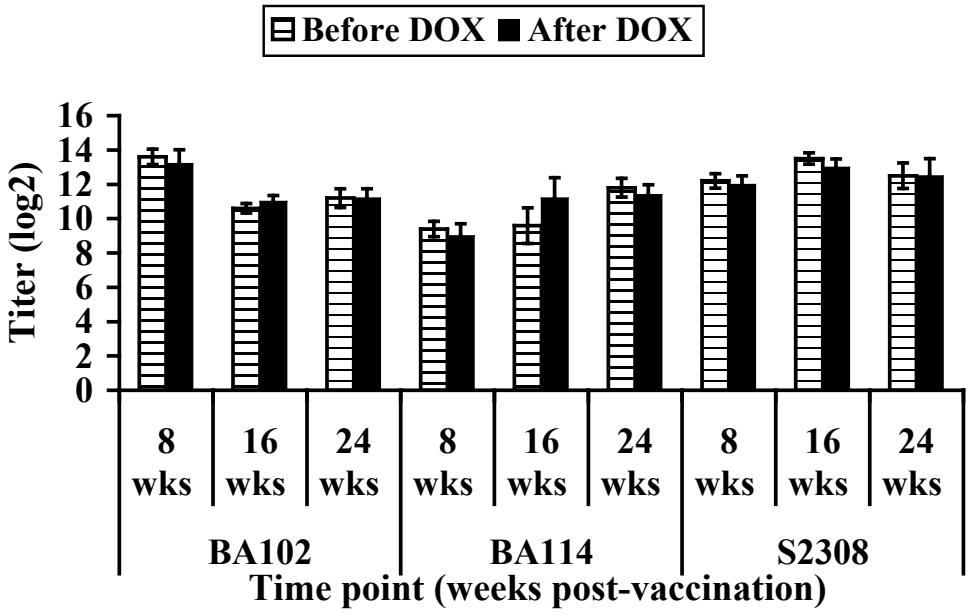



**Fig. 8.** Anti-*Brucella* antibody (IgG) in vaccinated BALB/c mice before and after doxycycline treatment. At 8, 16, and 24 weeks post-vaccination (Day 56, 113, and 168, respectively), sera from vaccinated mice were collected and stored at –70°C until use. Two weeks post-doxycycline treatment (Day 91, 148, and 203, respectively), sera were collected. Sandwich ELISA using phosphatase-labeled goat anti-mouse IgG(H+L) was performed and the values of IgG were calculated from the cutoff value of 0.737, which was generated from sera of five naïve control animals. Statistical analysis was performed using the Student's *t* test with one-tailed distribution and two-sample equal variance. The vertical bars indicate the standard errors.

**Table 5:** Detection of *Brucella*-specific IgG isotypes

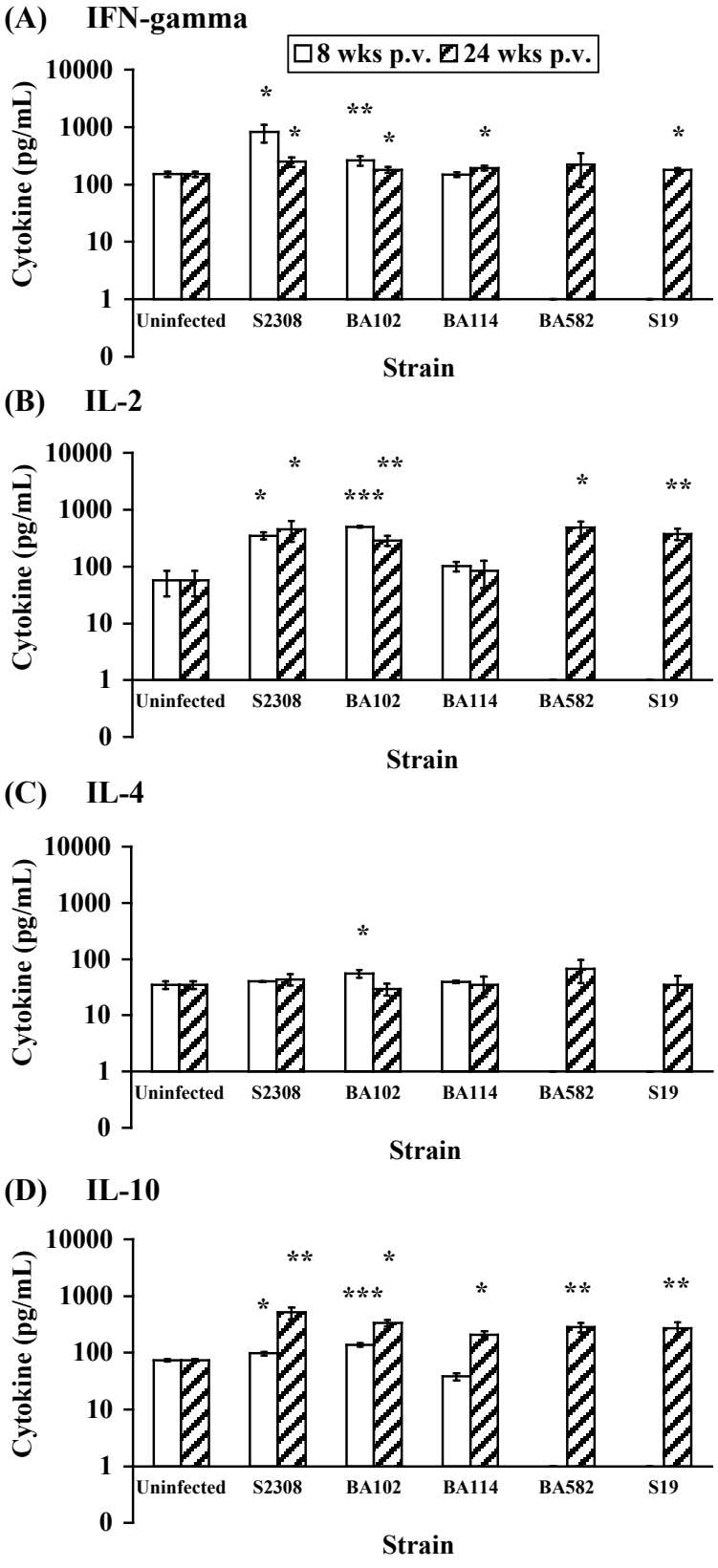
| | Naïve | BA114 | BA102 | BA114 | BA102 |
|---|---|---|---|---|---|
| [1]Before Challenge | | 8 wks p.v. | 8 wks p.v. | 24 wks p.v. | 24 wks p.v. |
| $IgG_{2a}/IgG_1$ | 0.7 | 1 | 1.03 | 0.8 | 1.1 |
| $IgG_{2a}$ | 11.2 | 11.4 | 17 | 12.2 | 15.6 |
| $IgG_1$ | 16 | 11.2 | 16.6 | [1*]15.6 | [1*]13.4 |
| [2]After Challenge | | | | | |
| $IgG_{2a}/IgG_1$ | 1.1 | [2*]1.3 | [2*]1.3 | [2*]1 | [2*]1.3 |
| $IgG_{2a}$ | 8.2 | 13.2 | 16.6 | 15.8 | 15.6 |
| $IgG_1$ | 7.6 | 12.4 | 14 | 16 | 12.4 |

[1]8 or 24 weeks post-vaccination (8, 24 wks p.v.) plus 21 days of doxycycline treatment and two additional weeks post-doxycycline treatment.
[2]One week after challenge.
[1]*Significant differences before challenge between the vaccinated groups of the 8-week post-vaccination and the 24-week post-vaccination times points; p-values: 0.0011 (BA114) and 0.0027 (BA102).
[2]*Significant differences after challenge between the vaccinated group and the control group (naïve animals).

**Cytokine production**. Cytokine induction (IL-2, IL-4, IL-10, and IFN-gamma) was evaluated using sandwich ELISA of supernatants from lymphocytes. By eight weeks post-inoculation, mice infected with the attenuated BA102 as well as S2308 exhibited significantly increased production of the $T_H1$ cytokines (IFN-gamma, and IL-2), yet no significant induction was observed for IL-4 in response to *Brucella* lysate (Fig.9A, B, C). However, by 24 weeks post-vaccination splenocytes from all vaccinated mice exhibited a slight change in cytokine profiles showing a significant increase in the $T_H2$-cytokine IL-10 (Fig. 9D). The increase in IL-10 does not appear to parallel the observed changes in the $IgG_{2a}$ and $IgG_1$, which showed a $T_H1$ memory response using sera obtained from vaccinated mice. It is not clear whether the difference could be the result of comparing *in vivo* and *in vitro* readouts in the protocols that employ viable organisms (*in vivo*) and either heat-killed organisms or lysates (*in vitro*).

**Lymphocyte proliferative responses.** The T cell-mediated memory immunity resulting from vaccination with attenuated mutants was evaluated in response to either heat-killed *B. abortus* (HKBA) or *B. abortus* cell extracts (BACL). All animals including all vaccinated groups, S2308-infected group, and the unvaccinated control group were given daily doses of doxycycline for three weeks in helping to modulate the bacterial load of the inoculated strain, particularly, the more persistent *gcvH* mutant, and were then left untreated for two weeks to permit the clearance of the antibiotic. Although no significant differences in lymphocyte proliferation response were observed between the BACL and HKBA-stimulated lymphocytes, HKBA-stimulation provided more

**Fig. 9.** Induction patterns of cytokines in BALB/c mice at 8 weeks and 24 weeks post-vaccination followed by 21 days of doxycycline (DOX) treatment (A)IFN-gamma, (B)IL-2, (C)IL-4, and (D)IL-10. Splenocytes were harvested at 2 weeks post-DOX treatment and cultured for 3 days. ELISAs for IFN-gamma, IL-2, IL-4, and IL-10 were performed using monoclonal antibody pairs. Statistical analysis was performed using the Student's *t* test with one-tailed distribution and two-sample equal variance. The vertical bars show the standard errors. The p-values indicated the significant differences in cytokine induction observed between the control (unvaccinated)/DOX-treated animals and the vaccinated/DOX-treated animals: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.


(A) IFN-gamma
8 wks p.v.
24 wks p.v.
Cytokine (pg/mL)
10000
1000
100
10
1
0
Uninfected
S2308
BA102
BA114
BA582
S19
Strain
(B) IL-2
Cytokine (pg/mL)
Strain
(C) IL-4
Cytokine (pg/mL)
Strain
(D) IL-10
Cytokine (pg/mL)
Strain

consistent results and will be discussed here. Lymphocytes from all groups of S2308-infected or attenuated mutant-vaccinated mice consistently responded to heat-killed brucellae (HKBA) over the course of the experiment (Fig. 10). At 24 weeks post-infection, T cell-mediated memory responses in animals vaccinated with two other strains, BA582 mutant and the vaccine strain *B. abortus* S19, were also evaluated. Both strains, BA582 and S19, are attenuated in mice with different rates of clearance from the spleens. All vaccinated animals and S2308-infected mice of the 24-week post-vaccination time point showed a strong lymphocyte proliferation response, despite the differences in the survival characteristics of each inoculum strain. The greatest proliferation response was observed in animals vaccinated with a longer-surviving mutant BA102 as well as in those infected with S2308. Mice vaccinated with S19, BA114, and BA582 also exhibited lymphocyte proliferation suggesting that these strains, which clear as quickly as 2 weeks post-inoculation, exhibit a sufficient burst during the expansion phase to elicit a long-term memory response.

**Discussion**

Since both *M. tuberculosis* and *Brucella* spp. are intracellular pathogens causing chronic diseases, it is tempting to speculate that chronic brucellosis may be similar to tuberculosis, i.e., with "non-replicating" organisms in the host. Lack of a functional enzyme of the glycine cleavage system renders the *gcvH* mutant (BA102) incapable of long term persistence in mice, yet has no effects on *in vitro* growth (69). This non-auxotrophic mutation resulted in an inability to maintain chronic infections *in vivo* and

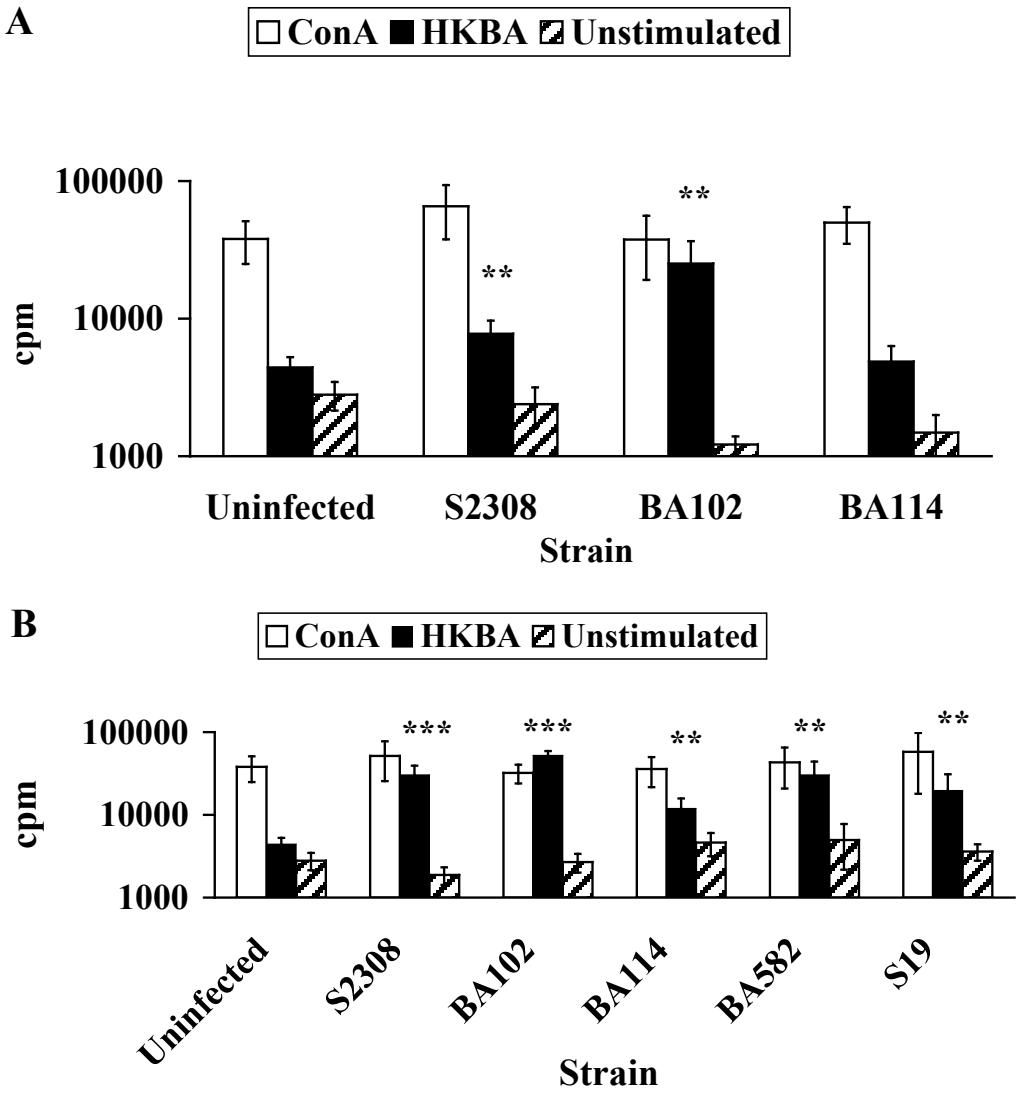



**Fig. 10.** Proliferation of splenocytes in BALB/c mice. (A)8 weeks post-vaccination, (B)24 weeks post-vaccination followed by 21 days of doxycycline treatment. Within each vaccinated/DOX-treated group of 4-5 mice, unstimulated splenocytes cultured in [3H]-thymidine were included as negative controls to compare to those that were stimulated with the heat-kileed S2308 (HKBA), or Concanavalin A (ConA) for 3 days. Results obtained from HKBA-stimulated splenocytes of the control group establish the threshold value for the proliferation response (4412.07$\pm$829.07 cpm). Statistical analysis was performed using the Student's *t* test with one-tailed distribution and two-sample equal variance. The vertical bars show the standard errors. The p-values indicate the significant differences between HKBA-stimulated control (unvaccinated) groups and HKBA-stimulated mutant-vaccinated groups: *, $p<0.05$; **, $p<0.01$, ***, $p<0.005$.

also prevented normal replication of the mutant in macrophage culture supporting the contention that chronic infections of hosts are a result of parasitism of macrophages (17) (74). However, differences in the intracellular survival of BA102 within the macrophage were not as apparent compared to those of the other *in vivo* attenuated mutants (Figure 1). All strains tested exhibited a decline in numbers (between 90 and 99%) over the first 24 hours of infection. But the differences were most apparent over the next 24 hours during which the *virB10* mutant (BA114) continued to decline while the parental strain S2308 recovered in numbers. This observation is in agreement with several studies of type IV secretion system (T4SS) in *Brucella*, in which the *virB* operon plays a critical role in the intracellular multiplication and virulence (79) (127). The T4SS of *B. abortus* has been shown to be required for modulation of the intracellular trafficking within HeLa cells (29), but not evasion of nitrosative or oxidative bactericidal killing by macrophages (136).

In contrast, inactivation of the *gcv* operon is expected to prevent adaptation to low oxygen environments. Consistent with an anaerobic state for *Brucella* is the observed requirement for cytochrome *bd* oxidase during intracellular growth (46). Under conditions of reduced oxygen found in inflammatory and necrotic tissues, glycine dehydrogenase may serve to regenerate NAD from a reduced form, which provides ATP to support the completion of a final cycle of DNA synthesis before the shutdown of replication, and thus may be a factor contributing to the latency of the disease (151) (149). This may explain why BA102 only persisted in the mouse spleens as long as 24weeks post-infection. The mutation in *gcvH* also appears to cause growth restriction

of the mutant in the macrophage, despite the presence of a functional T4SS. Such growth restriction has been observed in wild-type *E. coli* strains harboring non-functional selenolipoylated H-protein of the glycine cleavage system, in which both of the sulfur atoms of the protein were replaced by selenium atoms (57). Taken together, the presence of a functional glycine cleavage system is vital to the survival of *Brucella in vivo* and thus, is consistent with the need for additional factors for virulence.

Having two mutants with similar genetic backgrounds and possessed of different survival characteristics provided an opportunity to compare the vaccine potential with regard to the persistence of the strain. The efficacy of these live vaccine candidates to induce protection was also accompanied by a reduced splenomegaly. Both mutants carrying defined mutations, which led to reduced persistence in the host, have the ability to provide protection against wild type *B. abortus* and yet cause very minimal, if any, in the host.

To better understand the differences in protective immunity induced by these mutants, the fundamentals of host immune responses were examined and compared. The host antibody response showed no differences in IgG levels between animals vaccinated with the persistent BA102 and those vaccinated with the short-lived BA114. All vaccinated animals exhibited a constant level of anti-*Brucella* antibody, IgG, throughout the course of the study. This result indicates that the *Brucella* antigen remains in circulation for an extended period even after bacteria including those of the short-lived BA114 are cleared from the host. A similar observation has been documented in patients clinically cured of acute brucellosis, in which *Brucella*

antibodies persisted for more than 2 years after successful treatment and clinical cure (2).

Typically, during the course of an immune response, different immunoglobulin classes and subclasses are expressed. Initially, IgM is secreted and IgG levels subsequently increase. In the presence of IL-4, a product of $T_H2$ cells, B cells switch from IgM to $IgG_1$ secretion. In contrast, IFN-gamma, a product of $T_H1$ cells, is associated with switching from IgM to $IgG_{2a}$. Thus, stimulation of $T_H1$ or $T_H2$ cells results in expression of different immunoglobulin classes and isotypes. Live and heat-killed brucellae have been shown to elicit $T_H1$-type host responses in both humans and mice, (137) (156, 157).

In this study, the evaluation of the adaptive immune response focused on the development of $T_H1$ vs. $T_H2$ immune responses, through an examination of the $IgG_{2a}$ and $IgG_1$ levels and the cytokine profiles. Vaccination with either short-term or long-term surviving mutants appeared to favor a $T_H1$ memory response, which is characterized by the steady levels of $IgG_{2a}$ and thus, consistent with pattern in all *Brucella*-inoculated animals, i.e., $IgG_{2a}>IgG_1$. However, by 24 weeks post-vaccination, the $IgG_1$ levels in BA114-vaccinated animals were significantly increased. This sudden change observed in the $IgG_1$ isotype might be a result of the diminution of $T_H1$ response. A transient acquired cellular immunity has also been observed in C3H mice upon the clearance of bacteria from tissues, despite the fact that the C3H mouse model is more resistant to *Brucella* infection than the BALB/c model (106), (155). Recently, the diminution of $T_H1$ cytokine production in humans with chronic brucellosis was reported (60).

There are two possibilities that may help to explain the aforementioned switch in T helper cell response. First, at 24 weeks post-inoculation no live brucellae were present in the mice inoculated with the more rapidly cleared BA114, and thus, the host system may have resumed its original state of which the BALB/c mouse model has been shown to be a $T_H2$-driven response model (23), (52). The fact that the $IgG_{2a}/IgG_1$ ratio actually increased following challenge even at 24 weeks post-vaccination is consistent with a residual $T_H1$ memory response. Secondly, the absence of IL-2 and the late induction of IFN-gamma might be another major contributing factor that allows a more favorable shift towards a $T_H2$ response, as evident by a significant increase in the level of $IgG_1$ by 24 weeks post-vaccination ($IgG_1$ of 8 weeks vs. $IgG_1$ of 24 weeks, p-value: 0.0011). Although protection elicited by both mutants was still significant when compared to that of the unvaccinated animals and also the enhanced antibody production, which might augment the extracellular clearance of *Brucella*, the protection had decreased from a maximal level observed at 16 weeks post-vaccination. However, protection provided by BA102 was still significantly higher than that stimulated by BA114 at 24 weeks post-vaccination. Furthermore, the fact that the former also appears to have an elevated IL-10 response could be explained by the enhanced clearance of the organism by opsonizing antibody. Despite an increase in the synthesis of IL-10, the $IgG_{2a}$-to$IgG_1$ ratio measured from BA102-vaccinated animals at 24 weeks post-vaccination remains favorable towards a $T_H1$ response. Interleukin-10 was once clearly classified as a $T_H2$-associated cytokine that could down-modulate the $T_H1$ response, and now there is mounting evidence that IL-10 may act as an inhibitor of the $T_H2$ response both *in vivo* and *in vitro*

(156) (157). On the contrary, our findings suggest that IL-10 may act as an anti-inflammatory cytokine to buffer the host system during cellular attack, or that perhaps, the effect of IL-10 *in vitro* does not reflect events as they occurred one week after challenge.

In addition to cytokine profiles, results of lymphocyte proliferation suggest that *Brucella* can elicit a long-term memory response regardless of the length of persistence. Proliferative responses were evident even in lymphocytes from animals at 24 weeks after inoculating with a short-lived *Brucella* strain such as BA114. This is somewhat surprising when considering the decline in protection observed in response to vaccination with this mutant long after the bacteria have cleared out of the host. Taken together, our findings suggest that the combination of enhanced levels of $T_H1$-type cytokines (mainly IFN-gamma, IL-2, and IL-12) elicited by BA102 mutant is crucial for the development and the maintenance of $T_H1$ response in the host, particularly, in a *Brucella*-susceptible and non-$T_H1$-driven response host model such as the BALB/c mouse. The strongest protection against *B. abortus* not only requires a finely regulated balance of cytokines, is more dependent upon the timing of the cytokine response rather than on the absolute level of cytokine expression. Finally, our study has demonstrated that the attenuated *B. abortus gcvH* mutant, BA102, conferring the greatest cellular protection against wild type *B. abortus* without exerting unnecessary side effects such as splenomegaly on the host, is a good live vaccine candidate. Future work will be focused on investigating (1) the vaccine potential of unmarked deletion mutants of *Brucella abortus* and *Brucella melitensis gcvH* genes and *virB* operons in the natural hosts as well

as in the pregnant mouse and goat models, and (2) the intracellular trafficking of these mutants in murine macrophage cell lines and goat trophoblasts.

# CHAPTER IV
# THE ISOLATION OF THE *GCVH* HOMOLOGUE IN *BRUCELLA ABORTUS*

## Overview

A signature-tagged mutagenesis mutant, BA102, carrying a miniTn5-insertion that resides in the last 40 bp of the *gcvH* locus in *B. abortus* was identified as an attenuated mutant in a mouse model. The defect in persistence was further supported by the attenuation in intracellular survival, in which BA102 mutant exhibited decreased survival within the murine macrophage J774A.1. The attenuated *gcvH* mutant of *B. abortus* does not require glycine, serine, or threonine supplement for growth in minimal media. Nor does it show any *in vitro* growth defects in liquid media or on plates, when compared to the wild type *B. abortus* S2308. In order to determine whether the *in vivo* attenuated phenotype of the *gcvH* mutant (BA102) can be rescued and restored to that of the wild type *B. abortus* S2308, three complement strains were constructed from mutant BA102, resulting in complemented mutants carrying either the wild type copy of *gcvH*, *gcvP* or *gcvHP* in tandem on a broad-host range vector pBBR1mcs. Similar to the parental mutant strain BA102, all three complemented strains showed normal growth in liquid and on solid media. However, only the complemented mutant carrying both wild type *gcvH* and *gcvP* of *B. abortus* exhibited a small yet significant increase in bacterial load recovered from the infected macrophages. Thus, the result suggested that the intracellular survival defect of the complemented mutant strain carrying both wild type *gcvHP* was partially rescued. Unexpectedly, data from the mixed infection assays of the parental mutant strain revealed that bacteria of all three complemented strains were not

recoverable from spleens of animals eight weeks post-inoculation. The best explanation for this observation is the addiction module phenomenon, in which the stability of the plasmid in the host bacterial cells is maintained by the mechanism for killing plasmid-free bacteria provided by the addiction modules. The broad-host range pBBR1mcs vector, which was used as the backbone of all three constructs carrying wild type *gcvH*, *gcvP*, or *gcvHP*, might have contained a plasmid addiction system and thus the loss of the plasmid overtime from the complemented mutants resulted in the death of the bacterium. Therefore, the complementation of the *in vivo* attenuation of the BA102 mutant could not be determined. An alternative approach that involves constructing a chromosomal integration of wild type *B. abortus gcvH* and *gcvP* in an unmarked double deletional mutant of *B. abortus* is suggested.

**Introduction**

*Brucella* species are Gram-negative α-proteobacteria that reside intracellularly within their respective hosts. Brucellae are highly infectious pathogens that cause abortions and infertility in domestic and wild mammals, and a severe and debilitating zoonotic disease in humans (31). *Brucella melitensis*, *Brucella suis*, and *Brucella abortus* are potential biological warfare agents, and thus, they are a serious concern since there is presently no human vaccine available (55). The persistence of brucellae leads to the chronic phase of brucellosis. Chronic infection caused by the persistence of bacteria is a common trait that is also shared by a human pathogen known as the acid-fast *Mycobacterium tuberculosis*. In the chronic infection process, both species are

endocytosed into host cells where they adapt and survive for extended periods of time within the acidic membrane-bound compartments (32), (92), (94), (113). Under *in vitro* growth conditions, brucellae require 3-5 days to grow while mycobacteria may take several weeks before any sign of growth is visible (63), (145), (139). Thus, the shorter amount of time required for growth has made *Brucella* species an ideal model to study the molecular mechanisms responsible for chronic diseases, which caused by other pathogens that are genetically difficult to manipulate.

In the previous study, a bank of signature-tagged mutagenesis mutants was constructed from *B. abortus* S2308. Two groups of attenuated mutants were identified from a mouse model. One group carries inactivated genes that were necessary for establishing chronic infections, and the other carries inactivated genes that were essential for maintaining chronic infections. A total of fourteen STM mutants were confirmed to exhibit the *in vivo* attenuated phenotype. Four mutants that were unable to establish chronic infection carried homologues of the type IV secretion system, the purine biosynthesis pathway, and the O-antigen biosynthesis (69). The remaining ten attenuated mutants were defective in the ability to persist. They carried inactivated genes that have homologies to those encoding heat shock proteins, membrane-associated proteins and components of the metabolic pathways in other well-studied organisms including *E. coli*, *A. tumefaciens*, and *S. typhimurium* (TABLE I). Among the ten mutants, the *gcvH* mutant is of great interest.

In *E. coli* the *gcvH* gene resides in the middle of the three-gene operon, which encodes the multi-subunit glycine cleavage enzyme (GCV) complex. Numerous studies

have shown that the GCV complex consisting of GcvT, GcvH, and GcvP subunits, is involved in the $C_1$ biosynthesis (123). Others have shown that *E. coli* cultures at cell densities of 1.15 g/l and above exhibit an increase in the cleavage activity of the GCV complex when exogenous glycine was present (64). In addition, the rise in the uptake of exogenous glycine correlated with an increase in activity of the glycine transport protein, CycA permease. Thus, the *gcv* operon may be a component of the quorum sensing system or a quorum sensing-controlled virulence factor (125), (19), (64). Interestingly, the glycine dehydrogenase subunit of the GCV complex was found to be one of the three up-regulated enzymes in tubercle bacillus cultures during non-replicating stages (150), (149), (151). Researchers suggested that the glycine dehydrogenase might be involved in the coupling of the reductive amination of glyoxylate into glycine and the oxidation of NADH into NAD, which ultimately generates enough ATP to finish the last cycle of DNA replication before the shutdown of cellular metabolism when oxygen becomes limited. Hence it is not surprising that the enzymes encoded by the *gcv* operon of *B. abortus* may serve as cell-associated sensing elements that are involved in modulating the growth of brucellae within a nutrient-declining environment of the macrophage.

In this study, the *in vivo* attenuation of the *gcvH* mutant of *B. abortus* is further investigated. The location of the miniTn5-kanamycin cassette insertion in the mutant is identified and the arrangement of the *gcv* operon present in *B. abortus* is revealed. The *in vitro* growth characteristics of the mutant BA102 are evaluated. Finally, three complemented strains carrying the wild type *gcvH* and/or *gcvP* are constructed from the parental mutant BA102 and characterized for the ability to grow under *in vitro*

conditions and to survive intracellularly within the macrophage.

## Materials and Methods

**Bacteria and growth conditions.** Wild-type *B. abortus* S2308 was obtained from B. L. Deyoe, National Animal Disease Center, Ames, Iowa. Strain BA102, an attenuated mutant derived from the signature-tagged mutagenesis of *B. abortus* (69), contains a mini-Tn5(Km) insertion in *gcvH* of the glycine cleavage system. Complemented strains, BA102PCH18.1, BA102PCH19.1, and BA102PCH21.1, were derived from the parental strain BA102 but harboring a pBBR1mcs-based construct carrying *gcvH*, *gcvP* and *gcvHP*, respectively. All plasmid-bearing strains are resistant to both kanamycin and chloramphenicol. All bacterial strains used to prepare inoculum for infecting mice were grown for 3 days at 37°C and 5% $CO_2$ on *Brucella* agar or *Brucella* agar-Chloramphenicol (Cm, 30 μg/ml)-Kanamycin (Km, 50 μg/ml) plates. For the macrophage survival assay and the *in vitro* competitive growth assay, bacteria grown 3 days on plates were used to inoculate 5-ml TSB containing the appropriate antibiotics and grown for 24 h at 37°C in the rolling drum. The 24-h liquid culture was then used to prepare the 48-h culture (a 1:50 dilution of the 24 h culture). The 48-h culture was resuspended in PBS (pH 7.4) and adjusted to a concentration of $5X10^9$ CFU/ml (for the macrophage survival assay) and $1X10^9$ CFU/ml (for the *in vitro* competitive growth assay). For the λZAPIIS2308 library screen, overnight cultures of XL-1 Blue (*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F'*pro*AB*lac*$I^q$ZdΔ*M15Tn10*($Tet^r$)])

and SOLR (e14$^-$ (*mcr*A$^-$) Δ(*mcr*CB-*hsd*SMR-*mrr*)171 *sbc*C *rec*B *rec*J *uvr*C *umu*C::Tn5(Kan$^r$) *lac gyr*A96 *rel*A1 λ$^r$[F'*pro*AB*lac*I$^q$ZΔM15]Su$^-$(non-suppressing)) *E. coli* strains (Stratagene, La Jolla, CA) were grown at 37 °C in NZCYM broth (10 g NZ Amine A (casein hydrolysate), 5 g NaCl, 2 g $MgCl_2 6H_2O$, 5 ml 20% Casamino acids, pH 7.5) containing 0.2% (w/v) maltose.

**Cell line.** Macrophage-like cell line J774A.1 was obtained from ATCC (Manassas,VA) and maintained as frozen cultures in DMEM (Sigma, high glucose (4.5 g/L), 25 mM HEPES, without L-glutamine) containing 1% non-essential amino acids, 7.3 mM L-glutamine, 10% DMSO, and 50% fetal bovine serum at –180°C.

**Inverse PCR.** Genomic DNA of the mutant was digested with *RsaI* at 37°C for 3h, followed by Southern analysis using the kanamycin cassette of the miniTn5 as probe. Gel slicing corresponding to bands resulted from the Southern analysis were extracted to purify DNA fragments. Self-ligation reaction was performed at 14°C overnight, followed by transformation of *E. coli* DH5α (F$^-$ Φ80 Δ*lacZ* ΔM15 Δ[*lac*ZYA-*arg*F] U169 *end*A1 *rec*A1 *hsd*R17($r_k^- m_k^+$) *deo*R *thi*-1 *pho*A *sup*E44 λ$^-$ *gyr*A96 *rel*A1). Colony PCR was performed using inverse PCR primers, Sign-10 and Sign-11. Sign-10 primer (5'GCCGAACTTGTGTATAAGAGTCAG3') is complementary to the sequence of the O-end of the miniTn5 cassette and Sign-11 (5'AAAGGTAGCGTTGCCAATG3') is complementary to the sequence of the unique *Rsa*I site that resides in the middle of the cassette. The inverse PCR product was sequenced and the nucleotide data was searched

for homology via blastn. The inverse PCR product was also used as template DNA and along with Sign-10 and Sign-11 primers generating a "hot" probe to identify clones of the λZAPIIS2308 library.

**Isolation and sequencing of the *gcv* operon of *B. abortus*.** The first step involved screening λ phages carrying the *gcv* operon of *B. abortus*. Frozen stock of the λZAPIIS2308 library was thawed on ice and 10-fold serial dilutions of the frozen phage stock ($10^{11}$ PFU/ml) were performed in SM buffer (100 mM NaCl, 10 mM $MgSO_47H_2O$, 50 mM Tris-HCl (pH 7.5), 0.01% (w/v) Difco gelatin). Overnight *E. coli* XL-1 Blue culture was harvested by centrifugation for 10 min at 25°C, 4000 X g and resuspended in sterile 10 mM $MgSO_4$ to obtain a concentration of $1.6X10^9$ CFU/ml. The cell suspension was incubated at 37°C for 1 h with moderate agitation. Each serial dilution of λ phage was mixed with the *E. coli* cell suspension in a 1:2 volume ratio. The phage-bacteria mixture was incubated at 25°C for 30 min allowing the bacteriophage to adsorb to the bacteria. Molten (47 °C) top agar (0.7% Bacto-agar in NZCYM broth) was added and immediately poured onto a NZCYM-agar plate. The top agar was allowed to solidify at 25°C and then incubated at 37°C for 12-16 h (plaques began to appear after about 7 h of incubation). When plaques were about 1-1.5 mm (pinhead) in diameter, plates were removed from the incubator and refrigerated at least 1 h. A positively charged nylon filter circle (Amersham Hybond-$N^+$) was placed on top of the chilled top agar until it was completely wet. An 18-gauge needle dipped in India ink was used to mark filter and underlying agar before lifting it off with a blunt-ended forceps. The

membrane filter was soaked in the denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30-60 sec and the neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl) for 5 min. The filter was rinsed in 2X SSC buffer (0.3 M NaCl, 0.03 M trisodium citrate, pH 7.0), and blotted dry on Whatman 3MM paper. Hybridization was performed using $^{32}$P-labeled probe generated from the inverse PCR product prepared as described. Positive plaques were identified by aligning the resulting signals on the x-ray film with the original plate. Agar plugs were removed using a Pasteur pipette and placed in a microcentrifuge tube containing 500 μl of SM buffer and a drop of chloroform. The recovered phage in suspension was re-plated and re-screened until 100% of the phage hybridized with the hybridization probe. Then SM buffer containing 0.04% (v/v) chloroform was added to the plate and incubated overnight at 4°C (the phage suspensions are stable up to one year at 4°C). The second step involved the *in vivo* excision of the pBluescript phagemid from the λZAPS2308 library. Each overnight culture of *E. coli*, XL-1 Blue and SOLR strains, was used to inoculate fresh LB broth in a 1:100 volume. *E. coli* XL-1 Blue culture was incubated at 37°C for 2-3 h to mid log phase ($OD_{600}$=0.2-0.5). *E. coli* SOLR culture was grown to an $OD_{600}$=0.5-1.0 and then chilled to room temperature. Log-phase culture of XL-1 Blue cells were resuspended in 10 mM $MgSO_4$ to $OD_{600}$=1.0 (for single-clone excision). A mixture containing the XL-1 Blue suspension ($10^8$ CFU), the ExAssist helper phage ($10^9$ PFU), and the amplified phage stock ($10^7$ PFU) were incubated at 37°C for 15 min before adding LB broth and further incubating at 37°C for 2-2.5 h with shaking. The mixture was incubated at 70°C for 15 min and centrifuged at 4000 X g for 15 min. The supernatant containing the excised pBluescript phagemid was added to

freshly grown SOLR cells ($OD_{600}$=1.0) at 1:2 and 1:20 volume ratios (excised phagemid:SOLR) and incubated at 37°C for 15 min before adding LB broth. Incubation at 37°C was continued for 45 min before 200 μl of the phagemid-SOLR culture was plated on LB plates containing ampicillin (100 μg/ml), and plates were incubated overnight at 37°C. Colonies were restreaked on a fresh ampicillin-containing LB plate and incubated overnight at 37°C. Bacterial glycerol stocks containing 20% glycerol were stored at –80°C. Plasmid DNA purification was performed (QIAGEN miniprep kit). The sequence of the insert, which contains DNA fragment of *B. abortus* S2308, was obtained via sequencing using the reverse primer and the T7 primer.

**Construction of plasmid-bearing complemented strains from the parental mutant strain BA102.** For the preparation of complemented strains, three sets of primers were employed. Primer set 3 (5'CTCGAGCGGATTGGGTTATGGGAGTTTC3'and 5'CTGCAGAGGGTATTCAAATGTGCGGTATC3') were designed to amplify the 334 bp putative promoter region of the *gcv* operon; the 531 bp long DNA fragment including the open reading frame (ORF) of *gcvH* (377 bp) was amplified using primer set 4 (5'CTGCAGCGTCCACGCACTTCCCTT3' and 5'TCTAGATCCATGGAAGGAAGGCCG3'); the 2928 bp long DNA fragment including the ORF of *gcvP* (2798 bp) was amplified by primer set 5 (5'CTGCAGTCGGGTGATCGAATGACC3' and 5'TCTAGAGCATTATCCGACGCATCA3'); and the amplification of both ORFs of *gcvH* and *gcvP* in tandem was achieved by the forward primer of set 4 and the reverse

primer of set 5, resulting a product of 3327 bp in length. All PCRs were performed using High Fidelity Expand Taq Polymerase (Roche). Reaction amplifying the putative promoter region of the *gcv* operon was performed with a host start cycle and followed by a series of cycles (45 sec at 95°C, 1 min 48 °C, 3 min at 68°C, 10 cycles; 45 sec at 95°C, 1 min at 52°C, 3 min at 68°C, 25 cycles). Reactions amplifying *gcvH*, *gcvP*, and *gcvHP* in tandem were also performed with a hot start cycle and followed by a regimen of cycles (45 sec at 95°C, 1 min at 57°C, 4 min at 66°C, 5 cycles; 45 sec at 95°C, 1 min at 56°C, 4 min at 66°C, 4 cycles; 45 sec at 95°C, 1 min at 55°C, 4 min at 66°C, 3 cycles; 45 sec at 95°C, 1 min at 54°C, 4 min at 66°C, 2 cycles; 45 sec at 95°C, 1 min at 53°C, 4 min at 66°C, 25 cycles). All PCR products generated from primer sets as described above were purified following gel electrophoresis, and subcloned into pCR2.1 resulting as pPCH10, pPCH11, pPCH12, and pPCH20. Gel purified *Xba*I-*Xho*I fragment from pPCH10 carrying the putative promoter region of the *gcv* operon was ligated into the *Xba*I-*Xho*I digested pBBR1mcs resulting as the pPCH17 construct. Gel purified *Xho*I-*Pst*I fragments containing either *gcvH*, *gcvP* or both *gcvHP* in tandem were then ligated into the *Xho*I-*Pst*I digested pPCH17 to generate the following plasmids, pPCH18, pPCH19, and pPCH21, respectively. Each plasmid was electroporated into the mutant strain BA102, resulting as three complemented strains BA102PCH18, BA102PCH19, and BA102PCH21.

***In vitro* competitive growth assay.** A mixture of 48-hour cultures containing equal volumes of either wild type S2308 and parental mutant BA102 or wild type S2308 and

complemented mutant was used to inoculate LB broth in a 1:100 dilution. The inoculated media were incubated at 37°C, 250 rpm for 24 h. Ten-fold serial dilutions were performed for the 0 and 24 hour time points. Each dilution was plated on tryptic soy agar (TSA) plates (non-selective), and kanamycin-containing TSA plates (selecting for BA102), and kanamycin-and-chloramphenicol-containing TSA plates (selecting for complemented strains, i.e., BA102PCH18, BA102PCH19, and BA102PCH21). After three days of incubation at 37°C, colony counts were recorded. The results were tabulated from three independent trials. The number of CFU on TSA plates represents the total number of brucellae, whereas that on TSA containing antibiotics represents the bacterial number of the mutant or complemented strains. The input ratio is the inoculated ratio (0 hour time point), which is equal to the bacterial number of the wild type S2308 divided by that of the parental mutant or complemented mutant. The output ratio is the recovery ratio, which is equal to the bacterial number of the wild type S2308 after 24 h incubation divided by that of the mutant or complemented mutant after 24 h incubation. The competitive index (CI) represents the output ratio divided by the input ratio.

**Auxanography screening.** Plates were prepared from kanamycin (50 μg/ml)-containing *Brucella* minimal media (per liter, pH6.9±0.1**,** 7.5 g NaCl, 10 g $K_2PO_4$, 0.1 g sodium thiosulfate, 23.8 ml glycerol, 5 g lactic acid, 1.5 g glutamic acid, 42 μM $MgCl_26H_2O$, 0.18 μM $FeSO_47H_2O$, 0.19 μM $MnCl_24H_2O$, 4 μg/ml thiamine, 4 μg/ml nicotinic acid, 0.01 μg/ml calcium pantothenate, 15 g Bacto-agar) with the addition of

eleven different supplements [Davis, 1980 #9952]. Composition of supplemented media is described as follows. Media 1 (5 mM adenosine, 0.1 mM histidine, 0.3 mM phenylalanine, 5 mM glutamine, 0.32 mM thymine), media 2 (0.3 mM guanosine, 0.3 mM leucine, 0.1 mM tyrosine, 0.32 mM asparagine, 4 mM serine), media 3 (0.3 mM cysteine, 0.3 mM isoleucine, 0.1 mM tryptophan, 0.1 mM uracil, 5 mM glutamic acid), media 4 (0.3 mM methionine, 0.3 mM lysine, 0.3 mM threonine, 0.3 mM aspartic acid), media 5 (0.05 mM thiamine, 0.3 mM valine, 2 mM proline, 0.6 mM arginine, 0.13 mM glycine), media 6 (5 mM adenosine, 0.3 mM guanosine, 0.3 mM cysteine, 0.3 mM methionine, 0.05 mM thiamine), media 7 (0.1 mM histidine, 0.3 mM leucine, 0.3 mM isoleucine, 0.3 mM lysine, 0.3 mM valine), media 8 (0.3 mM phenylalanine, 0.1 mM tyrosine, 0.1 mM tryptophan, 0.3 mM threonine, 2 mM proline), media 9 (5 mM glutamine, 0.32 mM asparagine, 0.1 mM uracil, 0.3 mM aspartic acid, 0.6 mM arginine), media 10 (0.32 mM thymine, 4 mM serine, 5 mM glutamic acid, 0.1 mM diaminopimelic acid (DAP), 0.13 mM glycine), and media 11 (0.1 mM pyridoxine, 0.1 mM nicotinic acid, 0.1 mM biotin, 0.1 mM pantothenate, 0.47 mM alanine). Briefly, strain BA102 was streaked on plates and incubated for 3 days at 37°C, 5% $CO_2$.

## Results

**Isolation of *gcv* operon in *B. abortus*.** Prior to the availability of the *Brucella* genome sequences, isolation of *gcv* operon in *B. abortus* was achieved as follows. Several clones of the λZAPIIS2308 library were identified using $^{32}$-P labeled probe synthesized using inverse PCR products generated as described in Materials and Methods. Sequencing of

these clones revealed that the overall gene arrangement of the *gcv* operon of *B. abortus* is similar to that of *B. melitensis*, in which the operon is comprised of three genes arranged in the order *gcvT-gcvH-gcvP* presumably encoding a multi-subunit enzyme representing the glycine cleavage system (Fig. 11). In addition, sequencing of the inverse PCR product confirmed that the insertional site of miniTn5 in BA102 resides in the last 40 bp of the *gcvH* gene (Fig. 12A). As a result, transcription of *gcvH* was disrupted and that of *gcvP* was abolished.

***In vitro* growth characteristics of complemented mutants.** In order to determine whether the *in vivo* defect of BA102 mutant can be rescued and restored to the phenotype of the wild type *B. abortus*, three complemented mutants were constructed using a broad-host range chloramphenicol-based pBBR1.mcs vector. First, the putative *gcv* promoter region and the *gcvH* and/or *gcvP* genes were amplified, and subsequently cloned into the pBBR1.mcs vector. Then all constructs carry the putative *gcv* promoter region along with the *gcvH* and/or *gcvP*, generating pPCH18, pPCH19, and pPCH21, respectively (Figure 12B). The nucleotide sequences of the inserts were confirmed via sequencing. Finally, each construct was introduced into the kanamycin-resistant mutant strain BA102, resulting in complemented mutants that showed resistance to both kanamycin and chloramphenicol antibiotics. These three derivatives of *B. abortus* BA102 are known as BA102PCH18(*gcvH*), BA102PCH19(*gcvP*), and BA102PCH21(*gcvHP*).

584281 gcgtgaaatc gtcgattaga gacaataatt ccgcatgact tgtgcgccgg attgggttat
584341 gggagtttcg ttgggtgtcg gggcgtcata tcgtgtggtg ttcttgcact gtctgtttgc
584401 gggagagagc cgttaaggcc gccgaagggg aaaacgcccg aaatctctca ggtacaagga
584461 accgcaggcg ggtaagacaa ctctggaaag tcgggggcaa ctccgcgccg aaggtgtaag
584521 tatggcttta tatatagcca tgcgagtctc tcaggcctga gacagagggg cacgaaccaa
584581 ccgcattttg cggaaggcat acgtgcgtct ctggagttgt tatgggcgat accgcacatt
584641 tgaataccct gccgttgcag gatctgcatg aaaaggctgg cgcgcgcttc ggcggctttg
584701 ccggctggaa catgccaacc acctatccgc tgggtgtgat gaaggaacat cttcatacgc
584761 gcgaccatgc gggcctcttc gacatttccc acatgaagct ggtggaagtt tccggcgcgg
584821 atgccgccgc gcttctcgcc gagacctgcc cgctcgatcc gacgatcctg aagacgggcc
584881 agtcgaaata tacgttcttc ctcaatgata atggcggcgt gctggatgat ctcatcgtca
584941 cgcgccttgg cgaagaccgt ttcatggttg ttgccaatgc gggcaatgcc gatgccgaca
585001 tcgagcatct caatgaagcc gcgtcgggca aggccgtaaa ggttaatccg ctcgaccgtg
585061 ttttccttgc tttgcagggg ccggaagcag aagctgtcat caccgatgcc ggtctgccgg
585121 gcgccgatct tgccttcatg agcggtttcg agccgaaaca gagctggttc atgacccgtt
585181 cgggctatac cggcgaagac ggtttcgaga ttggcctgcc tgccgatgaa gcccgtgcgc
585241 tggccgaaaa gcttcttgcc gatgaacgtg tcgaatggat cggccttgcc gcccgcgatt
585301 ccttacgcct cgaagcgggc ctctgcctgc acggtcagga catcacgccg gaaaccgatc
585361 cggtttcggc tggcctcacc tgggcgatca ccaaggctgt gcgcgaaaaa gccgccttca
585421 acggcgcgaa agccgttctc gacgccattg ccaaaggcgc cagcgccaag cgcgtcggcc
585481 tcaagccgga aggccgccag ccggtgcgcg ccggggccga ccttttcgac gaaagtggcc
585541 gccagatcgg caccgtcacc tcgggcggct ttggcccttc cgccggtttc ccggtcgcca
585601 tgggctatgt cgaggcaagc ctcgccaccc ccggcacccg tgtttttgca gatgttcgcg
585661 gcaacaaggt tcccgttgac gtccacgcac ttcccttcac accgcatcgc tatcgcaaag
585721 gatgatttcc atggccaata tcctgttcac cgaagatcat gagtggatca acgtcgaaaa
585781 cggcgttgcc acggttggca tcacgatcca cgcacaggaa cagctcggcg acctcgtttt
585841 cgtcgaattg ccggaagtgg gccgcaccgt tgccaagggc gatggcgtgg tggttgtgga
585901 gtcggtcaag gccgcttccg acgtctacgc gccggtcgat ggcgaagtgg tggaagtcaa
585961 cgatgctgtc gcgagcgatc cttcccttat caatcaggct gccgaaggcg aaggctggct

Mini-Tn5 Km cassette insertion

586021 gttcaagctg aagcttgccg atgaaggcCA gctcacgggc ctgctcgaca aggccggtta
586081 tgaaaagctg atcgggtgat cgaatgaccg aatttcttcc ctttgtggcc cgccatatcg
586141 ggccgagaca tgaagacgag cgcgccatgc tggcagcgct cggccttcct tccatggaaa
586201 ccctcataac gcaggcggtt ccggcctcca ttcgcctcaa ccgcgcgctg aacctgccag
586261 cggcgctcag cgaagcggac gcacttgcgg aactgggcac gatcatgggc cgcaacgtgg
586321 tgaagaagag cttcatcggc gcgggctatc acggtgttca cacgccgccg gtcatccagc
586381 gcaacctgtt tgaaaacccg gcctggtaca cggcttatac gccctaccag tcggaaatca
586441 gccagggccg ccttgagctt ctgttccact tccagacact ggttgccgaa ctgaccggcc
586501 tgccggttgc ctgtgcctcg ctgctcgacg aggcgactgc cgttgccgaa gccatcggcg

**Fig. 11.** Position of the *gcv* operon of *Brucella melitensis* on chromosome II (584281-588960). Sequencing results of the inverse PCR product confirmed that gene arrangement of the *gcv* operon in *B. abortus* is similar to that of *B. melitensis* and also revealed the location of the mini-Tn5 kanamycin cassette insertion in *B. abortus* mutant strain BA102, residing at the position comparable to position 586049 of *B. melitensis* chromosome II as shown. *gcvT* (584622-585725), *gcvH* (585722-586099), and *gcvP* (586104-588902), the start codon of each is underlined. The putative promoter region of the *gcv* operon, which has been amplified and cloned into pPCH18, pPCH19, and pPCH21, is showing in red (584327-584621).

```
586561 ttgcctgccg ccatcatcgc gacaagcgca gccgcattct gcttgctggc gaactgcatc
586621 cgcagacggt cgatgtggtg aacacccgcg ctgaaccgct cggctgggag atcgccaccg
586681 gcagcgatgt tgacgacaac accgccgcaa tcgtggttcc atggccggat acgcgcggcg
586741 tctatggcga ttttgccaag gtcattgccg atgccaaggc caagggcgcg ctggttatcg
586801 ccgttgccga tccgctggcg ctcaccatca tggaagcgcc tgccaggtgg ggcgcggaca
586861 tggccgtcgg ctccatgcag cgctatggcg tgccgatggg ctttggcggc ccgcacgcgg
586921 cttatcttgc agtgtccgag gcgctcactc gcattattcc gggccgcatc gtcggccagt
586981 cggtcgatgc gcatggccgc gcggcctatc gtctggcgct ccagacccgc gagcagcaca
587041 tccgccgcga caaggcgacc tccaatatct gcaccgcgca ggcgcttctg gccaacatgg
587101 ccgcagcttt cgccatctgg catggcccgg ctggcttgca ggcgattgca acgcgcgtgg
587161 ccgctctggc cgcccgtttt gctgccgcgc tcaaggctgc cggtgttgaa attgcgggcg
587221 aaagcctgtt cgatacggta acggccaagg ttccgggcaa agccgcggca atcgcagccg
587281 aagccgacaa gggcggccgc ctgatccgca tcatcgatgc ggatacggtc ggcgtcactt
587341 tcgacgaaac ctcgacggaa gaggatctga cggccctggc atcgctcttc ggtgcgaagc
587401 tggttggcgg cgacacagtt cttgttccgg gcaaggaacg cggggaaggt ttcctcacgc
587461 aggaagtatt ccattcacat cgttccgaaa ccgagatgat gcgcttcctg cgtcgtctgg
587521 ccgacaagga tctggcgctc gaccgcgcga tgatcccgct tggttcctgc acgatgaagc
587581 tcaatgcggc ggcagaaatg atgccggtga gctggaatac ggttgcaaac ctgcatcctt
587641 ttgcgcctgc cgagcaggtg cagggttatg cgaaaatgac gtccgatctg gaagcatggc
587701 tttgcgaaat caccggtttt gcgggcgttt cattgcagcc aaatgccggt agtcagggtg
587761 aatatgccgg ccttatggcc atccgccact atcatcaggc gcgggggcag ggccatcgca
587821 acatctgcct tatcccgtcc tcggcgcatg gcaccaatcc ggccagcgcc tccatggcgg
587881 gcatgagcgt cgtcgtcgtc aattgccgcc cggatggcga catcgatatt gatgatctca
587941 aggccaaggc ggaaaagcat cgcgataatc tcgccgcctt catgattacc tatccgtcca
588001 cctatggcgt gttcgaggaa ggcatcaaag ccttctgcga gattgtgcat gacaatggcg
588061 gccaggtcta tttcgacggc gcgaacctca atgcgcttgt cggtctggcg cggcctgccg
588121 atatcggcgc ggacgtctgc cacatgaatc tgcacaagac cttctgcatc ccgcatggcg
588181 gcggtggtcc gggtgttggc ccgattggcg tggcgaaaca tctggtgcct tatctgccgg
588241 gccatgtgga agccggttcc gaacacgctg ttgccgcagc gcagttcggc agcgcatcca
588301 ttctggtcat tacgtggatg tatatccgca tgatgggcgg ggccggtttg aagaaggcga
588361 cggaagctgc aatcctcaat gccaattata ttgcgcatcg tctgaagggt gtttacccga
588421 tcctttatac cggcgcgcat gaccgcgtgg cgcatgaatg catcgtggat acgcgcgtat
588481 tgaaggacag tgcgggcatt accgtggaag acgtggccaa gcgcctgatc gactatggtt
588541 tccatgcgcc gaccatgtca tggccggttg ccggaacgct gatgatcgag ccgaccgagt
588601 ccgagcccaa gctggagatc gaccgtcttt gcgatgcgat gatcgcgatt gcaggcgagg
588661 cgaagaaggt tgccgatggc gtctggcctg cggatgacaa tccgcttgcc aatgcgccgc
588721 atacggcgag cgatacgctg gcaacggaat ggaagcatcc ctatacccgt gaggaagccg
588781 tattccccgg cggtgctttc gatccgacgg caaaatattg gccgccggtc agccgcgtgg
588841 ataatgtcgg cggcgaccgg aacctcatct gctcctgccc gcctgtcgca gcctatggct
588901 gacaggcgcg gctggagcat tttcgcacca aaagtgtgaa acgcctaagc ggggaaatca
```

**Fig. 11 continued.**

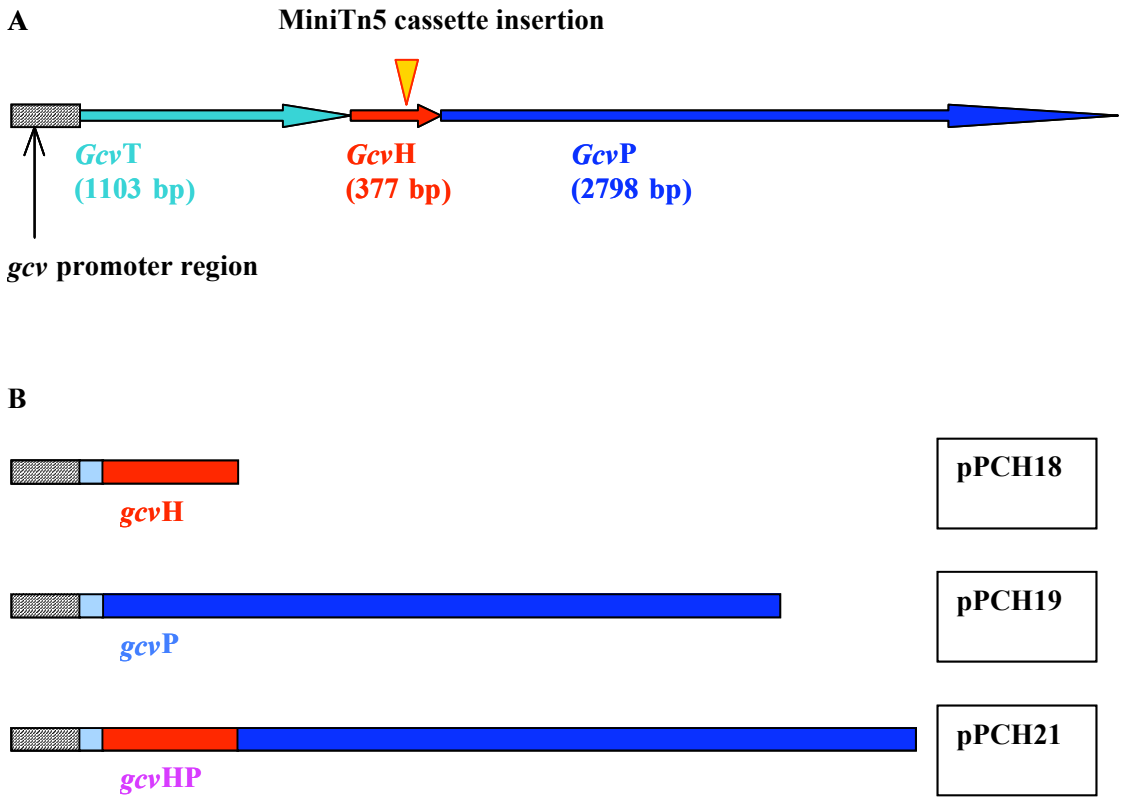



**Fig. 12.** Glycine cleavage system of *B. abortus*. (A)the *gcv* operon of *B. abortus* S2308 and the position of the mini-Tn5 kanamycin cassette insertion, (B)the pBBR1.mcs-based constructs carrying the putative *gcv* promoter region and the *gcvH* and/or *gcvP* of *B. abortus* generating pPCH18, pPCH19, and pPCH21, respectively.

The parental mutant strain BA102 exhibited no apparent *in vitro* growth defect when grown in rich media, such as tryptic soy broth (TSB). In an attempt to characterize the mutant more fully, growth was examined using *Brucella* minimal medium supplemented with glycine and/or serine. Neither the mutant BA102 nor any of the plasmid bearing strains exhibited any growth defects under the conditions used (Data not shown). This observation is similar to that of *E. coli gcv* mutant, in which the *gcv* mutant showed no requirement of any amino acid supplements for growth. On the contrary, the *serA/gcv* double mutant of *E. coli* required both glycine and serine supplements in the minimal growth media (130). When grown on plates, all strains including the parental mutant BA102 showed no changes in colony size. Thus, these results indicate that all three constructs are steadily maintained under *in vitro* conditions.

**Intracellular survival of plasmid-bearing strains in the macrophages.** The ability of each complemented strain to survive intracellularly in macrophages was compared to that of the parental mutant strain BA102 along with the short-lived *in vivo virB10* mutant, BA114 (Fig. 13). Complemented mutant harboring a wild type copy of *B. abortus gcvH*, showed no significant differences in the ability to survive within the macrophages when compared to that of the parental strain. On the other hand, mutant strain carrying a wild type *B. abortus gcvP* exhibited a continuous decrease in bacterial load 24 h post-infection of macrophages, although the reduction was not as severe as that of BA114. The complemented strain carrying both *gcvH* & *gcvP* showed a slight increase in numbers after the initial reduction, i.e, from 24 h to 48 h post-infection of

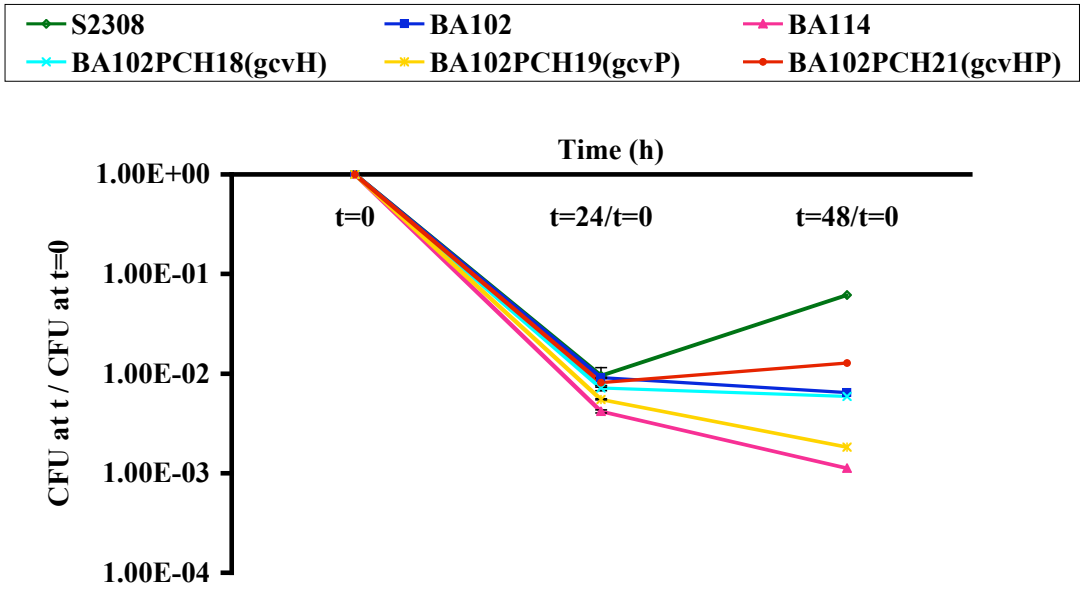



**Fig. 13.** Intracellular survival of *B. abortus* complemented mutant strains in the murine macrophage J774A.1. The graph is showing the average recovered bacterial load at t=24 and 48 h post-infection in relative to that at t=0. S2308, virulent *B. abortus*; BA102, parental *gcvH* mutant; BA114, *virB*10 mutant; BA102PCH18, complemented mutant carrying *gcvH*; BA102PCH19, complemented mutant carrying *gcvP*; BA102PCH21, complemented mutant carrying *gcvHP*.

macrophages. Compared to the recovery of the wild type S2308, the recovery of the complemented strain carrying *gcvHP* was small (t=24, $1.98X10^5$; t=48, $3.1X10^5$ CFU/mL), yet it was significant (p-value=0.0163) (Fig. 13).

***In vivo* survival of complemented strains**. The parental mutant strain BA102 exhibited an attenuated phenotype in the mouse model as the result of an insertional mutation in the 3' end of the second gene of the *gcv* operon in *B. abortus*. In order to determine whether the absence of a fully expressed *gcv* operon is responsible for the observed phenotype, BA102 was complemented with each of the three plasmids described earlier and the virulence/survival of each complemented strain was evaluated in competitive infection assays with the parental mutant BA102. Groups of 5 mice were inoculated with the mixture of complemented mutant and parental mutant BA102 (1:1 ratio) and sacrificed at eight weeks post-inoculation. Animals of the control group were inoculated with a mixture of mutant BA102 and wild type S2308 *B. abortus* and also sacrificed at the specified time. Bacteria were harvested from spleens on kanamycin-containing TSA plates as well as kanamycin/chloramphenicol-containing TSA plates. Unexpectedly, bacterial growth was only observed on kanamycin-containing TSA plates from spleens of mice inoculated with a mixture of complemented strain and mutant BA102, and those inoculated with a mixture of mutant BA102 and wild type S2308. Chloramphenicol-resistant colonies were not recovered from the spleens of those inoculated with the mixtures containing individual complemented strains bearing any of the recombinant plasmids, suggesting that only bacteria of the parental mutant strain BA102 were

recovered from spleens of animals inoculated with the complemented mutant-containing mixture.

**Discussion**

Similar to the glycine cleavage system (*gcv*) operon of *B. melitensis*, the *B. abortus gcv* operon consists of three genes arranged in the following order, *gcvT-gcvH-gcvP*. The insertion of the miniTn5-cassette into the *gcvH* of *B. abortus*, resulted a mutant strain exhibiting an attenuated phenotype in the mouse model. This *gcvH* mutant, designated strain BA102, persisted up to 24 weeks post-inoculation in the host. The mutant was exhibited reduced survival within macrophages, although the intracellular killing occurred at a much slower rate than that of the short-lived *virB10* mutant (BA114) (69). Sequence analysis revealed that the miniTn5 kanamycin cassette was inserted within the last 40 bp of *gcvH*, and thus the disruption of the *gcvH* may also abolish the expression of the *gcvP*.

In *E. coli*, the glycine cleavage complex plays an important role in the biosynthesis of glycine and $C_1$ units. During growth on glucose containing minimal medium, as much as 15% of the assimilated carbon in *E. coli* has been estimated to provide serine, glycine, and $C_1$ units via the serine-glycine interconversion pathway (117). The needs for glycine and one-carbon units are balanced by the action of the glycine cleavage system. The glycine cleavage enzyme complex (GCV) catalyzes the conversion of glycine into a $C_1$ unit and together with the serine hydroxymethyl transferase (*glyA* gene product) interconverts glycine and serine (Fig. 14). Serine and

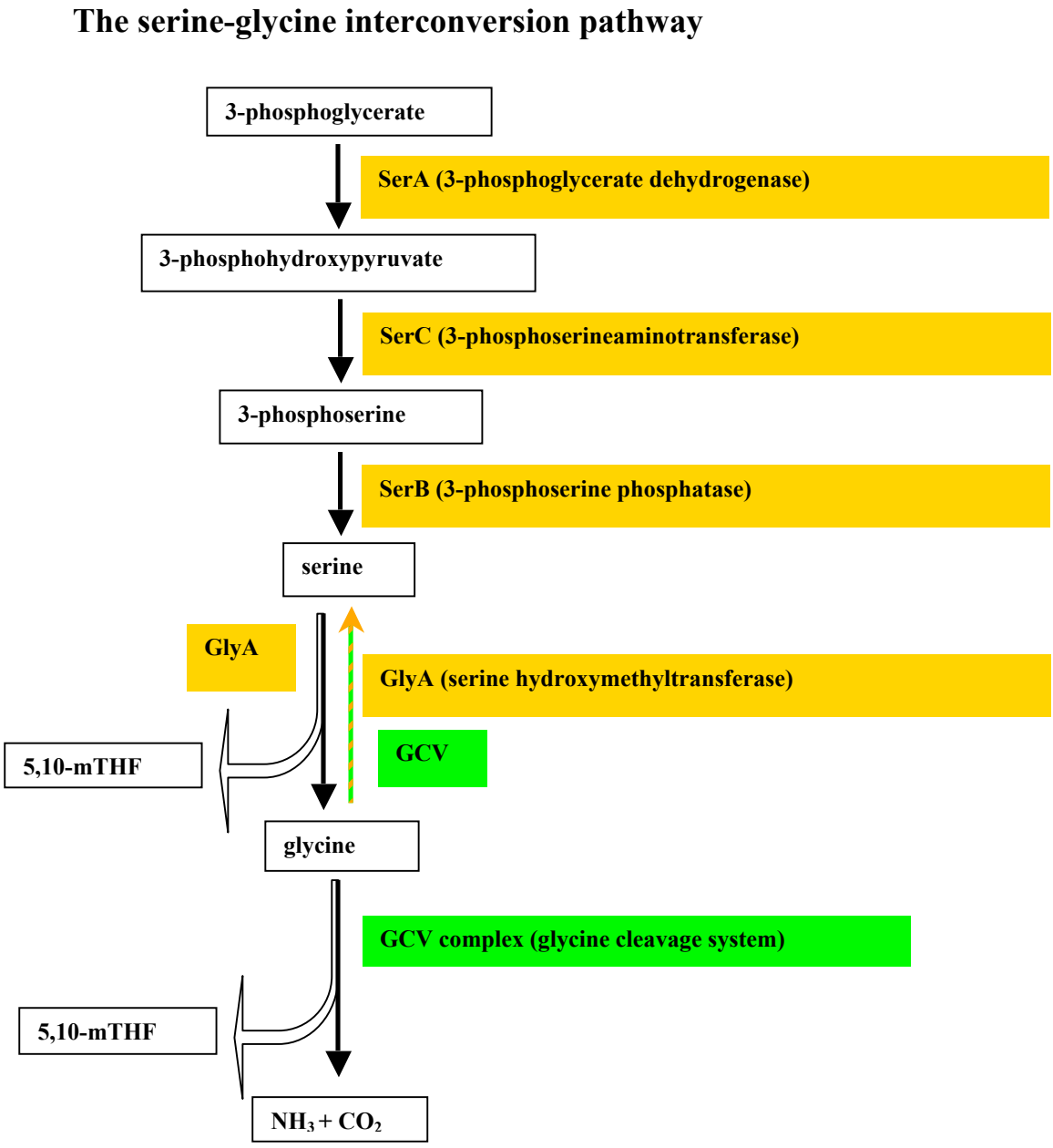



**Fig. 14.** The serine-glycine interconversion pathway in *E. coli* (117). The needs for glycine and one-carbon units are balanced by the action of the glycine cleavage system. The glycine cleavage enzyme complex (GCV) catalyzes the conversion of glycine into 5,10-mTHF, an important contributor of $C_1$ unit in cell metabolism, and together with the serine hydroxymethyltransferase (GlyA) interconverts glycine and serine. 5,10-mTHF, $N^5$, $N^{10}$-methylenetetrahydrofolate; open box, generated product; shaded box, enzyme involved in the reaction.

glycine are also derived from the alternate pathway known as the threonine utilization cycle (Fig. 15). In this pathway, threonine is converted to glycine through the catalytic reactions of threonine dehydrogenase (*tdh* gene product) and □-amino-□-ketobutyrate lyase (*kbl* gene product), and in turn, glycine is then converted to serine by the catalytic reaction of GlyA (56), (10). *E. coli* and *S. typhimurium* mutants carrying single mutations in *ser* genes (*serA*, *serC*, & *serB*) of the serine biosynthesis pathway required either serine or glycine supplement for growth while those carrying a mutation in *glyA* gene exhibited growth on glycine-supplemented minimal media (109), (130). However, all serine and glycine auxotrophs of *E. coli* and *S. typhimurium* have been shown to grow slowly in serine-and glycine-free minimal media provided that threonine was present (144). On the other hand, double mutants carrying a mutation in one of the known *ser* genes plus a mutation in *glyA*, *gcv*, or *tdh* did not exhibit growth on minimal medium supplemented with threonine (115). Taken together, it is not so surprising that the BA102 mutant resulted from a single insertional mutation in the *gcv* operon of *B. abortus* has no observed *in vitro* growth phenotype.

BA102 carrying a single insertional mutation in the *gcvH* gene exhibited normal growth on unsupplemented *Brucella* minimal media, suggesting that the GCV systems of *B. abortus* and *E. coli* may share a similar function. Ghrist and colleagues (59) showed that in *E. coli* the glycine transport system, CycA permease, regulates the *gcv* operon by transporting glycine into the cell, which in turn induces *gcv* expression endogenously. Interestingly, Han and colleagues (64) have reported evidence for cell-density-dependent glycine degradation in *E. coli*. In the presence of exogenous glycine,

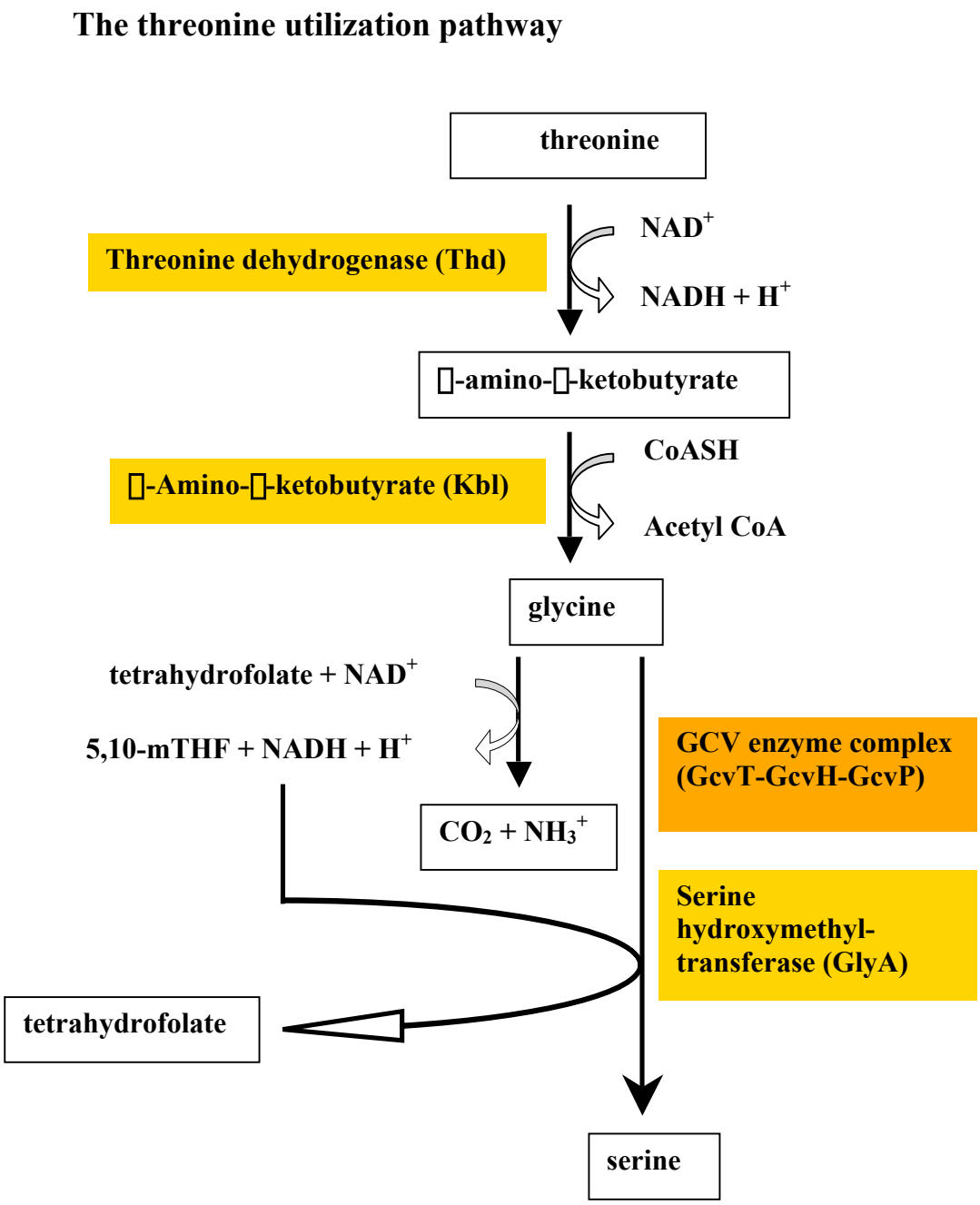



**Fig. 15.** Formation of glycine and serine from threonine in *E. coli* (56), (10). 5,10-mTHF, $N^5$, $N^{10}$-methylenetetrahydrofolate; open box, generated product; shaded box, enzyme involved in the reaction.

*E. coli* cells at cell densities below 1.15 g/l exhibited a repression on the biosynthesis of serine and glycine. Conversely, at cell densities above 1.15 g/l, the degradation of glycine was associated with an increased uptake rate of exogenous glycine, cleavage activity by GCV, and degradation of both glycine-derived serine, and glucose-derived serine to pyruvate. Taken together, it has been suggested that the switch in metabolism might be regulated by population-density dependence of quorum-sensing systems, i.e., at high densities, quorum sensing up-regulates the *cycA* expression allowing more glycine to be transported into the cell and hence up-modulating the cleavage activity of the glycine cleavage system in processing glycine into serine and $C_1$ units.

Quorum sensing (QS) allows bacteria to detect the density of their own species and alter their metabolism to take advantage of the density (19). Bacterial communication via quorum sensing is also important in the production of virulence factors, antibiotic resistance, and biofilm development. In *P. aeruginosa*, quorum sensing has been shown to regulate the production of virulence factors including proteases, exotoxin A, rhamnolipids, and pyocyanin, to induce inflammation, and to be involved in biofilm formation and development (37), (128), (114), (35). In addition, it has been implicated in antibiotic resistance (38). Other studies involved the use of models for both acute and chronic infection have further supported that the quorum sensing systems of *P. aeruginosa* contribute to its pathogenesis by enabling the bacteria to initiate infection and to persist in a host (122), (140). The bacterial cell-to-cell communication enables a bacterial population to mount unified response that is advantageous to its survival by improving access to complex nutrients or environment

niches and collective defense against other competitive microorganisms or eukaryotic host defense mechanisms, thus becoming better adapted to combating environmental threats. In this case, it is possible that brucellae rely on cell-to-cell communication for the successful adaptation to their intracellular habitat. Given these findings, the presence of this *in vivo* attenuated BA102 suggests that the *gcv* operon may be a component of the QS system or a QS-controlled virulence factor.

Another interesting phenomenon, which involves the *gcv* operon, has been observed in *Mycobacterium tuberculosis*. The glycine dehydrogenase encoded by *gcvP* of *M. tuberculosis*, has been identified as one of the three up-regulated enzymes found in a tubercle bacilli culture grown in a slow-stirred *in vitro* model, simulating an oxygen-depleted environment found in necrotic lung tissues of infected patients (150). Thus, it has been suggested that glycine dehydrogenase is involved in the coupling of the reductive amination of glyoxylate into glycine and the oxidation of NADH into NAD, in which this coupling reaction serves mainly to provide enough ATP to finish the last cycle of bacillary replication before oxygen limitation stops growth completely. This orderly shutdown is essential to continue the survival of *M. tuberculosis* in a quiescent form. Furthermore, the activity of the glyoxylate pathway, which bypasses the tricarboxylic acid cycle (TCA), has been shown to come into prominence during survival of mycobacteria under oxygen limitations (76), (15). Since both *Brucella* and TB are chronic disease-causing agents, it is possible that both pathogens utilize the enzymes encoded by the *gcv* operon for a similar purpose.

In order to complement the defect caused by the insertional mutation in *gcvH* of

*B. abortus*, three pBBR1mcs-based constructs carrying the wild type copies of *gcvH* and/or *gcvP* along with the putative *gcv* promoter were introduced into the mutant strain BA102, generating three complemented strains of BA102 (Figure 2B). All three strains have no *in vitro* growth defects. Complemented mutant strain carrying the *gcvH* showed a similar reduction of intracellular survival as the parental mutant strain BA102. However, the mutant strain harboring both wild type copies of *gcvH* and *gcvP*, exhibited a small yet significant recovery in bacterial load after the initial killing (Fig. 3), suggesting that the defect in survival within the macrophage was partially rescued. Interestingly, the presence of the wild type *gcvP* alone did not rescue the mutant strain from the macrophagic killing. Instead, the reduction in bacterial load of complemented mutant strain carrying *gcvP* was similar as to that of BA114. This result suggests that although the activity of glycine dehydrogenase encoded by the *gcvP* gene was restored, the GCV system remained incomplete without a functional GcvH enzyme. Thus, an excess of the partially processed product generated by the GcvP may have caused a disruption of metabolic balance in the bacterial cell and ultimately led to the death of the organism within the macrophage.

To further determine whether the attenuated phenotype of BA102 *in vivo* can be rescued and restored to the wild type phenotype of *B. abortus* S2308, all three plasmid-bearing strains were compared for their persistence in a mouse infection model. Unfortunately, bacteria of all three complemented strains were not recoverable after a mixed infection with the parental mutant BA102. One possible explanation is the existence of the addiction module in the pBBR1mcs vector, which may be similar to that

of the *par* locus of plasmid RK2/RP4 (47), which results in cell death to the plasmid-free progeny upon the loss of the pBBR1mcs-based construct. An alternative approach to resolve the instability of the plasmid is to construct an unmarked deletional mutant carrying a chromosomal copy of the gene of interest, so that the complementation is the result of gene expression from a single copy of the gene. In this case, a suicide vector-based construct carrying both wild type copies, *gcvH* and *gcvP*, along with a promoterless reporter gene is introduced via a single event of homologous recombination into the chromosome of an unmarked double (*gcvH*-and-*gcvP*) deletional mutant. The endogenous *gcv* promoter, thus, regulates the expression of these inserted genes. The expression level of *gcv* operon can then be monitored by that of the reporter gene under *in vitro* or *in vivo* conditions.

# CHAPTER V
# CONCLUSION

The genetic basis for the persistence of *Brucella* in the host remains poorly understood. In this manuscript, we identified genes of *B. abortus* required for chronic infections in the BALB/c mouse model. The approach employed signature-tagged transposon mutagenesis (STM) that permitted the screening of multiple mutants in a single passage and simplified the identification of the interrupted genes. The STM screen identified 27 mutants putatively attenuated for chronic infection. A statistically significant competitive-infection defect was confirmed in 14 of these mutants. Thus, the statistical significant evidence for attenuation was obtained for 8% of the mutants screened (14 of 178). Assuming that the mutagenesis was random and that the coding density of the 3200-kb *B. abortus* genome is similar to that of *E. coli*, our data suggest that as much as 257 genes may be involved in establishing and maintaining chronic infection in mice after intraperitoneal (i.p.) injection. In contrast, the STM screen of *S. typhimurium* in mice via i.p. injection revealed that only 3% of its 4400-kb genome is required for acute infection (66). The greater number of virulence genes required for chronic infection versus acute infection may reflect the requirements for additional adaptations to ensure long-term persistence, such as those that prevent clearance of *B. abortus* from the host immune system.

Our hypothesis of the study was that *B. abortus* possesses one group of virulence factors for establishing the initial infection, which is characterized by rapid bacterial growth, and another group that contributes to establishing and maintaining chronic

intracellular infection, in which little or no growth is observed (8), (14). Indeed, the 14 attenuated mutants fell into two groups. Four mutants were unable to establish the infection by either 2 or 8 weeks post-infection. The 10 remaining mutants were able to establish the infection at 2 weeks post-infection but failed to sustain chronic persistence at 8 weeks post-infection. The first group included mutants with transposon insertions in genes required for the O-antigen biosynthesis (BA184 mutant), the biosynthesis of aromatic amino acids (BA100 mutant), and the type IV secretion system (BA41 and BA114 mutants). Genes inactivated in these mutants are predicted to be involved early during infection. For example, BA184 (*wbk*A mutant) was highly attenuated and outcompeted by wild type S2308 by a 1000-fold at 2 weeks post-inoculation, suggesting that the mutant was eliminated early in the infection. This is expected since rought mutants are sensitive to complement-mediated killing mechanisms and thus may reach the spleen only in small numbers (1), (30), (43). The transposon-insertion in BA100 renders the mutant defective in the biosynthesis of aromatic amino acids as determined by auxanography. This biosynthesis pathway is also involved in the biosynthesis of 2,3-dihydroxybenzoic acid, the only siderophore known to be produced by *Brucella* (90). However, the siderophore was showed to be dispensable for the growth in mice (11). Thus, it is more likely that the attenuation of BA100 is the result of its inability to acquire aromatic amino acids in the host. Two mutants (BA41 and BA114) carrying transposon-insertion in the putative type IV secretion system in *B. abortus*, encoded by the *virB* genes, were also defective for initiation of chronic infection. Mutant BA114 (*virB*10) showed a higher competitive colonization defect than mutant BA41 (*virB*1). A

similar effect has been observed in *Agrobacterium tumefaciens*, a plant pathogen that is closely related to *Brucella*. Mutant of *A. tumefaciens* with inactivated *virB*1 exhibited a lower degree of attenuation than the one with a mutation in *virB*10 (12). In *B. suis*, *virB* mutants were unable to multiply in HeLa cells or macrophage cell line *in vitro* (103). Therefore, these data together with our findings that *virB*1 and *virB*10 mutants of *B. abortus* were unable to persist in mice after i.p. infection suggest that the attenuation of survival in the mouse model is the result of the inability of these mutants to grow intracellularly.

The second group of mutants that were unable to maintain chronic infection included mutants with inactivated genes involved in the glycine cleavage system (BA102 mutant), the production of glutamate synthase (BA152 mutant), the nutrient uptake (BA159 mutant), and several unknown functions (BA31, BA38, BA63, BA73, and BA122 mutants). As the first complete genome sequence of *Brucella* species became available, some of these unknown functions were inferred as glutamate decarboxylase beta (BA31), histidinol phosphate aminotransferase (BA38), glycine betaine (BA63), and a heat shock protein (BA122). Although the inactivation of genes in these mutants did not show an effect in the initiation of chronic infection, these genes were required for chronic persistence of the organism. Mutant BA102 carried a transposon insertion in a gene region showing homology to *gcvB* encoding glycine dehydrogenase in *Mycobacterium tuberculosis*. The activity of glycine dehydrogenase showed a 10-fold increase in tubercle bacillus cultures during non-replicating stages (150). Our finding that glycine dehydrogenase is required for the survival of *B. abortus*

in the mouse spleen suggests that both *Brucella* and *Mycobacteria* may share similar metabolic pathways for chronic persistence in the host. This result underlines the importance of research on the host-pathogen interactions of *B. abortus* in elucidating the molecular mechanisms of intracellular persistence, which may be shared by other chronic intracellular pathogens.

The presence of the two defined groups of attenuated mutants suggests that *B. abortus* uses distinct sets of virulence determinants for the initial establishment and the maintenance of chronic infection. Long-term survival of the organism in the host is the undesirable feature of the disease, yet the persistent nature of *Brucella* may be a great attribute for developing longer lasting live vaccines. To be effective, live attenuated vaccines must persist long enough to elicit protective immunity, yet they should be cleared as quickly as possible to avoid unnecessary side effects. Attenuated mutants with similar genetic background and possessed different survival characteristics are ideal for studying the vaccine potential with regard to the persistence of the strain.

*B. abortus gcvH* mutant, persisted up to 24 weeks post-infection in mice, showed a moderate attenuation for growth in macrophages while *B. abortus virB*10 mutant, cleared out as early as 8 weeks post-infection, exhibited a more marked defect in the intracellular survival. This observation of *virB*10 mutant is in agreement with several studies of type IV secretion system (T4SS) in *Brucella*, in which the *virB* operon play a critical role in the intracellular multiplication and virulence (79), (127). The T4SS of *B. abortus* is required for modulation of the intracellular trafficking with HeLa cells (29) but not evasion of nitrosative or oxidative bactericidal killing by macrophages (136).

Inactivation of the *gcv* operon, on the other hand, is expected to prevent adaptation to low oxygen environments. Consistent with the anaerobic state of *Brucella* is the observed requirement for cytochrome bd oxidase during intracellular growth (46). Under the conditions of depleting oxygen found in inflammatory and necrotic tissues, glycine dehydrogenase may serve to generate NAD from a reduced form, which provides ATP to support the completion of a final cycle of DNA synthesis before the shutdown of replication, and thus may be a factor contributing to the latency of the disease (148), (151). This may explain why BA102 only persisted in the mouse spleen as long as 24 weeks post-infection. The mutation of *gcvH* may also cause growth restriction of the mutant in the macrophage, despite the presence of a functional T4SS. Such growth restriction has been observed in wild type *E. coli* strains harboring non-functional selenolipoylated H-protein of the glycine cleavage system (57). The presence of a functional glycine cleavage system appears to be vital to the survival of *Brucella in vivo* and thus is consistent with the need for additional factors for virulence.

The vaccine potential of *virB*10 and *gcvH* mutants was investigated in the BALB/c mouse model for a period of vaccination exposure ranging from 8 weeks to 24 weeks. As a result, the persisting *gcvH* mutant induced significantly high levels of protection at 8, 16, and 24 weeks post-vaccination. Although protection against challenge began to decline from a maximal level observed at 16 weeks post-vaccination for both candidates, the protection elicited by both *virB*10 and *gcvH* mutants was still significant when compared to that of the control unvaccinated animals. In particular, the *gcvH* mutant still induced higher level of protection than the *virB*10 mutant. Since the

duration of bacterial colonization and the level of colonization of *virB*10 and *gcvH* mutants differed, it was difficult to relate the potential differences in protection solely to the level of colonization or the duration of colonization by the vaccine candidates exhibiting such a wide range of *in vivo* persistence and bacterial burden. Treatment with doxycycline for 21 days followed the vaccination exposure was implemented.

To better understand the differences in protective immunity induced by these mutants, the fundamentals of host immune responses were examined. The host anti-*Brucella* antibody, IgG, remained in circulation for an extended period even after bacteria including those of the early-clearing *virB*10 mutant were cleared from the host. A similar observation has been documented in patients clinically cured of acute brucellosis, in which *Brucella* antibodies persisted for more than 2 years after successful treatment and clinical cure (2). The adaptive cellular immune response showed a consistent pattern of host immune function in favor of $T_H1$ ($IgG_{2a}$>$IgG_1$) in all vaccinated mice except for those vaccinated with the *virB*10 mutant. At 24 weeks post-vaccination followed by 21 days of doxycycline, the $IgG_1$ levels in animals vaccinated with the *virB*10 mutant were significantly increased, suggesting that there is a diminution of $T_H1$ response. A transient acquired cellular immunity has also been observed in C3H mice upon the clearance of bacteria from tissues, despite the fact that the C3H mouse model is more resistant to *Brucella* infection than the BALB/c model (106), (155). The BALB/c mouse model has been shown to be a $T_H2$-driven response model (23), (52). Therefore, by more than 24 weeks post-inoculation, no live brucellae of the *virB*10 mutant were present in the mice, the host system may have resumed its

original state. The induction of two $T_H1$-associated cytokines, IFN-gamma and IL-2, was consistent and significantly high in all *Brucella*-inoculated animals except for those vaccinated with the *virB*10 mutant. In these vaccinated mice, the absence of IL-2 and the late induction of IFN-gamma observed at 24 weeks post-vaccination followed by 21-days of doxycycline treatment may be another contributing factor that allows a favorable change towards a $T_H2$ characteristic. Despite the host immune function in favor of $T_H1$ responses, the induction of IL-10 was significantly increased in all animals including those vaccinated with the *gcvH* mutant as well as those inoculated with virulent S2308. Interleukin-10 was once clearly classified as a $T_H2$-associated cytokine that could down-modulate the $T_H1$ response, and now there is mounting evidence that IL-10 may act as an inhibitor of the $T_H2$ response both *in vivo* and *in vitro* (157), (156). On the contrary, our findings suggest that IL-10 may act as an anti-inflammatory cytokine to buffer the host systems during cellular attack, or that the effect of IL-10 *in vitro* does not reflect events as they occurred one week after challenge. In addition to cytokine profiles, results of lymphocyte proliferation suggest that *Brucella* can elicit a long-term memory response regardless of the length of persistence. Proliferative responses were evident even in lymphocytes from animals at 24 weeks after inoculating with the short-lived *virB*10 mutant. This is somewhat surprising when considering the decline in protection observed in response to vaccination with the mutant long after the bacteria of the *virB*10 mutant have cleared from the host.

Taken together, our findings suggest that the combination of enhanced levels of $T_H1$-type cytokines elicited by the *gcvH* mutant is crucial for the development and the

maintenance of $T_H1$ response in the host, particularly, in a *Brucella*-susceptible and non-$T_H1$-driven response host model such as the BALB/c mouse. The strongest protection against *B. abortus* not only requires a finely balance of cytokines, is more dependent upon the timing of the cytokine response rather than on the absolute level of cytokine expression. Overall, the *gcvH* mutant conferred the greatest cellular protection against wild type *B. abortus* S2308 without exerting unnecessary side effects such as splenomegaly on the host, suggesting that greater persistence of vaccine strain correlates with greater protection.

Persistence has always been the hallmark of chronic brucellosis as well as human tuberculosis. *B. abortus gcvH* mutant was identified within a group of attenuated STM mutants that were unable to persist in mice. The inactivated gene of this *B. abortus* mutant was homologous to *M. tuberculosis gcvB* encoding glycine dehydrogenase, which was up-regulated in a culture of tubercle bacilli during non-replicating stages. This homology suggests that both organisms may depend on similar metabolic pathways for chronic persistence. A thorough sequence analysis revealed that the inactivated gene of this *B. abortus* mutant is homologous not only to *gcvB* of *M. tuberculosis* but also to *gcvH* of *E. coli*. The homology was further defined as the genome sequence of *B. melitensis* became available. The *gcvH* in *B. abortus*, mapped to the *gcv* operon of *B. melitensis*, is located in the middle of the *gcv* operon consisting of three genes in the following order, *gcvT-gcvH-gcvP*. In the *B. abortus gcvH* (BA102) mutant, the transposon insertion resides in the last 40 bp of *gcvH*. Thus, the disruption of the *gcvH* gene may also abolish the expression of the *gcvP* gene.

In an effort to restore or rescue the defect caused by the insertional mutation in the *gcvH* of *B. abortus*, wild type copies of *gcvH* and/or *gcvP* were introduced into the *gcvH* mutant, generating three complemented mutant strains. These mutant strains showed no *in vitro* growth defects. In macrophage J774A.1, the complemented mutant strain carrying the wild type *gcvH* exhibited reduced intracellular survival similar to the parental mutant strain. In contrast, the complemented mutant strain with both wild type *gcvH* and *gcvP* genes showed a small yet significant recovery in numbers after the initial macrophagic killing. Interestingly, the mutant strain harboring the wild type *gcvP* gene was severely attenuated in the macrophage as the short-lived *virB*10 of *B. abortus*. These results suggest that the expression of *gcv* operon may be needed for *Brucella* to persist intracellularly within the macrophage and that the accumulation of the partially processed product of the glycine cleavage system may cause a disruption of metabolic balance in the bacterial cell. The survival of the complemented mutant strains in mice was also examined. Unfortunately, bacteria of all three complemented strains were not recoverable after a mixed infection with the parental *gcvH* mutant strain. The best explanation for this observation is the addiction module phenomenon, in which the stability of low-copy number plasmids in the host bacterial cells is maintained by the mechanism for killing plasmid-free bacteria provided by the addiction module. In this case, an addiction module may exist in pBBR1mcs vector, which was used as the backbone of all constructs carrying *B. abortus gcvH* and/or *gcvP*, resulting in cell death to the plasmid-free progeny upon the loss of the plasmid. An alternative approach to resolve the instability of the plasmid is to construct an unmarked deletional mutant

carrying a chromosomal copy of the gene of interest, so that the complementation is the result of gene expression from a single copy of the gene. Thus, a suicide vector-based construct carrying both wild type *gcvH* and *gcvP* along with a promoterless reporter gene is introduced via a single event of homologous recombination into the chromosome of an unmarked double (*gcvH*-and-*gcvP*) deletional mutant. The endogeneous *gcv* promoter, thus, regulates the expression of these inserted genes. The expression level of *gcv* operon can then be monitored by that of the reporter gene under *in vivo* or *in vitro* conditions.

Virulence of *Brucella* is essentially the result of their capacity to survive and replicate within the phagocytic cells. However, many gaps remain in our understanding of this ability of brucellae to elude the bactericidal effects of host phagocytes, and basic questions remain unanswered. Identification of *Brucella* gene products, which are involved in the intracellular survival as well as those that contribute to the induction of protective immunity, is critical to elucidate the molecular mechanisms of the pathogenesis of the organism.

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