

## ABSTRACT

Title of document: CHARACTERIZATION OF BORRELIA  
BURGDORFERI GENE PRODUCTS  
CRITICAL FOR PATHOGEN  
PERSISTENCE AND TRANSMISSION

Manish Kumar, Ph.D., 2010

Directed by: Dr. Utpal Pal  
Assistant Professor  
Department of Veterinary Medicine

The pathogen of Lyme disease, *Borrelia burgdorferi*, persists in a natural tick-rodent infection cycle. An assessment of microbial transcriptome *in vivo* encoding selected membrane proteins shows that *bba52* is upregulated during pathogen transmission. Deletion of *bba52* in infectious *B. burgdorferi* did not alter the outcome of murine inflammation or long-term pathogen persistence in mice or ticks. However, the *bba52* mutant was impaired for transmission between feeding ticks and mice, phenotypic defects that could be rescued when *bba52* was genetically restored to the original genomic locus. I show that BBA52 is a surface-exposed outer membrane protein. As BBA52 is highly immunogenic, I next assessed whether BBA52 could serve as a potential candidate for transmission-blocking vaccines against spirochete infection. Passive transfer of BBA52 antibodies into the ticks did not interfere with microbial persistence in unfed ticks but blocked pathogen transmission from feeding ticks to murine

hosts. More importantly, active immunization of mice with recombinant BBA52 protein significantly blocked *B. burgdorferi* transmission from ticks to naïve murine hosts. As BBA52 antibodies lacked detectable borreliacidal activities, their interference with spirochete survival *in vivo* could result from the inhibition of BBA52 function, such as vector-pathogen interaction. By far-western analysis using tick gut proteins, I found that BBA52 interacts with a ~ 35 kDa tick gut protein. I also show that BBA52 forms distinct homo-oligomer in borrelial cells and also interacts with two proteins of *B. burgdorferi*. These BBA52 interacting proteins possess approximate molecular weight of 33 kDa and 25 kDa and are located in the outer membrane and protoplasm, respectively. Taken together, these studies suggested the remarkable existence of *B. burgdorferi* surface antigens that are differentially expressed *in vivo* and support microbial transitions between hosts and the vector. Identification and characterization of novel vector-specific and feeding induced borrelial antigens could contribute to the development of transmission-blocking vaccines against Lyme disease.

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CRITICAL FOR PATHOGEN PERSISTENCE AND TRANSMISSION

By

Manish Kumar

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**Advisory Committee:**  
Professor Utpal Pal, Chair  
Professor Siba K Samal  
Professor Charles Mitter  
Professor Yanjin Zhang  
Professor Ioannis Bossis

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## **Dedication**

I dedicate this dissertation to my parents. Their endeavours in cultivating me the best possible way to become a responsible human being cannot be put into words. I thank them from the bottom of my heart.

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## List of Abbreviations

µg	microgram
µl	microliter
°C	degrees Celsius
bp	base pair
BSA	Bovine serum albumin
BSK	Barbouer Stoener and Kelly media
cDNA	complement DNA
ml	milliliter
ELISA	Enzyme linked Immunosorbent Assay
EPS	Electroporation Solution
GST	Glutathione S-transferase
HRPO	Horserraddish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
KV	kilovoltage
LB	Luria Bertani

MCS	multiple cloning site
mm	millimeter
mRNA	messenger RNA
ORF	Open reading frame
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
rpm	revolution per min
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
TBS-T	Tris buffer saline-Tween 20
TGE	Tick gut extract
WT	wild type

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# Chapter 1: Introduction

**Background:** *Borrelia burgdorferi*, a bacterium that has been classified under the family *Spirochetaceae* is the causative agent of a highly-prevalent vector borne disease transmitted by bite of *Ixodes scapularis* ticks and known as Lyme disease or Lyme borreliosis. The disease has been named according to the place Lyme County in Connecticut, where the first detail study on this disease was initiated in early 1980. The scientific research on Lyme disease has proceeded at a rapid rate since the identification of the causative agent *B. burgdorferi* in 1982. *B. burgdorferi* has developed differential strategies to survive in the vector and mammalian host environment, for example, the pathogen alter its gene expression profiles as it navigate through tick-mammal infection cycle. With the recent advancement in technologies for assessment of gene expression, a number of genes have been identified to play critical roles in maintaining *B. burgdorferi* through natural enzootic cycle. This chapter will review the literature about past studies that identified the role of specific genes in the pathogenesis of Lyme disease.

## 1.1 Lyme disease

The incidence of Lyme disease came to light in the year 1975 when two mothers, Polly Murray and Judith Mensch, from Lyme, Connecticut reported about the number of incidence of juvenile rheumatoid arthritis. Thereafter, *Borrelia burgdorferi* was isolated by Willy Burgdorfer in the year 1982 from the *Ixodes* ticks (Burgdorfer et al., 1982). Shortly after, *B. burgdorferi* was cultivated from cerebrospinal fluids of patients suffering from Lyme disease (Barbour, 1984). Further investigations against Lyme

disease revealed that approximately one-quarter of the patients had developed erythematous cutaneous lesion that in some cases appeared to expand into reddish, often annular, concentric rings, known as *erythema migrans*, which is the only distinguished clinical symptoms of *Borrelia burgdorferi* infection in humans.

### **1.1.1 Geographical distribution**

Currently, Lyme disease is regarded as the most common vector borne disease in Northern hemisphere (Steere et al., 2004) and also in Eurasia (Piesman and Eisen, 2008a). According to the CDC, there has been increasing tendency of Lyme disease prevalence from the year 1995-2009 in the United States. Most cases occurred in the following 10 northeastern, mid-Atlantic, and north-central states: Connecticut, Delaware, Maryland, Massachusetts, Minnesota, New Jersey, New York, Pennsylvania, Rhode Island, and Wisconsin (Hanincova et al., 2008). The causative agent of Lyme disease in North America is caused by a wide range of *B. burgdorferi* isolates, collectively referred as *B. burgdorferi* sensu stricto complex. In Europe, major borrelial isolates includes 3 major strains, *Borrelia burgdorferi*, *B. garinii* and *B. afzelli* (Piesman and Gern, 2004), collectively known as *Borrelia burgdorferi* sensu lato complex. The trend of focal cases of Lyme diseases coincides with the increase in the prevalence of *Ixodes scapularis* and deer population in the area.

### **1.1.2 Clinical manifestation and treatment**

The spectrum of clinical manifestations resulting from infection with *B. burgdorferi* is generally termed as Lyme disease in United States and Lyme borreliosis in Europe and Asia (Rosa et al., 2005). Lyme disease typically begins with a unique skin

lesion, *erythema chronicum migrans* (ECM) which is caused at the site of tick bite and is described to be pathognomonic symptom (Stage 1). Patients with this lesion may also have a variety of non-specific clinical manifestations including headache, meningeal irritation, mild encephalopathy, multiple annular secondary lesions, malar or urticarial rash, generalized lymphadenopathy and splenomegaly, migratory musculoskeletal pain, hepatitis, sore throat, non-productive cough, conjunctivitis, periorbital edema, or testicular swelling (Steere et al., 1986). After a few weeks to months (Stage 2), few patients develop frank neurologic abnormalities, including meningitis, encephalitis, cranial neuritis (including bilateral facial palsy), motor or sensory radiculoneuritis, mononeuritis multiplex, or myelitis (Steere et al., 1986). At this time, some patients develop cardiac involvement primarily reflected as atrioventricular block, acute myopericarditis, cardiomegaly, or pancarditis (Steere et al., 1986). Throughout this stage, many patients continue to experience migratory musculoskeletal pain in joints, tendons, bursae, muscle, or bone. Months to years after initial disease onset (Stage 3), about 60% of patients develop arthritis, which may be intermittent or chronic. Thus, Lyme disease occurs in stages with different clinical manifestations at each stage, but the course of the illness in each patient is highly variable. Lyme disease can be efficiently treated at the early stage by the use of antibiotics. Treatment becomes more challenging as the disease progresses and reaches late stage. Antibiotic Doxycycline which is a Tetracycline derivative is routinely used to treat Lyme borreliosis (Pavia, 2003; Stricker et al., 2006). A 2 to 4-week course of antibiotics is generally effective therapy for the localized and early-disseminated stages of Lyme disease. However, a subset of patients requires longer antibiotic treatment and can develop chronic symptoms that are



unresponsive to antibiotics, possibly as post-infectious autoimmune sequelae (Steere, 2001). If untreated, Lyme disease can progress to Stage 3, or late-persistent disease, with chronic arthritis, neuroborreliosis and skin disorders. A recombinant vaccine against OspA was developed (Steere et al., 1998) but was retracted from the market by the manufacturer due to sales related issues. Vaccination with OspA caused potential autoimmune arthritis (Abbott, 2006; Nordstrand et al., 2000) and also was low in efficiency because people required frequent boosters to maintain effective antibody titers to confer protection.

## **1.2 General features of *B. burgdorferi***

*B. burgdorferi* spirochetes are Gram negative, highly motile, spiral-shaped and elongated with variable length (~10-30  $\mu\text{m}$ ), thin (~0.3  $\mu\text{m}$  in width) bacteria, which can be visualized under dark field microscopy (Goldstein et al., 1996). It has characteristic periplasmic 7-11 bipolar endoflagella attached to the poles of the bacterial cell (Barbour and Hayes, 1986; Goldstein et al., 1996; Motaleb et al., 2000), which plays an important role in cell shape (Motaleb et al., 2000) and chemotaxis (Shi et al., 1998) to different organs of host it invades. These flagella also attribute flat wave-like shape to the organism (Charon et al., 2009). *B. burgdorferi* is an obligate extracellular bacterium, not able to endure itself outside arthropod vector or vertebrate host, except for artificial laboratory culture medium. *B. burgdorferi* has fastidious growth requirements for *in vitro* cultivation. In laboratory, *B. burgdorferi* can be cultivated only in highly enriched medium, Barbour-Stoenner-Kelly II (BSK II) supplemented with the rabbit serum (Barbour, 1984). *B. burgdorferi* cultures are grown in a microaerophilic environment (1.5% CO<sub>2</sub>) at 34°C. The natural reservoir host for the *B. burgdorferi* is wild rodents,

most commonly the white footed mouse (*Peromyscus leucopus*). Humans and many other domestic animals are the accidental hosts that are infected by *I. scapularis* ticks. Ticks both acquire and transmit *B. burgdorferi* by feeding on a variety of small mammals that act as reservoir hosts (Magnarelli et al., 1988). Although *B. burgdorferi* is often referred as Gram-negative because they have both an outer and inner membrane, they share many features of Gram-positive bacteria. These include the low G+C content of the genome (28.6%) (Fraser et al., 1997b) and the close association of the inner membrane with a peptidoglycan cell wall. Unlike other Gram-negative bacteria, the *B. burgdorferi* outer membrane (OM) is very distinct in the sense that it lacks lipopolysaccharides (Takayama et al., 1987) and the membrane is very fluid and labile consisting of 45-62% protein, 23-50% lipid and 3-4% carbohydrate (Barbour and Hayes, 1986). *B. burgdorferi* OM also houses a lower density of membrane-spanning proteins but possesses a large number of membrane-anchored proteins that remain attached to outer or inner leaflet of the OM via amino-terminal lipid moiety.

### **1.3 Genome of *B. burgdorferi***

*B. burgdorferi* is classified under family *Spirochetaceae* due to its fascinating spiral shape. The members of genus *Borrelia* whose genome sequences have been determined till now are *B. burgdorferi*, *B. garinii*, *B. afzelii*, *B. duttonii* and *B. recurrentis*. Recently, whole genome sequences of 13 additional *B. burgdorferi* isolates were also derived (Schutzer et al., 2010). All the members of *B. burgdorferi* sensu lato identified till date harbors a linear chromosome in the range of about 900 kilobase pair (kbp) in length and a plethora of linear and circular extra chromosomal DNA elements or

plasmids. Lyme pathogens maintains highest number of known extrachromosomal plasmids known till now in any bacterium (Rosa et al., 2005). Among all these, *B. burgdorferi* strain B31, the *B. burgdorferi* type strain, has been studied in more detail, and harbours 12 linear and 9 circular plasmids that constitute about 612 kbp. The chromosomes harbors mostly homologous genes, whereas the plasmids are more variable, which also possess greater number of paralogous sequences, pseudo genes in addition to possession of certain essential genes. These features have been observed directly for eight of the *Borrelia* species, *B. burgdorferi*, *B. bissettii*, *B. garinii*, *B. afzelii*, *B. andersonii*, *B. valaisiana*, *B. lusitaniae*, and *B. japonica*, as well as the relapsing fever species *B. hermsii*, *B. turicatae*, *B. duttonii*, *B. recurrentis*, *B. coriaceae*, *B. parkeri*, and *B. anserina* (Casjens et al., 1995; Casjens and Huang, 1993; Davidson et al., 1992; Ferdows and Barbour, 1989; Kitten et al., 1993; Lescot et al., 2008; Ojaimi et al., 1994). The chromosome bears tightly packed genes with overlapping genes or with less intergenic sequences like other typical prokaryotes, while many of the linear plasmids have substantially lower gene densities with longer intergenic regions and many apparently represent decaying pseudogenes (Casjens, 2000). It is speculated that the stable chromosome encodes the proteome machinery required for basic cellular machinery. In contrast, the relatively unstable and evolutionarily variable plasmids encode most proteins that interact with the vertebrate and arthropod host environments encountered by the bacteria during its enzootic cycle. Pathogenic bacteria often carries plasmids encoding genes which are important for infection to the host but the plasmid encoding genes in *B. burgdorferi* have no similarity with other bacterial virulence genes (Casjens, 2000). This supports the notion that *B. burgdorferi* plasmids encode genes with

functions that are specific to spirochete infectious cycle, and the *B. burgdorferi* host-pathogen interactions. This fostered the hypothesis that *B. burgdorferi* host-pathogen interactions are different from those of other well-studied bacterial pathogens (Casjens, 2000).

*B. burgdorferi* extrachromosomal plasmids vary in stability, some of which could be lost after a few generation of *in vitro* culture while others are stably maintained (Byram et al., 2004; Grimm et al., 2003; Schwan et al., 1988; Xu et al., 1996). The loss of plasmids during *in vitro* growth results in difference in the multiplication of *B. burgdorferi* *in vivo* as some plasmids encodes for genes that are critical for survival in the natural host while the other plasmids have no effect on infectious cycle (Elias et al., 2002a; Labandeira-Rey and Skare, 2001; Purser and Norris, 2000). In genetic studies, as bacterial growth in culture cannot be avoided, care must be taken to assure that there is no plasmid loss during genetic manipulation (Rosa et al., 2005).

## **1.4 *B. burgdorferi* gene expression and development of genetic tools in spirochete research**

### **1.4.1 *B. burgdorferi* gene expression**

The advent of global transcriptional microarray analyses has provided important insight into how *B. burgdorferi* modulates its gene expression to various experimental conditions or environmental stimuli that either individually or collectively simulates a host-adapted condition. Although details of how majority of *B. burgdorferi* genes are transcriptionally regulated during tick-rodent infection cycle is still unclear, several regulatory proteins, including sigma factors, DNA-binding proteins, and transcriptional

activators/repressors that either directly or indirectly control the differential expression of many *B. burgdorferi* genes have been identified. *B. burgdorferi* has evolved a unique  $\sigma^{54}$ – $\sigma^S$  sigma factor cascade (the Rrp2–RpoN–RpoS pathway) that plays a central role in modulating a dramatic switch in the gene expression program as *B. burgdorferi* is transmitted from its arthropod to vertebrate host. There are two alternative sigma factors encoded in the *B. burgdorferi* genome, RpoN ( $\sigma^{54}$ , encoded by *ntrA* or *rpoN*; *bb0450*) and RpoS ( $\sigma^S$  or  $\sigma^{38}$ , encoded by *rpoS*; *bb0771*) (Fraser et al., 1997b). Hubner et al., 2001 generated an *rpoS* mutant and *rpoN* mutant in a low-passage infectious strain (*B. burgdorferi* strain 297) that resulted in the loss of OspC and DbpA/B, suggesting that RpoS and RpoN is required for the regulation of the genes encoding these surface lipoproteins. Subsequent studies, including microarray analyses, revealed that RpoS influences expression of more than 100 genes (Caimano et al., 2007; Fisher et al., 2005; Ouyang et al., 2008). Many RpoS-activated genes appear to be differentially expressed during tick feeding and some, including *ospC*, *dbpBA*, *bbk32*, *oppA-V*, *bba64*, and *bba66*, have been shown to be required for or associated with mammalian host infection (Clifton et al., 2006; Fischer et al., 2006; Fischer et al., 2003; Gautam et al., 2008; Gilmore et al., 2007; Grimm et al., 2004b; Maruskova et al., 2008; Medrano et al., 2007; Seshu et al., 2006; Shi et al., 2008; Weening et al., 2008). In addition, 59 genes were upregulated and 34 genes were downregulated by RpoS when spirochetes were cultivated within dialysis membrane chambers implanted into the peritoneal cavity of rats, which recapitulates the response to mammalian host signals (Akins et al., 1998; Caimano et al., 2007). Although the Rrp2–RpoN–RpoS pathway controls expression of a vast network of genes and operons, there are still an abundant number of differentially expressed genes that are not

regulated by this cascade (Boardman et al., 2008). This observation suggests that other DNA-binding proteins and/or transcriptional regulators must be expressed by *B. burgdorferi* and play important roles in regulating gene transcription.

#### **1.4.2 Development of genetic tools in *B. burgdorferi* research**

Genetic manipulation did not come underway until a decade following the isolation of *B. burgdorferi*. Genetic studies in *B. burgdorferi* have been extremely difficult due to its fastidious growing condition, segmented genome part of which could be lost during *in vitro* propagation, lack of selectable markers, extremely low DNA transformation efficiency and evolutionary distance from other genetically tractable bacteria. The first successful *B. burgdorferi* transformation was reported by Samuels and coworkers in the year 1984 which is considered as a seminal contribution in the Lyme disease research. Recently, molecular genetics approach has been applied with growing success to study the biology and the virulence of *B. burgdorferi* (Rosa et al., 2005; Sartakova et al., 2001; Tilly et al., 2000; Yang et al., 2009; Zhang et al., 2009). However, the generation of isogenic mutant and complemented clones still remains technically challenging, primarily due to the unstable nature of some of the essential plasmids, multifactorial barriers to the uptake and incorporation of exogenous DNA and highly efficient restriction modification system. Polar effects following insertional inactivation and allelic exchange can arise as a consequence of genetic manipulation, especially in cases of *B. burgdorferi* chromosome, which contains many genes with overlapping transcriptional and termination signals (Fraser et al., 1997b). The presence of paralogous gene families encoding products with similar functions and with overlapping transcriptional signals, in *B. burgdorferi* also can complicate the

interpretation of mutant phenotypes (Casjens et al., 2000a). Despite critical challenges, substantial progress has been made in utilizing a genetic approach to study factors involved in the biology and pathogenesis of *B. burgdorferi*.

In *B. burgdorferi*, the gene of interest is inactivated mainly by three methods, allelic-exchange, plasmid integration or random genome-wide transposon mutagenesis. Historically, *ospC* was the foremost gene to be inactivated in *B. burgdorferi* by allelic-exchange (Tilly et al., 1997) by means of the selective marker  $GyrB^r$ , a mutated form of the chromosomal *gyrB* gene conferring resistance to coumermycin A1 (Samuels et al., 1994). Yet, coumermycin as a selectable marker is imperfect due to high frequency of recombination with the endogenous *gyrB* gene (Tilly et al., 1998). Lately, an efficient selectable marker was developed by engineering the *B. burgdorferi* flagellin A (*flaA*) or flagellin B (*flaB*) promoter with the kanamycin-resistance gene from Tn903 (Bono et al., 2000). This strategy of linking *flaB* gene promoter to the selectable markers is now an established technique to genetically manipulate *B. burgdorferi* and routinely used by several laboratories. Additional selectable markers have now been developed including *ermC* (conferring resistance to erythromycin) (Sartakova et al., 2000), *aacCI* (gentamycin) (Elias et al., 2002b), *aadA* (spectinomycin and streptomycin) (Frank et al., 2003).

The next confront is to delete genes in infectious *B. burgdorferi*. Apparently, low passage, infectious *B. burgdorferi* isolates grow slowly, exhibit lower plating efficiency and transformation frequencies are 100-fold lower than those of high passage, non-infectious isolates (Tilly et al., 2000). Amongst *B. burgdorferi* isolates, genetic manipulation studies often use the sequenced prototype isolate B31 and 297 whereas

some other isolates, such as N40 is relatively difficult to transform. Interestingly, just as the loss of plasmids lp25, lp28-1, and lp28-4 correlates with loss of infectivity of *B. burgdorferi*, the absence of lp25 correlates with an increase in transformation efficiency. The lower transformation frequency is coupled with restriction-modification system in infectious *B. burgdorferi* which is encoded on the lp25 and lp56 plasmids and is missing or non-functional in *in vitro* cultivated *B. burgdorferi* (Lawrenz et al., 2002). Additionally, the nicotinamidase gene *pncA* (BBE02) positioned on lp25, also potentially effects transformation efficiency of *B. burgdorferi*. Deletion of BBE02 increased the transformation efficiency for infectious *B. burgdorferi* B31, providing a valuable tool for determining virulence factors contributing to Lyme disease (Kawabata et al., 2004).

Genetic complementation based on the use of shuttle vectors for trans-complementation and vectors for cis-complementation has been developed for *B. burgdorferi*. The first shuttle vector introduced in *B. burgdorferi* was pGK12 with an *ermC* gene conferring resistance to erythromycin (Sartakova et al., 2000). Another vector, pBSV2 including a region of *B. burgdorferi* cp9 and a kanamycin resistance marker, was successfully transformed in low passage *B. burgdorferi* N40 (Stewart et al., 2001) and served as the basis for the most widely used shuttle vector pBSV2G, containing a gentamicin resistance (GmR) marker (Elias et al., 2003). Additional shuttle vectors and other complementation techniques for *B. burgdorferi* are also available (Eggers et al., 2002; Hubner et al., 2001; Pal et al., 2008b; Promnares et al., 2009; Sartakova et al., 2001; Yang et al., 2009; Zhang et al., 2009).



## **1.5 Role of outer membrane proteins in *B. burgdorferi* infectivity and transmission**

Outer surface protein (Osp) A, OspB and OspC are amongst the first known borrelial proteins and are considered as the most abundant lipoprotein expressed by *B. burgdorferi*. An OspA/B gene is organized under a single operon and is so far the most extensively studied lipoprotein (Barbour, 1988; Bergstrom et al., 1989). OspC which is encoded on cp26 (Fraser et al., 1997b) is homologous to variable small proteins (Vsps) of relapsing fever *Borrelia* (Carter et al., 1994). The expression of OspA/B and OspC are reciprocally regulated, undergoing a switch during *Borrelia* transition from its tick vector to the mammalian host. In unfed ticks, *B. burgdorferi* express high amounts of OspA and OspB and almost undetectable levels of OspC, whereas during the blood meal and upon mammalian infection, OspA/B synthesis is down-regulated and OspC production is up-regulated (de Silva AM, 1996; Schwan et al., 1995). Immunization with OspA confers protective immunity and protects the host against subsequent *B. burgdorferi* infection (Wallich et al., 1996). However, use of OspA as a vaccine is questionable because of possible side effects (Lathrop et al., 2002) amongst other reasons. OspA may function as an adhesin in the tick midgut epithelium (Pal et al., 2001; Pal et al., 2004b) and binds to tick gut receptor TROSPA (Pal et al., 2000; Pal et al., 2004a). OspA was also shown to bind to plasminogen (Coleman et al., 1997) and may provide protection for other surface proteins, such as P66, by shielding against possible protease digestion (Bunikis and Barbour, 1999). OspC might facilitate the invasion of *B. burgdorferi* in tick salivary glands (Fingerle et al., 2007; Gilmore and Mbow, 1999; Pal et al., 2004b). OspC binds to tick salivary protein Salp15 (Ramamoorthi et al., 2005), and OspC-Salp15 interaction

facilitates establishment of spirochete infection of the host and movement to the mammalian host (Gilmore and Mbow, 1999; Pal et al., 2004b). In addition, OspC synthesis correlates with migration from the tick midgut to the salivary gland (Fingerle et al., 2002; Gilmore and Mbow, 1999; Leuba-Garcia, 1998; Schwan and Piesman, 2000). However, in another study *B. burgdorferi* OspC function was shown to be strictly required to infect mice, but did not seem to be necessary for spirochete dissemination to the salivary gland (Grimm et al., 2004a).

The multicopy lipoproteins (Mlp) family is one of many paralog protein families and there are upto ten *mlp* genes which are differentially expressed in response to *in vitro* culture and host-induced environment, encoded on cp32 and cp18 plasmid (Caimano et al., 2000; Porcella et al., 1996). Thus, Mlp expression is differentially up-regulated in harmony due to either elevated temperature (37°C versus 23°C), increased spirochete cell density, reduced pH (6.8 versus 8.0) of the culture medium, and by unknown host factors (Ojaimi et al., 2003; Ojaimi et al., 2005; Porcella et al., 2000; Yang et al., 1999; Yang et al., 2003). These references circuitously suggest that Mlp proteins might also be involved in *B. burgdorferi* infections of the mammalian host. Lp6.6, a small lipoprotein encoded by *bba62*, is drastically down-regulated throughout mammalian infection and its function might be coupled with *B. burgdorferi* encountering the tick environment (Lahdenne et al., 1997). Lp6.6 has recently been demonstrated to facilitate transmission from tick to mice and is involved in maintaining stable protein complexes in the outer membrane (Promnares et al., 2009). The RevA protein is expressed during mammalian infection, and is up-regulated immediately after the *B. burgdorferi* infected tick feeds on the mammal (Gilmore and Mbow, 1998; Gilmore et al., 2001). Many more new *B.*

*burgdorferi* outer surface exposed lipoproteins have been identified such as BB0323, BB0210, BB0329, BB0365, BB0668, BB0681, BB0844, BBA03, BBA07, BBA25, BBA34, BBA36, BBB19, BBI42, BBK07, BBK13, BBK32, BBK53, BBL40, BBM27, BBO40, BBP39, BBQ03 BB0689, BBA36, BBA66, and BBA69 that are actively expressed and immunogenic during *B. burgdorferi* infections. These membrane antigens can be targets of vaccine candidates to prevent the Lyme disease (Barbour et al., 2008; Brooks et al., 2006; Yang et al., 2009).

The roles of outer membrane proteins as microbial adhesin have been studied in detail in *B. burgdorferi*. A number of borrelial membrane antigens are identified that binds components of host extracellular matrix (ECM). The surface-exposed protein ErpX was identified as having affinity for laminin, and is the first laminin-binding protein to be identified in a Lyme disease spirochete (Brissette et al., 2009). An additional *B. burgdorferi* protein that binds specific ECM molecules are the surface lipoprotein decorin-binding proteins A and B (DbpA/B) (Guo et al., 1998; Guo et al., 1995; Hanson et al., 1998), which is hypothesized to be involved in tissue tropism of Lyme disease spirochetes. Expressions of these two proteins are temperature-regulated and are induced at 35°C rather than 23°C (Ojaimi et al., 2003). Antibodies against DbpA can thwart an infection (Hagman et al., 1998; Hanson et al., 1998), which shows the importance of tissue adhesion in the pathogenesis of Lyme disease.

*Borrelia* glycosaminoglycan-binding protein (Bgp) is a surface hemagglutinin of *B. burgdorferi* and can bind to heparan sulphate and dermatan sulphate. Although, its classification as a lipoprotein is undetermined (Parveen and Leong, 2000), the adaptation of spirochetes to the mammalian host environment leads to enhanced glycosaminoglycan

(GAG) binding, thus playing a significant role during mammalian infection (Parveen N, 2003). Moreover, Bgp expression was up-regulated during conditions that mimicked tick feeding (Ramamoorthy and Philipp, 1998). Secretion of Bgp into extracellular environment has always been noticed signifying a possible role as an immune response decoy (Cluss et al., 2004). Bgp is a rare example of borrelial proteins that are known to be secreted into the extracellular milieu.

BBA64, BBK32, VlsE, OspC and BmpA, OM proteins are important diagnostic tools for early Lyme disease, being recognized by sera from *B. burgdorferi* infection and are also expressed *in vivo* and up-regulated at 35°C versus 23°C (Bryksin et al., 2005; Fikrig et al., 2000; Indest and Philipp, 2000; Magnarelli et al., 2002; Ojaimi et al., 2003). More recently, another OM protein BBK07 has been characterized as a novel serodiagnostic marker for Lyme disease (Barbour et al., 2008; Coleman and Pal, 2009). Although these OM proteins are induced during mammalian infection, in most cases their functions remain as an enigma. However, BBK32 binds fibronectin (Probert and Johnson, 1998) and promotes *B. burgdorferi* attachment to GAGs (Fischer et al., 2006). Furthermore, inactivation of *bbk32* in low-passage, infectious *B. burgdorferi* leads to significant attenuation of borrelial virulence in the mouse model of infection (Seshu et al., 2006). Thus, the borrelial fibronectin-BBK32 interaction appears to be an important pathogenic mechanism of *B. burgdorferi*, particularly for dissemination and persistence (Norman et al., 2008; Seshu et al., 2006). In contrast, another study concluded that genetic inactivation of *bbk32* failed to influence *B. burgdorferi* infectivity in mice (Li et al., 2006).

Many lipoproteins have also been studied for being involved in serum resistance. OspE and OspF are the predominant members of OspE/F related proteins (Erp) encoded on both cp32 and cp18 plasmids. *B. burgdorferi* can carry as many as nine different *erp* loci per cell and all can be expressed simultaneously (Akins et al., 1999; Casjens et al., 2000a; Casjens et al., 1997; Stevenson et al., 1998; Stevenson et al., 1996). Many Erps can bind complement inhibitory factor H helping *B. burgdorferi* to circumvent host-mediated serum killing (Hellwage et al., 2001; Stevenson et al., 2002). Fascinatingly, another group of lipoproteins possess OspE/F-like leader peptides (Elp). Even though they are unrelated to OspE/F, few of them are transcriptionally associated (Akins et al., 1999; Hefty et al., 2001). Gene rearrangements within the Erp/Elp paralog families have contributed in sequence assortment, nurturing the parasitic strategy and immunoevasiveness of the Lyme disease pathogen (Akins et al., 1999; Caimano et al., 2000). Members of Erp and Elp families are differentially expressed during *B. burgdorferi* movement in tick and transmission to mammalian host (Akins et al., 1995; Hefty et al., 2001; Stevenson et al., 1998). Host-specific signals were found to alter the expression patterns and cellular localization of these lipoproteins (Hefty et al., 2002).

*B. burgdorferi* is known to express up to 5 complement regulator-acquiring surface proteins (CRASPs). CRASP-1 is a multifunctional protein of *B. burgdorferi* that binds to several human extracellular matrix proteins and plasminogen (Hallstrom et al., 2010). These interactions may contribute to organ tropism, bacterial colonization, adhesion and spreading of *B. burgdorferi* in the host. CRASPs of *B. afzelii* also bind complement inhibitory factor H and factor H-like protein to avoid complement activation (Kraiczky et al., 2001), although a recent study demonstrated that the inactivation of one

of the factor H binding borrelial protein, BbCRASP-2, had insignificant effects on borrelial infectivity (Coleman et al., 2008). Different strains of *B. burgdorferi* can be classified as sensitive, intermediate-sensitive or resistant to the bactericidal effects of human serum (Kraiczky et al., 2001), which might associate with heterologous function CRASPs and OspE.

In conclusion, a set of borrelial genes are identified that play important role in supporting spirochete persistence in the mammalian host or arthropod vector or their transitions, such as *ospC*, *rpoS*, *rpoN*, *rrp2*, *vlsE*, *bb0210*, *dbpBA*, *ospA*, *bptA*, *napA/dps*, *bba62*, *bb0690* and *bb0365* (Caimano et al., 2007; Fisher et al., 2005; Grimm et al., 2004b; Li et al., 2007b; Pal et al., 2008a; Pal et al., 2004b; Promnares et al., 2009; Revel et al., 2005; Shi et al., 2008; Weening et al., 2008; Yang et al., 2009; Yang et al., 2003; Yang et al., 2005; Yang et al., 2004). These studies lend further support to the speculation that *B. burgdorferi* modifies its gene expression in a coordinated manner to adapt to different microenvironment(s) of hosts and the vector.

## 1.6 Hypothesis

An experimental reproduction of infectious cycle of *B. burgdorferi* in the laboratory using mouse model has enabled us to carefully analyze the timing and mechanics of spirochete transmission from the tick to mouse (Piesman, 1993, 1995; Piesman et al., 1987; Piesman et al., 1990; Ribeiro et al., 1987). However, literature search suggests that till date most of the investigations for understanding *B. burgdorferi* gene expression *in vivo* or virulence have been focused on the mammalian side. Although OspA has been approved by FDA for human use as a Lyme disease vaccine, it

was withdrawn from the market within two years of FDA approval, due to several reasons including poor sales by the manufacturer, possible autoimmune disorder and relatively rapid waning of OspA antibody titer in vaccinated individuals. Therefore, identification of novel vaccine candidate and development of new preventive measures remains a primary focus of Lyme disease research. Here I employed high throughput quantitative reverse transcriptase PCR (qRT-PCR) method to study expression of novel membrane proteins of *B. burgdorferi* in ticks. I optimized a high-throughput qRT-PCR based assay to compare borrelial sub-genomic transcriptome between feeding ticks and murine hosts to identify genes that are primarily expressed in the arthropod vector. I focused on the study of *B. burgdorferi* membrane antigens that are encoded by stable and conserved genetic elements. In this dissertation I solely focused on a *B. burgdorferi* protein BBA52, which is expressed in ticks but is not produced during mammalian infection. In fact, I discovered that BBA52 is a vector-specific and feeding-induced gene product that is also highly immunogenic. I hypothesized that *B. burgdorferi* OM proteins, if found to be critical for borrelial persistence and transmission between ticks and mammalian hosts, could be used as a novel antigenic target to develop new transmission-blocking Lyme disease vaccines.

## **Chapter 2: Analysis of *B. burgdorferi* transcriptome during pathogen transmission from feeding ticks to naïve hosts**

### **Abstract**

*B. burgdorferi*, the pathogen of Lyme borreliosis, persists in a natural tick-rodent transmission cycle. Assessment of *in vivo* and *in vitro* microbial transcriptome encoding selected membrane proteins by high throughput qRT-PCR, showed *bba62*, *bba52*, *bb0323*, *bba74*, *bbe31*, *bbg01* are predominantly expressed during vector-specific phase of *B. burgdorferi* life cycle compared to mammalian host. In addition, *bba52* and *bba62* are expressed exclusively in vector, and have no detectable expression in the mammalian host. Further assessment of these proteins may shed new light on vector-pathogen interaction and *B. burgdorferi* persistence through a tick-rodent cycle.

### **2.1 Introduction**

*B. burgdorferi* is unique spirochete which requires obligate blood-feeding arthropods for the transmission and maintenance in susceptible vertebrate host populations (Canale-Parola, 1984). The four principal hard tick species from the family *Ixodidae* that transmit Lyme disease spirochetes (*Borrelia burgdorferi* sensu lato) include *Ixodes scapularis* in eastern North America, *I. pacificus* in western North America, *I. persulcatus* in Asia, and *I. ricinus* in Europe. *B. burgdorferi* does not readily infect most tick, suggesting that the interactions between *Ixodes* are highly specific. A few past



studies have shed light on the pathogen-vector interaction (de Silva AM, 2009; Fikrig and Narasimhan, 2006; Munderloh and Kurtti, 1995; Tsao, 2009). The first report of a functional genomics approach to assess differential expression of *B. burgdorferi* genes cultivated in different environments involved a microarray based analysis (Revel et al., 2002a). Subsequently other studies also described transcriptional profiles of *B. burgdorferi* genes regulated either by temperature (Ojaimi et al., 2003) or mammalian host factors including spirochete cultivation in dialysis membrane chamber (DMC) implanted under the host skin or exposure of cultured *B. burgdorferi* to blood (Brooks et al., 2003; Tokarz et al., 2004). Along with microarray, other tool like DECAL (differential expression with a customized amplification library) has been used to understand transcriptome of *B. burgdorferi* in fed tick (Narasimhan et al., 2002b; Pal et al., 2008a). However, the host environments are likely to be too complex to be reproduced *in vitro*. Therefore, genes that are expressed *in vivo*, especially in the vector are required for a better understanding of *B. burgdorferi* infection cycle. In fact identification of antigens that are expressed in the vector has important implication for identification of vaccines against tick-borne disease like Lyme disease. As antigens expressed in the host are, often, not effective vaccine candidates because pathogen evolve defensive mechanisms to evade the sophisticated immune system of mammals, including antigen variation via variable recombination and differential gene expression (De Silva and Fikrig, 1997; Zhang et al., 1997; Zhang and Norris, 1998). On the other hand, the vector lacks an adaptive immune system, the antigenic variation mechanisms of *B. burgdorferi* appear to be minimally active (Indest et al., 2001; Nosbisch and de Silva, 2007) and the antigens expressed by *B. burgdorferi* are primarily conserved in vector.

Here in this chapter, I have optimized and employed a high throughput quantitative reverse transcriptase PCR (qRT-PCR) analyses to measure the *B. burgdorferi* gene expression in feeding ticks and compared it with corresponding gene expression in spirochetes that are either *in vitro* grown or isolated from dermis of infected mice. I was interested to identify the *B. burgdorferi* gene products that are exclusively expressed in ticks during the enzootic infection cycle of spirochetes. Further identification of *B. burgdorferi* antigens that are essential for spirochete persistence and transmission through the vector could potentially contribute to the development of a transmission-blocking vaccine.

### **2.1.1 Differential expression of *Borrelia burgdorferi* transcriptome in mammalian host and tick vector**

*B. burgdorferi* has evolved strategies to differentially express its gene products including surface antigens that likely plays important role in pathogen survival in two significantly different niches of rodent host and arthropod vectors. Mammals and tick possess radically different microenvironment for *B. burgdorferi* in terms of immune system, nutrient content, oxidative state, pH and temperature amongst other factors. Being unique in its genome content among all other bacterium, *B. burgdorferi* is able to maintain a segmented genome with 21 extra chromosomal plasmids and regulate genes in a tight temporal and spatial pattern in order to sustain itself in the enzootic cycle. This differential expression of *B. burgdorferi* has been a special interest by the researcher community to exploit these genes that are induced at a given time and tissue location, and evaluated their role in preventing the disease transmission from mammals to tick and vice versa.

The recent advancement in the molecular techniques for manipulating *B. burgdorferi* genome has helped scientists to identify many conundrums of spirochetal infection in human and animal populations. Techniques like microarray, DECAL and quantitative PCR analysis have proved to be central tools to identify the catalog of *B. burgdorferi* differentially expressed genes during the pathogen persistence in the mammals or in ticks. *B. burgdorferi* has developed strategies to survive in two different niches by orchestrating the expression of various genes in response to the changing environment (Pal and Fikrig, 2003). As example, *B. burgdorferi* synthesizes fibronectin-binding protein (BBK32), decorin-binding protein (DbpA), a family of anticomplement proteins (Erp's), and VlsE proteins in mammals, and these proteins facilitate spirochete dissemination and survival within the mammal (Barthold et al., 1990; Fikrig et al., 2000; Guo et al., 1998). In contrast, outer surface protein A (OspA) and OspB are expressed mainly by *B. burgdorferi* in ticks (Li et al., 2007b; Schwan and Piesman, 2000). BB0690 is highly expressed in *B. burgdorferi* residing in the gut of the quiescent intermolt *I. scapularis* and likely plays a role in protecting spirochetes during dormancy in unfed ticks (Li et al., 2007b).

A variety of animal models have been identified to study *B. burgdorferi* infection. Amongst these models, murine models are most popular; in particular C3H/He mice are most widely used. Inbred C3H/He mice can readily be infected with *B. burgdorferi* via needle challenge or tick feeding (Anguita et al., 2003; Barthold et al., 1990; Barthold et al., 1993). The spirochete disseminates to various murine tissues like skin, heart, joint, bladder and cause severe arthritis and carditis. Similarly, analysis of *B. burgdorferi* transcriptome in *I. scapularis* during feeding on *B. burgdorferi*-infected mice provides

evidence for their survival strategies in the vector. During the first blood meal of larval ticks on infected mice, the bacterium first enters the gut of the tick and resides in the gut throughout the long intermolt periods of low metabolic activity (Pal and Fikrig, 2003). The spirochete exits the gut during subsequent blood meal, passes to salivary gland and is transmitted to vertebrate host (Fikrig and Narasimhan, 2006). During the entire voyage in ticks, *B. burgdorferi* undergoes marked changes in the expression of surface proteins for its successful transmission to another vertebrate host.

Recent studies have identified a number of *B. burgdorferi* genes that are upregulated in the vector which plays significant role in survival of spirochete in ticks (Munderloh and Kurtti, 2005). As example, *ospA* and *ospB* genes facilitate *B. burgdorferi* colonization in tick gut (Neelakanta et al., 2007; Pal et al., 2001; Yang et al., 2004). Similarly, *ospC* is involved in the transmission of *B. burgdorferi* through ticks (Pal et al., 2004b) and establishment of infection in the mammalian host (Grimm et al., 2004b). Additional gene expression profile has been studied in arthropod vector and includes *erpT* (Fikrig et al., 1999), *bbk32* and *bbk50* (Fikrig et al., 2000), *rev*, *mlpA*, *erpa/i/n*, and *erpb/j/o* (Gilmore et al., 2001), *bb0690* (Li et al., 2007b). Although some genes are dramatically and specifically upregulated in *B. burgdorferi* life cycle, gene deletion studies suggested they are functionally redundant and does not play critical role in pathogen persistence and transmission (Coleman et al., 2008; Hubner et al., 2003; Li et al., 2007a; Stewart et al., 2008).

## **2.2 Materials and Methods**

### **2.2.1 Oligonucleotide synthesis of hypothetical genes of *B. burgdorferi***

About 140 genes were selected based on their predicted localization to the spirochete membrane, according to the database annotation, and PSORT prediction *in silico* analysis (Yang et al., 2009). The primers used for qRT-PCR reaction was designed using OligoPerfect Primer design software (Invitrogen) based on the *B. burgdorferi* B31 M1 genomic sequence (Casjens et al., 2000a; Fraser et al., 1997b). All qRT-PCR primer pairs with a similar annealing temperature (60°C) and an amplicon size ranging from 100-300 base pairs were designed for each of the selected *B. burgdorferi* genes. Each primer pair was tested for efficiency and non-specific amplification by melt-curve analysis using *B. burgdorferi* genomic DNA as a template in the iQ5 real-time thermal cycler using iQ™ SYBR Green Supermix according to the manufacturer's instruction (Bio-Rad laboratories) (Livak and Schmittgen, 2001).

### **2.2.2 Laboratory infection of the *Ixodes* tick with B31A3 infectious strain of *Borrelia***

A group of five C3H mice was infected with *B. burgdorferi* intradermally ( $10^5$  spirochete/animal) by needle inoculation. For this, *B. burgdorferi* B31A3 (Elias et al., 2002b) strain was grown at 34°C incubator in the laboratory prepared BSK medium by reculturing from the glycerol stock of *B. burgdorferi*. After four to five days of culture, the concentration of *B. burgdorferi* was counted on a Petroff Hausser Counting Chamber under a dark field microscope. The number of bacteria per ml was calculated by counting the four outside corners of the central square millimeter and by multiplying the average of the four corners count by  $16 \times 5 \times 10^4$ . If the culture is collected from the late log or stationary growth phase with higher concentration of bacteria, the culture can be diluted

in sterile PBS or BSK in 1:10 for more accurate counting by the microscope. Once the counting was done, mice were restrained appropriately and the injection site was cleaned with 70% ethanol. Using 1 ml tuberculin syringe with 25 G needle, mice were injected a single injection intradermally  $10^5$  spirochetes in 100-200  $\mu$ l of BSK media for each animal. For feeding studies, unfed larvae were obtained from our collaborator in Connecticut Agriculture Experiment Station. After 14 days of infection, 30-40 *Ixodes* larvae were fed on each C3H mouse separately. For set up of tick feeding, the mice was first anaesthetized using Ketamine and Xylazine combination (Ketamine: 120 mg/Kg and Xylazine: 16 mg/Kg) and the dose rate was 0.1 ml/10g body weight intraperitoneally. The cage containing the unconscious mice were placed on a sticky mat (Adhesive entryway, Fisherbrand<sup>®</sup>) to avoid possible escape of larvae. Mice were infested with larvae by gentle brushing with a light paint brush. Mice were allowed to regain its consciousness and in the mean time, mice cage box were prepared. The mice cage contains dry raised wire mesh that holded mice comfortably and underneath which, there was water to collect larvae after repletion. There was an additional barrier to prevent accidental escapes of larvae- entire mouse cage was placed within a larger rabbit cage with a thin layer of contained water. The whole mouse cage setup was then allowed to stand over 2.5×2.5 feet sticky mat (Adhesive entryway, Fisherbrand<sup>®</sup>) placed on the racks. It is mandatory to keep one mice per cage as they have social instinct to remove larvae from each other. After repletion, the ticks were collected as fed *B. burgdorferi*-infected larvae. The larvae were stored in a properly humidified chamber, maintained at 25°C after being received. I used a commercially-available environmental chamber that was set at 24°C with 16h/8h light/dark photoperiod regimen and 95% humidity. In this

condition, fed larvae usually molt to nymphs within 4-5 weeks following engorgement. This group of newly molted infected ticks could be used for laboratory transmission of *B. burgdorferi* to the naïve mice. *B. burgdorferi* infectivity in the molted ticks was tested by qRT-PCR using *flaB* gene primer, a housekeeping gene of the *B. burgdorferi* for one of the flagellar proteins.

### **2.2.3 Experimental infection of naïve mice with the infected *Ixodes* Nymph and comparative expression analysis of *B. burgdorferi* gene in the vector, host and *in vitro* culture using quantitative RT-PCR.**

After molting of the infected larvae to nymph, a group of 5 mice was fed with 10 infected nymphs separately on each mouse with the same cage settings as discussed before with the larvae feeding. Five ticks was collected forcefully at different time point from the mice after 24 h, 48 h and 96 h of transmission using fine forceps and stored in liquid nitrogen until RNA was extracted. Simultaneously, mice was sacrificed after two weeks of *B. burgdorferi* infected tick infestation by atlanto-occipital joint dislocation and mice tissue skin, heart, joint (ankle) and bladder were collected under sterile condition and stored in liquid nitrogen until RNA extraction. RNA was extracted from the tick sample using the Qiagen RNA extraction kit followed by DNase treatment (NEB). cDNA synthesis was performed using Stratagene cDNA synthesis kit using random primer as per the protocol. For the mice tissue, RNA was extracted after homogenizing the tissue sample in liquid nitrogen using pestle and mortar. The RNA extraction was performed using the Trizol reagent (Invitrogen). Briefly, pooled tissues were

homogenized by crystallizing the samples using liquid nitrogen in pestle and mortar. Tissues were homogenized in 1 ml of Trizol reagent per 50-100 mg of tissue using pestle and mortar. The homogenized samples were incubated at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. Thereafter, 0.2 ml of chloroform per ml of Trizol was added in the Eppendorf tube and was vigorously shaken for 15 sec and incubated at room temperature for 5 min followed by centrifugation at  $12000\times g$  for 15 min at  $4^{\circ}C$ . Following centrifugation, the aqueous phase was separated carefully in another Eppendorf tube and RNA was precipitated with 0.5 ml of Isopropanol for 10 min at room temperature and then centrifuged for 10 min at  $12000\times g$  at  $4^{\circ}C$ . The RNA pellet obtained was washed with 1 ml of 75% ethanol per 1 ml Trizol. RNA obtained was further treated with DNase. Briefly, about 10  $\mu g$  RNA was treated with 2 units of DNase in a 20  $\mu l$  reaction at  $37^{\circ}C$  for 10 min followed by heat inactivation of DNase at  $75^{\circ}C$  for 10 min after adding 5 mM final concentration EDTA. First strand cDNA synthesis was performed from these tissues RNA using Stratagene cDNA synthesis kit as follows. About 2  $\mu g$  of RNA in 15.7  $\mu l$  of water and 3  $\mu l$  of random primer (0.1  $\mu g / \mu l$ ) was incubated together at  $65^{\circ}C$  for 5 min followed by  $25^{\circ}C$  for 10 min. Thereafter, a mastermix consisting of 2  $\mu l$  of  $10\times$ AffinityScript RT buffer, 0.8  $\mu l$  of dNTP mix (25 mM each dNTP), 0.5  $\mu l$  of RNase Block Ribonuclease Inhibitor (40 U/ $\mu l$ ) and 1  $\mu l$  of Affinity Script Multiple Temperature Reverse Transcriptase was prepared. A total of 20  $\mu l$  of final reaction volume was prepared and the reaction was set up at  $25^{\circ}C$  for 10 min,  $42^{\circ}C$  for 60 min followed by termination of the reaction at  $70^{\circ}C$  for 15 min and then to  $4^{\circ}C$ .



At the same time, another group of five mice was challenged with single intradermal injection with  $10^5$  spirochetes/mouse. At day fourteen of infection, mice were sacrificed by atlanto-occipital joint dislocation for RNA extraction from skin sample. For isolation of RNA from *in vitro* grown cells, *B. burgdorferi* were grown in BSK medium at 34°C to a concentration of  $10^8$  per ml. RNA was extracted by Trizol reagent from  $10^8$  cells, followed by DNase treatment and first strand cDNA synthesis as discussed before. A sensitive quantitative RT-PCR (qRT-PCR) approach was used to compare spirochete transcriptomes in tick tissues, mice skin and *in vitro* grown *B. burgdorferi* in BSK medium. The primers used for the qRT-PCR reaction were designed using OligoPerfect Primer design software (Invitrogen) with the same annealing temperature (60°C) and a similar amplicon size (~200 bp), using the *B. burgdorferi* B31 M1 genomic sequence. To further ensure specific amplification of *B. burgdorferi* cDNA in tissue samples, thermal cycling in each well was followed by melt-curve analysis, and wells showing non-specific amplification were discarded from the data analysis. For expression screening of *B. burgdorferi* genes, I simultaneously assayed several candidate genes in a 96-well PCR plate, using duplicate wells of template cDNA (cultured spirochetes, infected skin and tick samples) with parallel positive (*B. burgdorferi* genomic DNA) and negative (no template) controls. Transcripts levels of individual gene in murine and tick samples were calculated using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001) normalized against *flaB* transcripts and represented as gene expression levels relative to the *flaB* expression in corresponding tissues derived from two independent experiments. For measuring gene-specific transcripts of spirochete using qRT-PCR, tick cDNA of different time points was pooled together and fold increase in

the expression of individual genes in each of the murine, tick sample and *in vitro* grown *Borrelia* was calculated based on threshold cycle (Ct) values using the  $2^{-\Delta\Delta Ct}$  method, normalized against *flaB* Ct values (Yang et al., 2009). The cyclic reaction condition that was used for quantifying the gene expression of *Borrelia* was as follows:

Cycle 1×1	Step 1	95°C	3 min
Cycle 2×50	Step 1	95°C	10 sec
	Step 2	60°C	20 sec
	Step 3	72°C	20 sec

Briefly, qRT-PCR reaction was set up in 20 µl reaction volume consisting of 10 µl of 2×SYBR Green, 1 µl of primers (Forward and Reverse, 10 µM), 8 µl of water, and 1 µl of cDNA.

### 2.3 Results

*B. burgdorferi* faces radically different tissue environments during host-vector transitions and presumably alters antigenic expression to complete the transition and persist in the new environment. To identify microbial genes that are differentially expressed in feeding ticks during transmission, the transcript levels of selected spirochete genes in nymphs and murine dermis were compared using qRT-PCR analysis. The genes were selected because of their putative membrane localization, as determined by database annotation and *in silico* analysis for extracellular exposure. Naive mice (5 animals/group) were infested with *B. burgdorferi*-infected nymphs (25 ticks/group), and ticks were collected at 24–96 h of feeding and then pooled together. Dermis samples were collected from groups of mice (5 animals/group) at 2 weeks after infection. Both

murine and tick samples were subjected to qRT-PCR analysis performed as detailed in section 2.2.3. The results are represented as the fold increase in individual gene transcript levels relative to *flaB* expression. *bba52*, along with a few other *B. burgdorferi* genes, is highly expressed in feeding ticks during transmission, in comparison with its transcript levels in mice (FIGURE 1). I choose to focus on *bba52*, on the basis of previous studies showing likely tick-specific expression (Ojaimi et al., 2003; Tokarz et al., 2004), its annotation as a nonparalogous and outer membrane protein of unknown function (Casjens et al., 2000a; Fraser et al., 1997b), and its unique genomic location as an insertion into the stable plasmid lp54, which otherwise contains many redundant sequences (Casjens et al., 2000a).

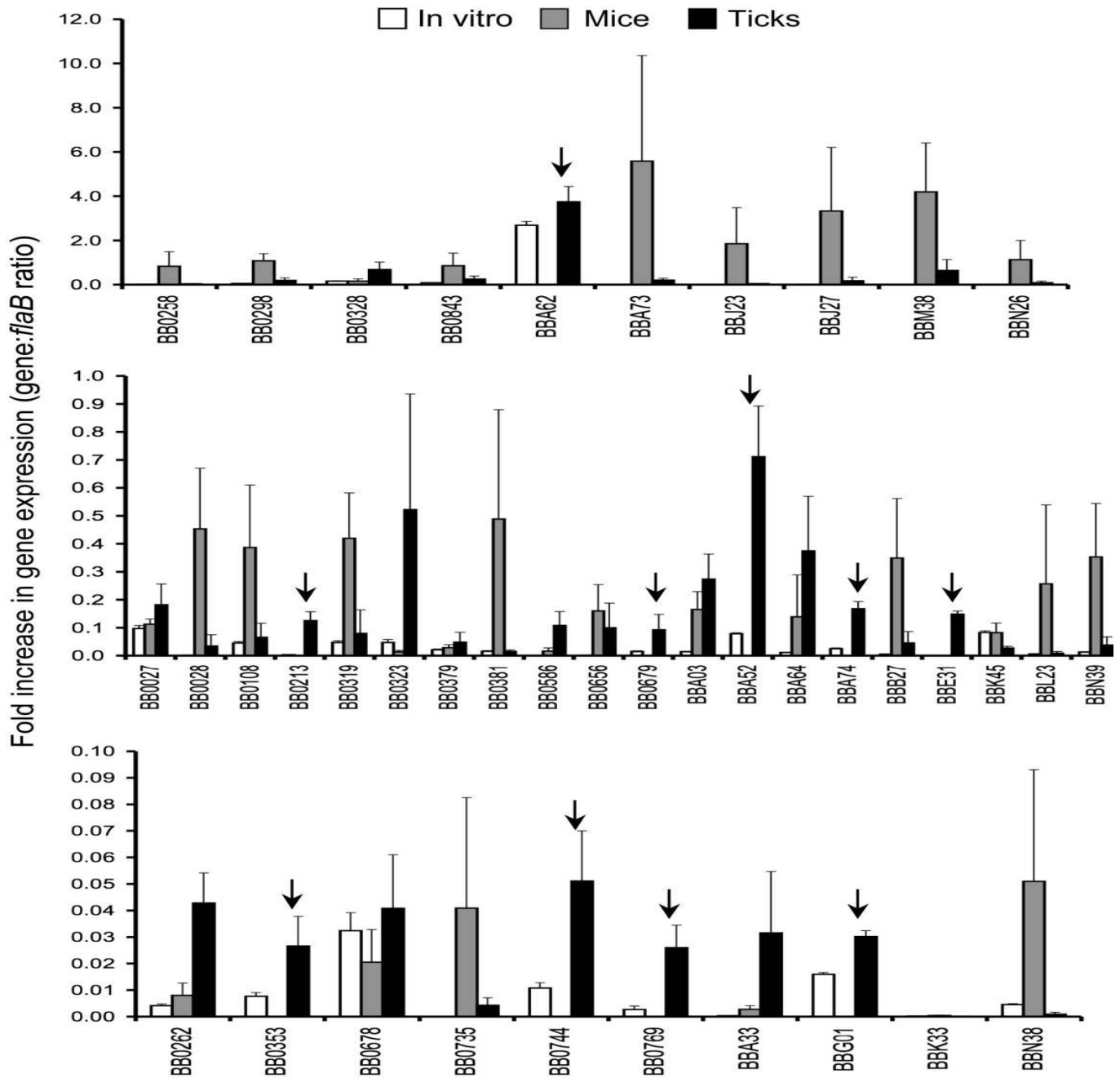


Figure 1. Relative expression levels of selected *Borrelia burgdorferi* genes in feeding ticks during transmission.

Total RNA was isolated from cultured spirochetes; from pooled *B. burgdorferi*-infected nymphs collected at 24, 48, and 96 h of feeding on naive mice; and from murine skin after 2 weeks of *B. burgdorferi* infection. Total RNA was converted to complementary DNA for measuring gene-specific transcripts by use of quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The fold increase in expression levels of individual genes in each of the tick or murine samples was calculated based on threshold cycle (Ct) values, by use of the  $2^{-\Delta\Delta Ct}$  method and normalized against *flaB* Ct values. *Upper*, *middle*, and *lower panels*, genes with the highest, moderate, and lowest expression ratios, respectively, relative to that of *flaB*. Bars denote the mean  $\pm$  standard deviation from 4 qRT-PCR analyses of 2 independent infection experiments. Arrows denote genes that are highly expressed in feeding ticks but remain undetectable in the murine dermis.

**Table 1:** Oligonucleotide primers used in the current study

ORF	Putative Function or Gene Symbol	Paralog Family	Sequence (5' → 3': Forward, Reverse)
BB0019	CHP		CGACTTCCAGTGGCAATTTT, AATTGCAATTCATTCCGCTAT
BB0027	CHP		CCGCAGCCAATATTGATAGG, TATTGCCCCAATACCTCCTG
BB0028	LP		ACATTGCAGCCATGCATTTA, TAGCCATCTCTCCTACGG
BB0108	BMP		TGGGAACTGTAGGGATTAGCTC, CCTTGCTTTGAAGCCTCTTG
BB0144	ABC transporter, binding protein		AGCCTCATTGGGCTTTTTCT, CAACAAATCAACCGCATTG
BB0155	LP		GGAACACTTCCTGGTGAAT, TTTTTAAATCGGGTTTGTTTT
BB0213	LP		TGATTCTAGCCTTGAAAGGA, TGCCCTTATCAAAAAGACGAA
BB0258	Undecaprenol kinase		TACCGGAGTTGTTGGGACTT, GCCCCATAAAAATTCCTGCT
BB0262	M23 peptidase domain protein	2	GGGAAGATTGCCAAAGATT, CCGCATTAATCCTGCAGTT
BB0298	TPR repeat domain protein, LP		CTTGGTGATATGGGTGAGGA, TGCTCTCAGTTGAAGCCTTT
BB0319	Exported protein		GCGAGTGGATAGAAATGCTTG, CAGCATTTCCAAAGCCAGAT
BB0323	LysM domain protein		ATATGGATCCCGCTGGAAT, AGCCGCTTCAAGTGCTTTTA
BB0328	Solute-binding proteins, family 5, LP	37	GCGATGGAGTTGCCATTAAT, ATGCCAAGCTCAGATTCAGG
BB0353	Membrane protein, putative		TGCAAAAAGTTGTGGCCTTT, TTCGCTCTGCTCATTGTCTTT
BB0379	Protein kinase C1 inhibitor		TCCTTAACTGTTGGGCATACTC,

			GCCTCTCTTAAACCAAAACCA
BB0381	Trehalase		GTGGGCATGACCTTTAGAGC, GCAACGCTTCTACCAGCTTC
BB0469	IspA		TTTCTTGCAATGCCTATTTTCA, GGCCATCTATCAAGTCCAAAA
BB0586	FemA protein		AGCGATGTTCTTGGCAAAT, TTTGCGGGTTGTATGTCATC
BB0656	Coproporphyrinogen III oxidase,		GCATGAGAGCAGGCACAATA, AAGAGCCCTCAAGCTGACAC
BB0678	ABC transporter, permease protein	130	GCAGGTCTTGCTGGTGCTAT, TGAAGATGGAAGGCCCATTA
BB0679	ABC transporter, permease protein	130	TTTAGCAGGGGTTTCAGGTG, GCCAAAACAATTGCCAAAGT
BB0735	Rare lipoprotein A		CATGGTATGGCGAAGCTTTT, CTCAGCGGCATATTTTGACA
BB0744	Borrelia P83/P100 antigen		AAGCACTCAGCAAGGCATTC, GAAAATTTGGCCAATCTCCA
BB0769	Glycoprotease family		GCCTGAGATTGCCTCAAGAC, GTGCATTAAGGGGCGTAAA
BB0843	Arginine-ornithine antiporter		TTGGTGGATGCAAGAAATGA, AACCTTTCACAAACGCATCC
BBA03	OMP,BbK2.14 (297)		GAAAAATCGCAAGCCAAATC, TTCGTCTCCACCGCTATTTT
BBA33	LP		CAGCGAGATTAAAGTTGGCAA, CTGGCTGTGGATCAAGAACA
BBA52	OMP		CCAAAAGCCCACAAGGTGTA, TCTCTTCCCATCATCTGG
BBA62	Lp6.6		TGTTGCTTGCGAAACTACAAG, TGTCTGGCATTGACTTCATCA
BBA64	P35 antigen	54	TAATGCCAACAATGCTCCAA, GTGGCTGTCAAATGCACTG
BBA73	Putative antigen P35, LP	54	CGCAAACATCAAGCTCAAAC, CCGAGAATTTTCCATCAGC
BBA74	OMS28	171	GCTGTTTCTGTTGCTGGTGA,

			ATCTCTTGCGCCTTGAGCTA
BBB27	LP		GGTGCATCTTTTAAGGCAGAA, TCCATCTTTACATTCGCCAAG
BBE31	Putative surface protein, LP	60	ATTTGGCACTTTTTGCAACC, TTCCGTTAAATGAGATTCAAGC
BBG01	LP	12	GACACCGCTCAGAAAAGTT, TGCCTCACTTAAAGCCTGTC
BBI16	VraA	60	CTGTTTTAAGCCCCCTGGAT, GCTTTTCCTGCAACACTTCC
BBJ23	TPR repeat domain protein	106	TTATTCGGGCAAAATTGAGG, TCCTGCAATATCGCTTTGA
BBJ27	ABC transporter, permease protein		TGCAGGTGAGTTTTTGCTTG, CTCCACCCTCAAATGCAAAC
BBK33	CHP	65	GATTGGAATCAAATGAAACAAAA, AACCTTTTAGTTGTAACAGGCACA
BBK45	Immunogenic protein P37, LP	75	AAACACAAACCCTCCGTTG, GGCAATAAGGTTGGGTTAGC
BBL23	blyA-8	109	TGATTTTTGTAACAGTGCTGGTTT, TGTGATTTTTGCCATTACCA
BBM38	Holin, BlyA family, erpK, LP	164	TGCAAGAATTACGCAAGTGG, TTCAGCAACACCTTGAACCA
BBN26	Orf D-9	143	AACCCGCCAATTATTCCAAC, CGCAGTTATGGAGTGTTCGT
BBN38	ErpP, LP	162	GTTTAAACGCTGGGGGACAT, ACGCAATATGTTTCAGCACCA
BBN39	ErpQ, LP	163	GTGCTGTTTTTGCGCTGATA, TCGCCCTGCATTAATTCTTC

Abbreviations: CHP, conserved hypothetical protein; HP, hypothetical protein; LP, lipoprotein; OMP, outer membrane protein; MSP, membrane-spanning protein. Designations of the open reading frame (ORF), abbreviated gene function or symbol and paralog family information are according to the annotations in the database ([www.tigr.org](http://www.tigr.org)).

## 2.3 Discussion

*B. burgdorferi* undergoes remarkable changes in antigenic composition as it invades and colonizes diverse tissues in arthropods and mammals (Fingerle et al., 2002; Liang et al., 2002b; Narasimhan et al., 2003; Narasimhan et al., 2002b; Schwan et al., 1995; Tilly et al., 2008; Yang et al., 2009). These changes are mediated at least in more than 10 percent of all *B. burgdorferi* genes by regulatory networks involving Rrp2-RpoN/RpoS or Rrp1-Hpk1 TCS and c-di-GMP (Boardman et al., 2008; Caimano et al., 2007; Hubner et al., 2001; Rogers et al., 2009a; Yang et al., 2003), in addition to the intergenic recombination-based mechanism involving the *vlsE* locus (Coutte et al., 2009; Zhang et al., 1997). Microarray analyses of transcriptional alterations in cultured spirochetes identified a large number of genes that are differentially expressed, including *bba52* which responded to physiochemical alterations, including variations in temperature, the addition of blood, or growth in a dialysis membrane chamber (DMC) implanted within the murine host (Brooks et al., 2003; Caimano et al., 2007; Ojaimi et al., 2003; Tokarz et al., 2004). In agreement with these findings, my data show that selected *B. burgdorferi* genes are also variably expressed *in vivo* and highly transcribed in feeding ticks during transmission. The expression pattern of many of these genes *bb0323*, *bba52*, *bba62*, *bba74*, and *bbe31* agreed with previous studies of cultured spirochetes that predicted preferential expression in ticks (Brooks et al., 2003; Caimano et al., 2007; Ojaimi et al., 2002; Tokarz et al., 2004). Specifically, *bba74* (Mulay et al., 2009) and *bba62* (Promnares et al., 2009) were recently identified as being expressed in feeding ticks. Another gene, *bbg01* has been reported to be upregulated by several fold in *B. burgdorferi sensu stricto* (designated N40 strain) by tick transmission after being



passed for 75 times, showing possible role in pathogenesis (Adusumilli et al., 2010).

The majority of these genes, however, encoded proteins of unknown functions that are possibly relevant for pathogen transmission from feeding ticks or for the establishment of early mammalian infection.

# **Chapter 3: Characterization of a selected tick-induced target gene, *bba52* for its role in pathogen persistence, pathogenesis and vector-host transitions**

## **Abstract**

*B. burgdorferi*, the pathogen of Lyme borreliosis, persists in nature through a tick-rodent transmission cycle. A selective assessment of the microbial transcriptome, limited to gene encoding putative membrane proteins, reveals that *bba52* transcription *in vivo* is strictly confined to the vector-specific portion of microbial life cycle with highest expression levels in feeding ticks and swift downregulation in mice. *bba52* deletion did not affect murine disease as assessed by the genesis of arthritis and carditis or long-term pathogen persistence in mice or ticks. However, *bba52* deficiency did impair microbial transitions between hosts and vector, defects that could be fully rescued when *bba52* expression was genetically restored to the original genomic locus. These studies establish that BBA52 facilitates vector-host transitions by the pathogen and as such, is a potential antigenic target for interference with *B. burgdorferi* transmission from ticks to mammalian hosts.

### 3.1 Introduction

Lyme borreliosis, caused by *B. burgdorferi*, is a vector-borne zoonosis prevalent in North America and Europe (Piesman and Eisen, 2008a, b). When feeding on an infected host, immature *Ixodes* ticks acquire the pathogen and during a subsequent blood meal, transmit the pathogen to a new host. Infected humans develop serious clinical complications including arthritis, carditis and a variety of neurological disorders (Steere et al., 2004). As wild rodents are the natural reservoir hosts of *B. burgdorferi*, certain inbred mice, such as C3H mice are considered excellent models of pathogenesis and are used to study the transmission cycle of spirochetes (Barthold, 1992). Genome sequencing of *B. burgdorferi* (Casjens et al., 2000b; Fraser et al., 1997a), studies on the expression and regulation of borrelial genes (Blevins et al., 2009; Brooks et al., 2003; Caimano et al., 2007; Fisher et al., 2005; He et al., 2008; Liang et al., 2002a; Narasimhan et al., 2003; Ojaimi et al., 2002; Revel et al., 2002b; Rogers et al., 2009b; Tokarz et al., 2004; Yang et al., 2003) and advances in genetic manipulation techniques (Rosa et al., 2005) have all greatly contributed to our understanding of the unique biology and enzootic infection cycle of this spirochete. However, a human vaccine against *B. burgdorferi* is currently unavailable, and thus, the development of effective preventive measures remains one of the major focus of Lyme disease research.

*B. burgdorferi* may persist in a host or vector for months to years, shuffling between locations during short episodes of tick feeding (de Silva et al., 2009). During migration from an infected tick to a host, *B. burgdorferi* invades salivary glands and transmits along with tick saliva (de Silva et al., 2009). Many salivary gland proteins (Salps) are feeding-induced, soluble and can influence the spirochete transition between

vector and host (Das et al., 2001; Rosa, 2005). A few *I. scapularis* Salps have been identified (Narasimhan et al., 2007; Ramamoorthy et al., 2005; Tyson et al., 2007) that play important roles in spirochete infection cycle. Further characterization of the interactions of Salps with borrelial antigens and their contributions to infectivity will aid in our understanding of poorly-understood aspects of borrelial transmission.

It is clear that the identification of borrelial antigens that play important roles in spirochete survival in ticks or enable vector-host transitions is the key to blocking pathogen transmission. As extracellularly exposed membrane proteins may directly interact with different environments during transmission or dissemination thus contributing to pathogen adaptation, I sought to assess the expression of selected putative membrane proteins in feeding ticks and mice. I further studied one of these genes, *bba52*, which displayed vector-specific expression. BBA52, annotated as an outer membrane protein of no assigned function (Casjens et al., 2000b; Fraser et al., 1997a), is encoded by the linear plasmid (lp) lp54, which is a stable extra-chromosomal element and is considered to be a necessary part of the spirochete genome (Terekhova et al., 2006). I show that BBA52 facilitates vector-host transitions of *B. burgdorferi* and is a potential antigenic target to interfere with transmission of Lyme borreliosis.

## **3.2 Materials and Methods**

### **3.2.1 Animal and tick infection with *B. burgdorferi***

A group of five mice was infected with  $10^5$  spirochete / mouse intradermally by needle inoculation. After 14 days of infection, each mouse was infested with 10 naïve nymphs or 50 larvae. Nymphs were collected after 66 h of feeding on mice while larvae were collected after complete feeding. The samples were collected and stored in liquid nitrogen until RNA extraction. For tick transmission, experiment was set up on a group of five mice. Mice were anaesthetized using a combination ketamine and xylazine as described in section 2.2.2. Once the mouse become unconscious, 10-20 *B. burgdorferi* infected ticks were allowed to parasitize on each mouse. The ticks were applied on mice using a fine paint brush. At 42 h and 66 h of feeding, partially-fed ticks were forcefully removed from mice using fine forceps. After collection from mice, ticks were immediately dissected under a high resolution dissecting microscope to separate the tick gut and salivary glands. Dissection was accomplished by immersing the ticks under a drop of sterile PBS on a slide, which is necessary to prevent tick tissues from air drying. RNA was extracted from the dissected sample by Trizol as detailed in chapter 2 and following additional DNase treatment; the cDNA was synthesized using random hexamers.

### **3.2.2 Generation of recombinant proteins and antisera**

#### **3.2.2.1 Cloning of BBA52 open reading frame for protein expression in *E.coli***

A primer set for BBA52 ORF was designed with *Bam*HI and *Xho*I restriction enzymes (Table 2) for directional cloning after deleting the signal peptide using B31 A3 genome data base. Genomic DNA of *B. burgdorferi* B31-A3 was isolated using the Qiagen DNA extraction kit for Gram negative bacteria as per the manufacturer's

protocol. Using the *B. burgdorferi* genomic DNA, BBA52 without the sequence coding for signal peptide, was amplified by PCR at 58°C annealing temperature. The BBA52 PCR product was resolved using agarose gel electrophoresis and the amplicon purified from the excised gel slice using Qiagen gel purification kit as detailed in manufacturer's protocol. About 1 µg of PGEX-6P-1 (Amersham-Pharmacia Biotech) vector and the insert, BBA52 amplicon was digested with restriction enzymes *Bam*HI and *Xho*I (NEB) at 37°C for overnight. Both the vector and the insert DNA fragments were again resolved using agarose gel electrophoresis and purified, as described above. Ligation was performed for 2 h at room temperature in 10 µl reaction solutions consisting of 2 µl of vector, 6 µl of insert, 1 µl of 10 × ligation buffers and 1 µl of DNA ligase (NEB). Five µl of ligated product was used for transformation of chemically competent DH5α *E. coli* cells (Invitrogen). The cells were removed from -70°C freezer, and after addition of ligated product, the cells were left on ice for 30 min. Thereafter, the cells were quickly moved to 42°C water bath to provide a heat shock for 45 sec and immediately kept on ice for additional 10 min. One ml of SOC medium was added and the transformed *E. coli* was kept for 1 h at 37°C on a shaking platform at 250 rpm. After 1 h, 100 µl of transformed *E. coli* was plated on agar plate containing 100 µg/ml of ampicillin and left overnight at 37°C incubator. Next day, about 10 clones were picked up from the agar plates to check for positive clones by colony PCR and were run for agarose gel electrophoresis. Two of the positive clones selected by colony PCR were grown in 10 ml of LB medium for overnight and plasmid were extracted using Qiagen plasmid extraction kit as per the manufacturer's instruction. One ml of each clone was cryopreserved in -70°C in LB media containing 20% glycerol. One microgram of the plasmid was used to

digest the insert using restriction enzymes *Bam*HI and *Xho*I and resolved on the agarose gel electrophoresis to confirm the expected size of the cloned insert. The plasmid clone was further sequenced to rule out any mutation during the DNA engineering. To check the expression of BBA52 protein, the clone was grown overnight in LB media containing ampicillin and in the morning further diluted in 1:100 in fresh antibiotic-containing LB medium and grown until OD<sub>650</sub> of 0.5. The culture was then induced with 1mM IPTG for 2 h. The cell lysates were run on a SDS-PAGE and stained with Coomassie brilliant blue for 1 h. The gel was then destained using the destaining solution for 1 h to visualize the overexpressed BBA52 in *E.coli*. In the similar fashion another *B. burgdorferi* protein BBA62 was also cloned and produced in DH5 $\alpha$  *E.coli* cells without the signal peptide, using primers as shown in Table 2.

### **3.2.2.2 Overexpression and purification of GST-BBA52 and GST-BBA62 tagged protein**

*E.coli* clone expressing BBA52 and BBA62 was retrieved from the glycerol stock and grown overnight in 10 ml LB medium containing ampicillin at 37°C shaking incubator. Next morning, the culture was inoculated in two flasks each containing 500 ml media and allowed to grow until the OD<sub>650</sub> reached 0.5. The expression of the protein of interest was induced by adding 1 mM IPTG for 2 h. Thereafter, cells were centrifuged using Sorvall centrifuge at 5000 rpm for 30 min. The pellets were treated with 20 ml of 1% Triton-X-100 in PBS, left for 20 min on ice and then sonicated for 6 min under ice cold condition. The lysates were then centrifuged at 12000 $\times$ g for 30 min at 4°C to remove the insoluble protein fraction. In the mean time, about 700  $\mu$ l of GST beads (Glutathione Sepharose™ 4B, GE healthcare) were added in the column and washed

twice with PBS to remove ethanol preservatives. Finally, the column was equilibrated and plugged with stopper. The clear supernatant after centrifugation was poured into the column and was allowed to bind with the beads for around 10-15 min. The unbound protein was drained of the column and the beads were washed three times with PBS. Finally, the protein was eluted in Eppendorf tubes with Tris buffer pH 8.0 containing 10 mM glutathione in 1 ml aliquots and stored at -70°C until use.



**Table 2:** Oligonucleotide primers used in the current study

Sequence (5' – 3')	Purpose
GGGAGCTCAAAGACAAAATCGCTTTGC	Primer P1, 5' PCR of the left arm for constructing <i>bba52</i> mutant. A <i>SacI</i> site (italicized) is attached for the purpose of cloning.
AAGGATCCAATATTCTCCTAATATTTAGATGT	Primer P2, 3' PCR of the left arm for constructing <i>bba52</i> mutant. A <i>Bam</i> H1 (italicized) is attached for the purpose of cloning.
GGGTCGACTGATTTGCTTTGGAAGTTTT	Primer P3, 5' PCR of the right arm for constructing <i>bba52</i> mutant. A <i>salI</i> site (italicized) is attached for the purpose of cloning.
GCGGTACCTTAATCCTTTTGCGGAGTT	Primer P4, 3' PCR of the right arm for constructing <i>bba52</i> mutant. A <i>KpnI</i> site (italicized) is attached for the purpose of cloning.
GGTTGCATTGATTCTGTT	Primer P5, upstream 5' PCR primer for the detection of intended integration of <i>pflaB-Kan</i> cassette in <i>bba52</i> locus
AAGTAAAATCACCTCATCTTCTGCTGTT	Primer P6, downstream 3' PCR primer for the detection of intended integration of <i>pflaB-Kan</i> cassette in <i>bba52</i> genomic locus
AGTGTTGCAAGACCATTTGATTTTA	Primer P7, <i>bba52</i> forward primer
TTAATAAACTGATCTTCAAGAGAA	Primer P8, <i>bba52</i> reverse primer
ATGAATAAGCAAGAGATTGCGAC	Primer P9, kanamycin internal forward primer
ATTCGACTCGTCCAACATC	Primer P10, kanamycin internal reverse primer
GTAAGCTCAGCCCGTGCA	Forward primer for RT-PCR of <i>bba51</i>
GCTGTAATAAACCCCGAGATTAA	Reverse primer for RT-PCR of <i>bba51</i>
TGACGAAGAGATTGCAGTCAA	Forward primer for RT-PCR of <i>bba53</i>
CTACCTTTGCTTTTTGGCTTT	Reverse primer for RT-PCR of <i>bba53</i>
GCTCAAATAAGAGTTTGTC	Forward primer for RT-PCR of <i>flaB</i>
ATTCCAAGCTCTTCAGCTG	Reverse primer for RT-PCR of <i>flaB</i>

TTGCTGATCAAGCTCAATATAACCA	Forward primer for Quantitative RT-PCR of <i>flaB</i>
TTGAGACCCTGAAAGTGATGC	Reverse primer for Quantitative RT-PCR of <i>flaB</i>
AGAGGGAAATCGTGCGTGAC	Forward primer for Quantitative RT-PCR of mouse $\beta$ - <i>actin</i>
CAATAGTGATGACCTGGCCGT	Reverse primer for Quantitative RT-PCR of mouse $\beta$ - <i>actin</i>
GGTATCGTGCTCGACTC	Forward primer for Quantitative RT-PCR of tick $\beta$ - <i>actin</i>
ATCAGGTAGTCGGTCAGG	Reverse primer for Quantitative RT-PCR of tick $\beta$ - <i>actin</i>
CCAAAAGCCCACAAGGTGTA	Forward primer for Quantitative RT-PCR of <i>bba52</i>
TCTCTTTCCCATCATCTGG	Reverse primer for Quantitative RT-PCR of <i>bba52</i>
CGGAATTCTTAAATAAACTGATCTTCAAGAG	Primer P11, 3' PCR of the left arm for constructing <i>bba52</i> complemented isolate. A <i>EcoRI</i> site (italicized) is attached for cloning.
CGGAATTCCGAGCTTCAAGGAAGA	Primer P12, Forward primer for amplification of <i>flgB-aadA</i> cassette in pKFSS1 vector. A <i>EcoRI</i> site (italicized) is attached for cloning.
CGCGGATCCATTATTTGCCGACTACC	Primer P13, primer for amplification of <i>flgB-aadA</i> cassette in pKFSS1 vector. A <i>BamHI</i> site (italicized) is attached for cloning.
GAGGATCCAGTGTTGCAAGACCATTTGATTTA	Forward primer for recombinant BBA52 production. A <i>BamHI</i> site (italicized) is attached for cloning.
GGCTCGAGTTAAATAAACTGATCTTCAAGAGAA	Reverse primer for recombinant BBA52 production. A <i>XhoI</i> site (italicized) is attached for cloning.
CGGGATCCATGCACCACCACCACCACAGTGTGGCAAGACCATTTGATTTA	Forward primer for recombinant BBA52 production with 6His-tag at N-terminal in baculovirus. A <i>BamHI</i> site (italicized) is attached for cloning.
CCGCTCGAGTTAAATAAACTGATCTTCAAGAGAA	Reverse primer for recombinant BBA52 production in baculovirus. A <i>XhoI</i> site (italicized) is attached for cloning.
AAGGATCCGAAACTACAAGAATTCAG	Forward primer for recombinant BBA62. A <i>BamHI</i> site (italicized) is attached for cloning.
AACTCGAGTTACTTTTTTCATTGACTTTGT	Reverse primer for recombinant BBA62. A <i>XhoI</i> site (italicized) is attached for cloning.

### **3.2.2.3 Cloning of BBA52 open reading frame for protein expression in Baculovirus expression system**

A full-length BBA52 protein was made by using Bac-to-Bac expression system (Invitrogen). Briefly as per manufacturer's protocol, a site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) is propagated in *E. coli*. The bacmid contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lacZ $\alpha$*  peptide from a pUC-based cloning vector. Inserted into the N-terminus of the *lacZ $\alpha$*  gene is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-attTn7) that does not disrupt the reading frame of the *lacZ $\alpha$*  peptide. The bacmid propagates in *Escherichia coli* DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG. Recombinant bacmids (sometimes referred to as composite bacmids) are constructed by transposing a mini-Tn7 element from a pFastBac™ donor plasmid to the mini-attTn7 attachment site on the bacmid when the Tn7 transposition functions are provided in trans by a helper plasmid. The helper plasmid confers resistance to tetracycline and encodes the transposase. The mini-Tn7 in a pFastBac™ donor plasmid contains an expression cassette consisting of a Gm<sup>r</sup> gene, a baculovirus-specific promoter, a multiple cloning site, and an SV40 poly(A) signal inserted between the left and right arms of Tn7. The plasmid pFastBac™ was used to generate viruses which will express unfused recombinant proteins. Genes to be expressed are inserted into the multiple cloning site of a pFastBac™ donor plasmid downstream of the baculovirus-specific promoter.

Insertions of the mini-Tn7 into the mini-attTn7 attachment site on the bacmid disrupts expression of the *lacZ* $\alpha$  peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid. Recombinant bacmid DNA can be rapidly isolated from small scale cultures and then used to transfect insect cells.

Briefly, BBA52 ORF without the signal peptide sequence and with a 6 $\times$ His tag at N-terminus was amplified by PCR using two primers containing *Bam*H1 and *Xho*1 sites, and *B. burgdorferi* genomic DNA as template, as shown in Table 2. The amplicon was cloned into the pFastBac TOPO donor plasmid at *Bam*HI and *Xho*I site. The recombinant donor plasmid was transformed into competent DH10 Bac *E.coli* cells provided in the kit. *E.coli* containing the recombinant Bacmid (LacZ negative) was selected for obtaining pure miniprep of the recombinant Bacmid. The true recombination clone was further confirmed by doing PCR with M13/pUC forward and reverse primers, as described in the manufacturer's protocol.

#### **3.2.2.4 Sf9 cell culture, transfection and protein purification**

Vials containing Sf9 cells (commercially obtained from Invitrogen) were immediately stored in liquid nitrogen. Sf-900<sup>TM</sup> III SFM medium (GIBCO) was warmed to the room temperature. In the mean time Sf9 cells were thawed at 37°C in water bath under sterile condition. Immediately after thawing of cells, vial was sprayed with 70% ethanol to maintain the sterility. About 2 ml of fresh Sf-900<sup>TM</sup> III SFM medium was added to the cell and transferred to another tube containing 8 ml of Sf-900<sup>TM</sup> III SFM medium. Cells were pelleted at 1000 rpm for 5 minute and this washing step was

repeated twice. Finally, cells were diluted with Sf-900<sup>TM</sup> III SFM medium at a density of about  $1-2 \times 10^6$  cells per ml and are grown in tissue culture flasks in an incubator maintained at 27°C.

One microgram of recombinant Bacmid containing BBA52 ORF was transfected to  $8 \times 10^5$  Sf9 cells in six well plate along with help of Cellfectin<sup>®</sup> II reagent as per the standard protocol. After 4 days, generation of recombinant virus was confirmed by western blot analysis with the Sf9 lysate obtained from the six-well plates and using anti-His HRPO conjugated antibody. For large-scale production of the protein, approximately  $2 \times 10^9$  Sf9 cells were grown in spinner flasks and were infected with 40 ml of a high-titer recombinant baculovirus stock in 500 ml of Sf-900<sup>TM</sup> III SFM medium (GIBCO). After 96 h of incubation at 27°C, the cells were harvested, and collected by centrifugation at 2,000 rpm (Sorvall FH18/250 rotor) for 5 min. The cell pellet was resuspended in extraction buffer (50 mM Tris pH 8.0, 300 mM NaCl, 0.5% Triton X-100, 10 mM imidazole, 1 mM PMSF) and sonicated. The lysates were clarified by centrifugation at 20,000×g for 30 min. The cell extract was incubated with Ni-NTA agarose resin (Invitrogen) for 2 h, then loaded into a column, washed with washing buffer (50 mM Tris pH 8.0, 300 mM NaCl, 0.5% TritonX-100, 40 mM imidazole), and the protein was eluted with PBS containing 1 M Imidazole, and stored at -70°C until further use.

#### **3.2.2.5 Generation of Murine and Rabbit antibody:**

Recombinant GST-tagged BBA62 and BBA52 along with recombinant N-terminus 6×His tagged BBA52 proteins were used to generate antibodies in mice. Briefly, about 10 µg of each protein were separately used to immunize a group of mice (5

mice/group). For primary immunization, antigen was prepared by making emulsions with equal amount of Freund's complete adjuvant (FCA) from Sigma. About 200 µl of the emulsion containing about 10 µg of antigen was used to inject each mouse intradermally. After 10 days, first booster was given with the same amount of antigen in each mouse using Freund's incomplete adjuvant (FIA). Second booster was given in the next 10 days of first booster and in another 7 days of the second booster all mice were sacrificed for serum collection. Serum titer was determined by ELISA with the BBA52 coated plates.

Polyclonal rabbit antibody against BBA52 was also produced commercially (Medicore, Inc.). I also generated additional BBA52 antibodies against a carboxyl terminal peptide of the mature protein. Using a commercial source (GenScript Corporation), affinity-purified polyclonal antibodies against a BBA52 peptide sequence (EFLDDPSQESDELEC) of predicted immunogenicity was generated in rabbits. As a control, affinity purified IgGs were also isolated from normal rabbit serum.

### **3.3 Construction of BBA52-pXLF10601 mutagenesis plasmid for target deletion of *bba52* complete ORF of *B. burgdorferi*.**

#### **3.3.1 Construction and Isolation of BBA52-pXLF10601 mutagenesis plasmid**

For *B. burgdorferi* gene manipulation, plasmid pXLF10601 was used to construct the mutagenesis plasmid as described (Coleman et al., 2008; Kumar et al., 2010; Promnares et al., 2009). This plasmid harbors two multiple cloning sites (MCS1 and MCS2) flanking upstream and downstream of the kanamycin cassette driven by *flaB*

promoter of *B. burgdorferi*. This plasmid also contains another ampicillin resistance cassette, required for selection in *E. coli*. In order to create the mutagenic plasmid for creation of *bba52* deficient *B. burgdorferi*, two sets of primers were designed that encompass about 1500 bp, flanking upstream and downstream of BBA52. The upstream and downstream of BBA52 gene was amplified by PCR and cloned at MCS1 and MCS2 sites using the primers mentioned in Table 1. P1 and P2 are the primers used to amplify the downstream flanking region of BBA52 gene and cloned at MCS1 site while P3 and P4 are the primers used to amplify upstream of BBA52 gene and cloned at MCS2 site. The construct was verified by sequencing of the insert and by digestion of the insert with the specific restriction endonuclease (NEB). After confirmation of the desired construct, the recombinant plasmid was purified in larger quantities by using a maxiprep kit (Qiagen). Finally, the plasmid was eluted in nuclease free water to a concentration of 4-5 µg/µl.

### **3.3.2 Preparation of competent *B. burgdorferi*:**

*B. burgdorferi* were grown in 100 ml of BSK medium at 34°C incubator until a density of  $5 \times 10^7$  cells/ml. The cells were centrifuged in two 50 ml falcon tubes at 4000×g for 20 min at 4°C. Supernatants were decanted and the pellet obtained was resuspended in 12 ml of sterile chilled phosphate buffer saline (PBS) and finally centrifuged at 3000×g for 10 min at 4°C. This step was repeated twice. The pellet was resuspended again in filter sterilized 4 ml of chilled EPS (93 g/L sucrose, 15 % glycerol) and centrifuged at 2000×g for 10 min at 4°C. The supernatant obtained was decanted and the pellet was resuspended in 1.5 ml of EPS solution and centrifuged at 2000×g for 10 min at room temperature. Finally, the pellet was resuspended and equally distributed

in two 50 µl of chilled EPS aliquots. About 25-30 µg of plasmid DNA was added to the resuspended pellet and electroporation was performed using 0.1 cm gap cuvette at 1.8 KV, 25 µF, 400 Ω electroporation setup in an electroporator (BioRad). After electroporation, *B. burgdorferi* cells were quickly recovered from the cuvette in a sterile condition, in 10 ml of BSK medium and left overnight at 34°C. Next day, the transformed *B. burgdorferi* were placed on 96 well plates (Cellstar) after adding 10 ml of another fresh BSK medium with 350 µg /ml of kanamycin for the selection of transformants. Plates were tightly sealed with plastic wrap and stored in humidified incubator maintained at 34°C. After 10 day of incubation, each well was duplicated in another 96 well plate to replenish the kanamycin. *B. burgdorferi* clones recovered after duplication, usually 5-7 days, were recultured in another 5 ml of BSK medium with kanamycin, and the generation of intended mutant was confirmed by immunoblot analysis against BBA52 protein, and also by PCR and RT-PCR analysis of DNA and cDNA, respectively isolated from each mutant clones. Depending on the number of transformants that grow in selection medium, I usually assess 10-20 clones for further analysis. The endogenous set of plasmids contained in the parental B31A3 isolate was also assessed in the mutant isolates as described earlier (Zhang et al., 2009). One of the *bba52* mutant *B. burgdorferi* clones that retained the same set of plasmids as the wild type *B. burgdorferi* was selected for additional experiments. Finally, to rule out, if targeted genetic manipulation of *bba52* had exerted unwanted polar effect on the expression of immediately downstream gene, the expression of surrounding genes, such as *bba51* as well *bba53* was assessed by RT-PCR and qRT-PCR analysis.

### **3.3.3 Complementation of *bba52* gene in the mutant *B. burgdorferi***



I implemented the similar protocol to transform *bba52* mutant for complementation of *bba52* gene as described before in section 3.3.2. First, I constructed a shuttle plasmid pKFSS1 with *bba52* native promoter for complementation in *trans*. Repeated attempts to transform spirochetes with this plasmid failed to generate a complemented isolate. So, I next engineered a cis-construct in pXLF14301 with native *bba52* promoter which complements the gene at chromosomal location between *bb0445* and *bb446* gene as previously described (Zhang et al., 2009). The native promoter of *bba52* is undefined and there is a 40 bp intergenic region between the upstream gene *bba51* and *bba52*. When this 40 bp DNA element was used as a native promoter to drive expression of *bba52* in the transformed spirochetes, I failed to obtain any complemented isolates. Similarly, I further constructed both *cis* and *trans* constructs using pXLF14301 and pKFSS1 plasmids, respectively where *bba52* ORF is driven by the *flaB* promoter, however transformation with these new series of constructs failed to achieve *bba52* complementation.

I therefore, decided to implement a new strategy to restore *bba52* expression in the deletion mutant by the replacement of wild type copy of *bba52* in the native locus. To accomplish this, I engineered a new construct using the *bba52* mutagenesis plasmid pXLF10601-*bba52*. A new primer set, P1 and P11, was designed to extend the P1P2 flanking region incorporating the ORF of *bba52*. Simultaneously, I amplified by PCR the *aadA* cassette with the *flgB* promoter using a set of primers P12 and P13 and pKFSS1 as a template. The P1P11 amplicon was ligated with the *flgB-aadA* cassette (P12P13 amplicon) using the plasmid (pECFPC-1) having compatible multiple cloning sites (MCS3) in the sequence of *SacI*, *EcoRI* and *BamHI*. The insert, P1P13 was digested out

from this plasmid (MCS3) and replaced with the old P1P2 flanking region of pXLF10601-*bba52* constructed for mutagenesis. This new plasmid is referred as the *bba52* complemented construct. *bba52* deletion mutant was transformed with 25 µg of *bba52* complemented construct, as described before in section 3.3.2. All other procedures for *bba52* complementation were followed in the similar manner as described for deletion of *bba52* gene. The transformants were selected using kanamycin (350 µg/ml) and streptomycin (100 µg/ml). PCR analysis confirmed that one of the *bba52*-complemented isolates retained all endogenous plasmids, except for the loss of the nonessential plasmid lp5.

### **3.3.4 Confocal microscopy**

Confocal immunofluorescence of tick salivary glands was performed using an LSM-510 laser-scanning microscope (Zeiss), as detailed elsewhere (Pal et al., 2004a; Pal et al., 2004b). Samples for each time point of the analysis were dissected from a minimum of 5 ticks, and whole organs were scanned at 0.6-mm intervals through the full tissue thickness. Spirochetes were detected using fluorescein isothiocyanate–labeled anti-*B. burgdorferi* goat immunoglobulin (Ig) G (KPL), whereas tick salivary glands were labeled with Texas-Red phalloidin (Invitrogen).

## **3.4 Results**

### **3.4.1 Gene expression of *bba52* during representative experimental enzootic cycle of *B. burgdorferi* in ticks and mice**

I assessed *bba52* expression in detail during representative phases of the *B. burgdorferi* infection cycle. Mice (10 animals/group) were infected with *B. burgdorferi* ( $10^5$  cells/mouse). Total RNA was isolated from skin, joint, heart, and bladder samples at 1, 2, and 3 weeks after infection and was pooled by tissue type. Ticks were parasitized on parallel groups of mice that had been infected for 2 weeks (25 nymphs or 30 larvae/mouse), and engorged ticks were isolated. Fed intermolt nymphs were analyzed at 25 days after feeding, whereas larvae were allowed to molt to nymphs. Newly molted infected nymphs were allowed to feed on naive mice (10 ticks/mice) and were collected after 12–48 h of feeding. Skin samples were also collected from mice after 5 days of tick engorgement. Total RNA was prepared and subjected to qRT-PCR analysis to measure *bba52* transcripts and was normalized against *flaB*. The results showed that *bba52* transcripts were undetectable in mice during persistent infection or early tickborne infection, but they were obvious at all tested stages of *B. burgdorferi* infection in ticks, with the highest levels noted during tick feeding (FIGURE 2).

### **3.4.2 *B. burgdorferi* mutant with deleted *bba52***

I next generated *bba52* deletion mutant as detailed in Materials and Methods. Transformation of wild type *B. burgdorferi* was performed using mutagenesis plasmid construct pXLF10601-*bba52* to replace entire BBA52 ORF with kanamycin cassette driven by *flaB* promoter using allelic exchange. I obtained about 5 transformant clones, in which *bba52* gene was successfully deleted. For confirmation of the desired integration of mutagenic construct, PCR was performed using a variety of primer combination. Primers P5 and P6 were designed downstream of P1 and upstream of P4.

Primer P5 and kanamycin reverse primer P10 were used to amplify DNA template of mutant and wild type *B. burgdorferi* (FIGURE 3B). Primer P5 was designed upstream of P1 primer sequence to differentiate the amplicons originated from mutagenic plasmid contamination or from the mutant genome. In the similar manner, primer P6 and kanamycin forward P9 were used to amplify the wild type and mutant genome. The ORF of kanamycin can be amplified only in the mutant and the ORF of BBA52 can only be PCR amplified from the wild type genomic DNA. Next plasmid profile of one of the mutants, which was similar to wild type *B. burgdorferi*, was used for the rest of the study. To accomplish this, the 21 endogenous plasmids contained by the parental isolate were checked by PCR amplification using plasmid specific primers as described before (Zhang et al., 2009). Mutants maintaining all the plasmids except lp5, which is known to be not essential for infectivity of *B. burgdorferi* (Li et al., 2006) was selected for further study. RT-PCR was also performed with *bba52* primer and its adjacent gene *bba51* and *bba53* to verify for potential polar effects of gene manipulation (FIGURE 3D). To compare the protein profile of *bba52* mutant and wild type, lysates were resolved parallel to each other on a SDS-PAGE gel and then stained with coomassie brilliant blue. After staining, the gel was destained for an hour using destaining solution. The protein profiles of the wild type and the mutant were similar (FIGURE 3D). Immunoblot was next performed using the full length BBA52 antibody and FlaB as a positive control after resolving the wild type and mutant lysates on a SDS-PAGE followed by its transfer onto a nitrocellulose membrane. A clear band at 33 kDa was visible in the wild type and absent in the mutant, whereas FlaB bands showed equal load of wild type and mutant *B. burgdorferi* lysates (FIGURE 3D).

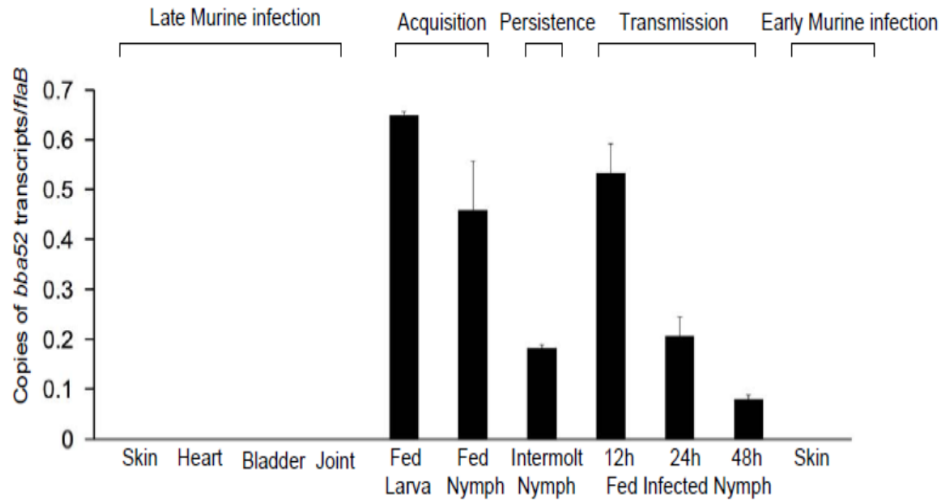


Figure 2. *bba52* expression is vector-specific.

*bba52* expression was analyzed at various stages of murine and tick infectivity. RNA was isolated from mice (10 animals/group) at 1, 2 and 3 weeks after *B. burgdorferi* infection and pooled by tissue types (Skin, heart, joint and bladder). Naïve larvae and nymphs were allowed to feed on *B. burgdorferi*-infected mice (25 ticks/mice) and collected at 96 h (fed larva and fed nymph) or 25 days following feeding (intermolt nymph). *B. burgdorferi*-infected nymphs (10 nymphs/mouse) were allowed to feed on naïve mice and collected at 12 h, 24 h, and 48 h of feeding. Murine skin samples were collected following 5 days of tick engorgement. RNA samples from murine and tick samples were analyzed by qRT-PCR and presented as copies of *bba52* transcript per copy of *flaB* transcript. Error bars represents the mean±SEM from four qRT-PCR analysis of two independent murine –tick infection experiments.

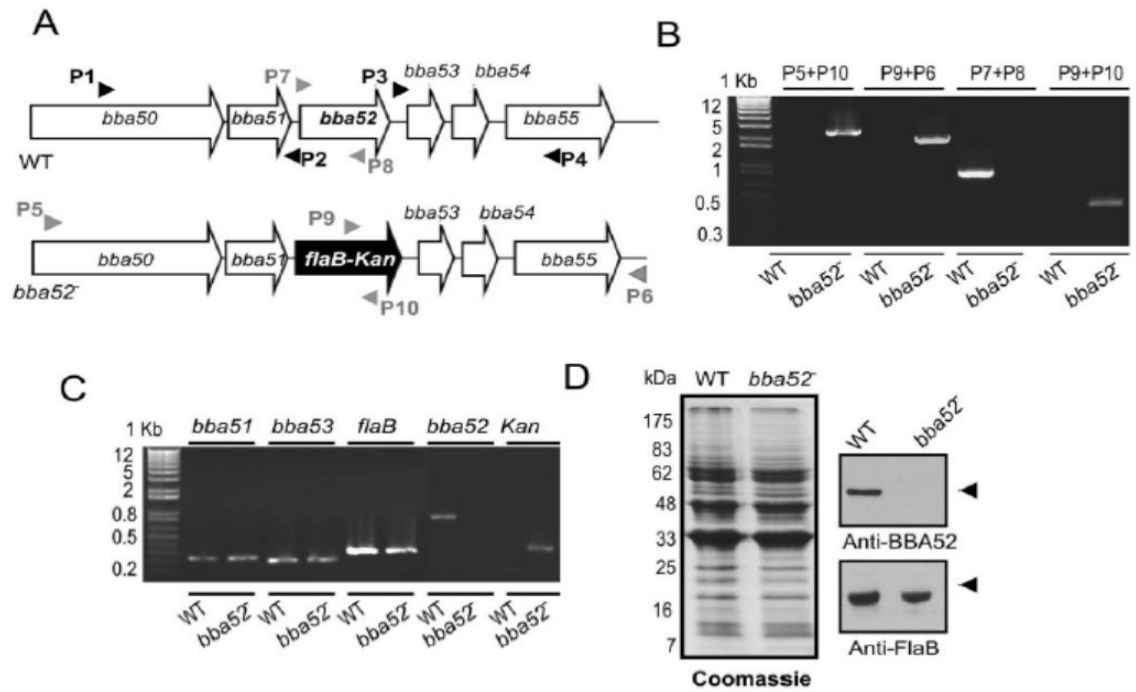


Figure 3. Construction and analysis of *bba52* mutant *B. burgdorferi*.

(A). Schematic representation of wild type (WT) and *bba52* mutant (*bba52*<sup>-</sup>) *B. burgdorferi* at the *bba52* locus. Genes *bba50*-*bba55* (white box arrows) and the kanamycin-resistance cassette driven by the *B. burgdorferi* *flaB* promoter (*flaB*-*Kan*, black box arrow) are indicated. The regions up- and down-stream of the *bba52* locus were amplified using primers P1-P4 (black arrow-heads) and ligated on either side of the *flaB*-*Kan* cassette to obtain the mutagenic construct, as detailed in the text. (B) Integration of the mutagenic construct, *flaB*-*Kan*, in the intended genomic locus. Primers 5-10 (gray arrowheads, positions indicated in figure 3A) were used for PCR analysis using isolated DNA from wild type (WT) or mutant *B. burgdorferi* (*bba52*<sup>-</sup>) and subjected to gel electrophoresis. The combination of primers used for PCR is indicated at the top, and migration of the DNA ladder is shown on the left. (C) Reverse-transcription PCR assessment of *bba52* transcripts and the polar effects of mutagenesis. (D) Protein analysis of wild-type *B. burgdorferi* (WT) and *bba52* mutant (*bba52*<sup>-</sup>). Equal amounts of protein were separated on an SDS-PAGE gel, and either stained with Coomassie blue (left panel) or transferred onto a nitrocellulose membrane and probed with BBA52 and FlaB antibodies (right panels). Migration of protein standards is shown to the left in kDa.

### 3.4.3 Growth of wild type and mutant *in vitro*

I assessed for the deletion effect of *bba52* by comparing the growth of mutant and wild type spirochetes in BSK medium at 34°C. Spirochetes were cultured starting from a density of  $10^5$  to  $10^8$  cells per ml. Cells were counted by using Petroff-Hausser cell counting chamber under dark field microscopy in triplicates on every 24 h for 140 h (FIGURE 4). There was no significant difference in the growth of the mutant lacking *bba52* gene compared to that of wild type *B. burgdorferi*.

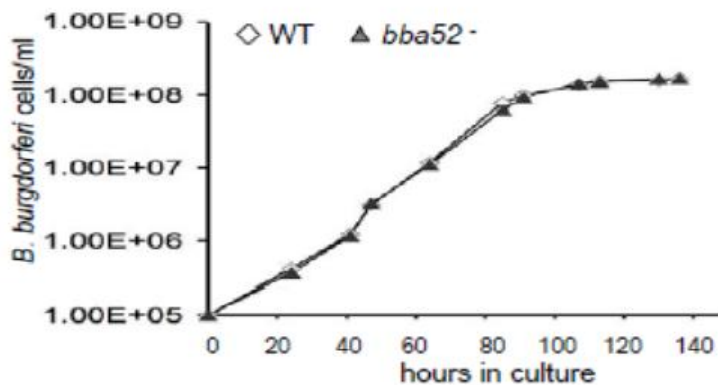


Figure 4. Comparative *in vitro* growth curves of WT and *bba52* mutant.

Spirochetes were diluted to a density of  $10^5$  cells/mL and grown at 34°C in BSK-H medium. Triplicate samples were counted under a dark field microscope by use of a Petroff-Hausser cell counter. Differences between the numbers of WT and *bba52* mutants were insignificant at all times of growth ( $P > .05$ ).

### 3.4.4 Assessment of *bba52* deletion mutant for its stability to persist in an experimental enzootic life cycle of *B. burgdorferi*

I first compared the ability of *bba52* mutants to persist in mice. Groups of mice (3mice/group) were injected with the *bba52* mutant or wild type spirochetes ( $10^5$

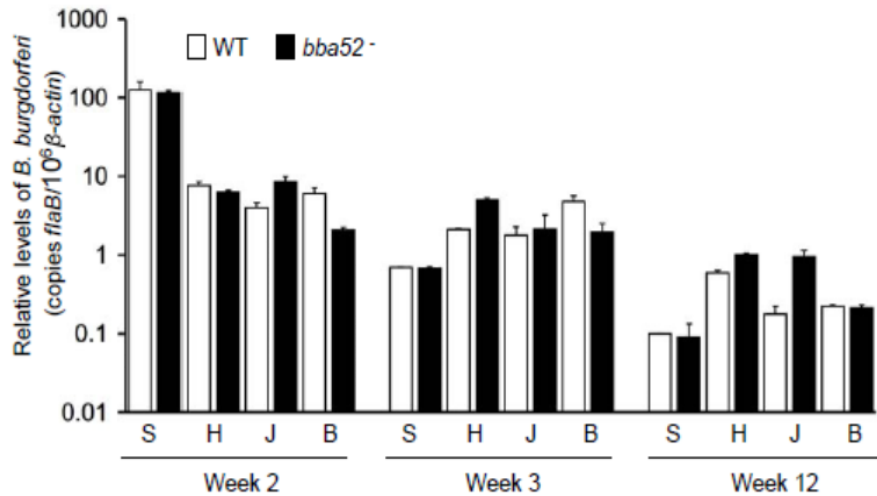


Figure 5. Full infectivity of *bba52* mutant *B. burgdorferi* in mice.

The pathogen burdens in multiple tissues of infected mice are shown. Mice (15 animals/group) were infected with wild-type (WT) or *bba52* mutant (*bba52*<sup>-</sup>) isolates, and spirochete burdens were analyzed in skin (S), heart (H), joint (J), and bladder (B) samples, by measuring copies of *B. burgdorferi flaB* RNA at 2, 3, and 12 weeks of infection. Amounts of murine  $\beta$ -actin in each sample were determined and used to normalize the quantities of spirochete RNA. Bars denote the mean value  $\pm$  standard error (SE) of quantitative reverse-transcription polymerase chain reaction analyses from 2 independent infection experiments. The difference between WT and *bba52* mutant levels was statistically insignificant at all time points and in all tissues ( $P > 1 .05$ ).

spirochetes/animal). Animals were sacrificed after 1,2,3,4 and 12 week of infection. Tissues such as skin, heart, joint and bladder were collected and pooled together for extraction of RNA using Trizol as detailed in section 2.2.3. *B. burgdorferi* burden in tissues were determined by qRT-PCR by using *flaB* primers and mouse  $\beta$ -actin primers for normalization of spirochete load (FIGURE 5). The results expressed as copies of *flaB* per  $10^6$  copy of the mouse  $\beta$ -actin. I did not observe any significant difference in the *B. burgdorferi* load in any tissues compared to the wild type isolate. I also assessed for the difference in the disease



pathogenesis, between mutants versus wild type infection. To assess this, swelling in the ankle joints between weeks 0 - 4 of infection was measured using a digital caliper and isolated joints were analyzed for histopathological signs of Lyme arthritis using published procedures (Zhang et al., 2009). There was no difference in the intensity of joint inflammation developed between wild type and mutant infection (FIGURE 6). This data was in agreement with the histopathology of the mice ankle joint as well. The outcome of the result showing a lack of phenotypic defects of *bba52* mutation generally supports the *bba52* expression pattern, where I could not detect the *bba52* transcripts in mice. Instead this protein is exclusively expressed in the vector and thus likely required spirochete enzootic cycle where it is expressed, such as in the vector. Thus, I was interested to assess the phenotype of the mutant in ticks during spirochete acquisition in ticks, their persistence throughout the intermolt stages and transmission from infected ticks to naïve hosts.

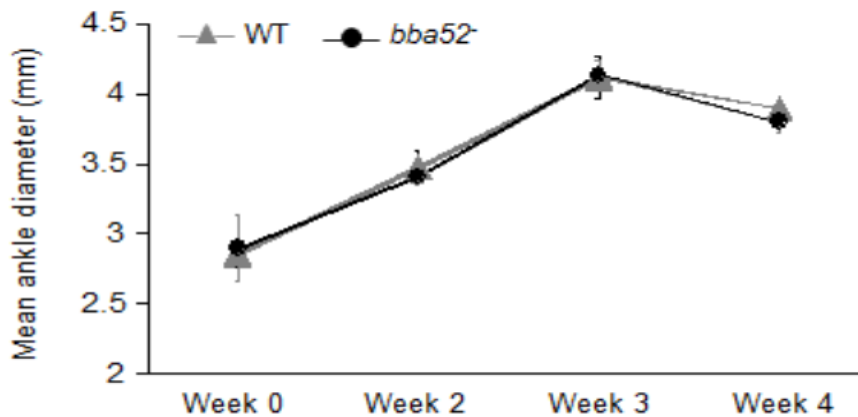


Figure 6. Assessment of joint swelling in *B. burgdorferi*-infected mice.

Groups of mice (3 animals/group) were infected with WT or *bba52*<sup>-</sup> and were examined for joint swelling, by use of a digital caliper, at 0, 2, 3, and 4 weeks after spirochete challenge. Data denote the mean  $\pm$  SE from 2 independent infection experiments. No difference in the ability of the WT and *bba52*<sup>-</sup> to induce joint swelling was recorded ( $P > 1 .05$ ).

### 3.4.5 Complementation of *bba52* gene in mutant

As complementation is necessary to follow molecular Koch's postulate showing any observed phenotypic defects of *bba52* gene deletion is due to the loss of target gene, I intended to complement the gene. My repeated attempt to transform the mutant to replace the *bba52* gene back to the mutant was unsuccessful. Therefore, I developed a novel strategy to replace, the *bba52* gene at the original locus. Our conventional approaches to construct mutagenic constructs to complement the *B. burgdorferi* as previously described (Promnares et al., 2009; Yang et al., 2009; Zhang et al., 2009) failed to recover any transformant. Although, the complementation construct where *bba52* expression was driven by the *flaB* promoter was able to produce recombinant BBA52 in *E.coli*, but failed to isolate a BBA52-producing complemented clone in *B. burgdorferi*. The final approach to complement *bba52* gene at the same locus was achieved using the same mutagenesis plasmid pXLF-10601, as described in detail in section 3.4.2 (FIGURE 7). The isolated complemented strains, which were able to grow in streptomycin and kanamycin retained all the parental plasmids and expressed *bba52* mRNA and protein. RT-PCR analysis using the RNA isolated from wild type, mutant and complemented isolates confirms comparable transcripts synthesis (FIGURE 8B). As expected, the genetic manipulation process did not introduce polar effects in complemented isolates, as assessed by the transcription of the surrounding genes *bba51* and *bba53* (FIGURE 8B). FIGURE 8C shows the comparative protein analysis of wild type, mutant and complemented isolates, as detected by staining with Coomassie brilliant blue. Immunoblot was also performed using anti-BBA52 antibody that indicated re-production

of BBA52 in the complemented isolate. As a positive control, FlaB antibody was used to show equal load of of wild type and mutant protein for Western blot analysis (FIGURE 8C).

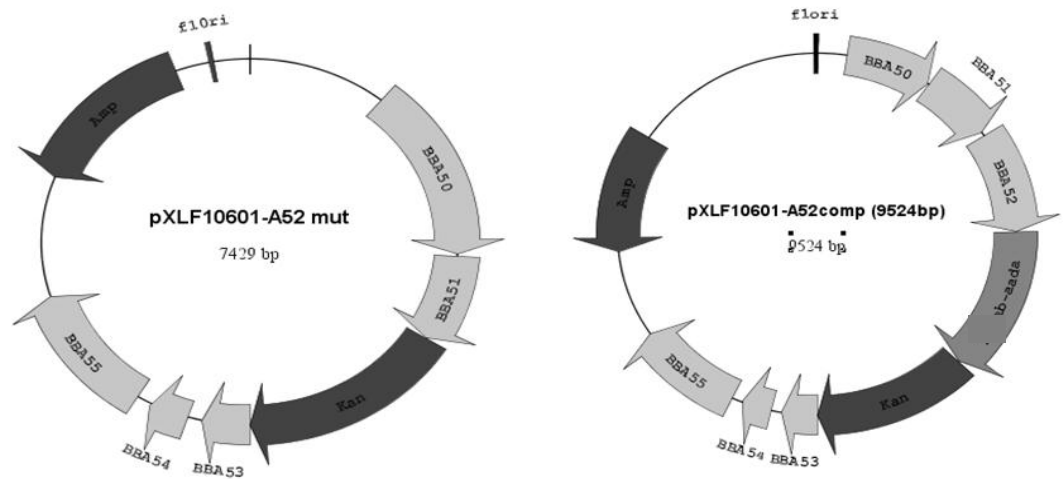


Figure 7. Schematic diagram of construct pXLF10601-A52 mutant and construct pXLF 10601-A52 complement.

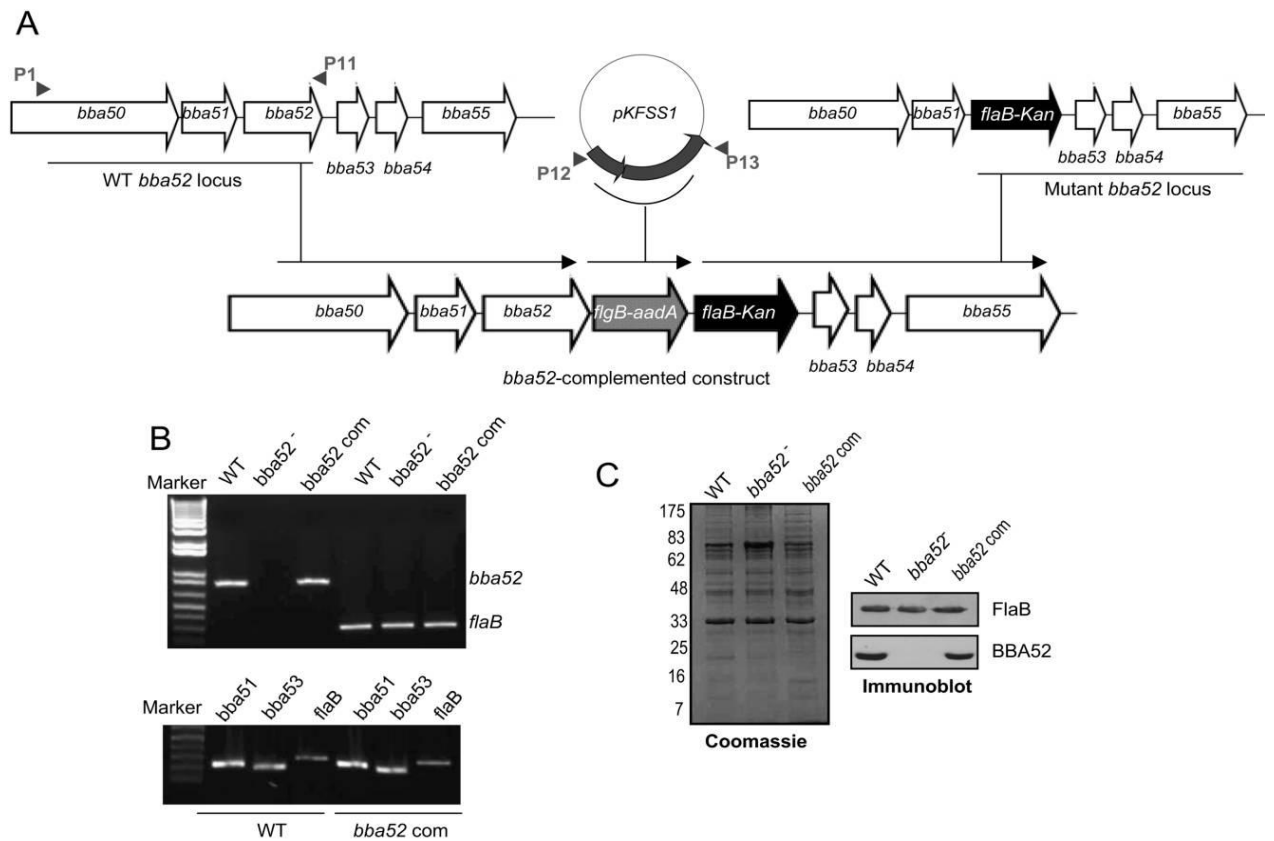


Figure 8. Genetic complementation of *bba52* mutant *Borrelia burgdorferi*.

(A) Construction of the *bba52*-complemented construct for reinsertion of *bba52* in cis, in the original gene locus of the lp54 plasmid. A new 5' arm was generated using primers P1–P11 and P12–P13, which were used to amplify and assemble 2 DNA inserts surrounding *bba52* and *aadA* cassette with the *flgB* promoter, respectively. The insert representing P1–P13 amplicon (new 5' arm) was fused with the *flaB-Kan* cassette carrying the old 3' arm (as generated using P3–P4) (Figure 3A) to obtain *bba52* complemented construct, and it was integrated in the *B. burgdorferi* lp54 locus via homologous recombination. (B) Reverse-transcription polymerase chain reaction (PCR) analysis of the *bba52* complemented isolate. Total RNA was isolated from either the wild-type (WT), *bba52* mutant (*bba52*<sup>-</sup>) or *bba52*-complemented *B. burgdorferi* (*bba52* Com); converted to complementary DNA; subjected to PCR analysis with *flaB* and *bba52* primers; and analyzed on a 1.5% agarose gel (upper panel). *bba52* complemented isolates did not display polar effects on the transcription of genes surrounding *bba52* locus (*bba51* and *bba53*) (lower panel). (C) Production of BBA52 protein in the complemented *B. burgdorferi*. Spirochete lysates were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, stained with Coomassie blue (left panel), or transferred to nitrocellulose membrane and probed with BBA52 and FlaB antibodies (right panels).

### **3.4.6 Phenotype of *bba52* mutant *B. burgdorferi* in ticks during spirochete acquisition, persistence and transmission.**

Although both wild-type spirochetes and *bba52* mutants persisted at similar levels in the murine dermis throughout the infection (FIGURE 5), the mutant was significantly impaired in its ability to transmit to naive ticks. To ensure the specificity of the result, I sought to complement the mutants with a wild-type copy of the *bba52* gene in *cis* and use this isolate in mouse-tick transmission studies. I then assessed whether BBA52 is required for *B. burgdorferi* entry and persistence in and transmission through ticks. To examine the effect of the *bba52* deletion on spirochete acquisition by ticks, larval and naive nymphal ticks were allowed to parasitize mice that had been infected with wild-type, *bba52* mutant, or *bba52*-complemented isolates. The generation of larvae to Nymphs has been schematically represented in FIGURE 10. Partially fed nymphs were forcibly removed at 24 and 48 h after the onset of feeding, and parallel groups of larvae or nymphs were collected as fully engorged ticks. The spirochete burden was assessed by qRT-PCR analysis of *flaB* normalized against tick  $\beta$ -*actin* levels. Compared with wild-type or *bba52*-complemented isolates, the levels of *bba52* mutants were significantly lower in feeding ticks analyzed at 24 h ( $P < .002$ ) and 48 h ( $P < .02$ ) of host attachment (FIGURE 9A). However, analysis of fully engorged larvae or nymphs at 7 and 25 days after feeding (FIGURE 9B) showed similar burdens of wild-type and mutant spirochetes, suggesting that *bba52* deletion only transiently affected *B. burgdorferi* acquisition by ticks, without having significant influence on microbial persistence in the ticks. I then compared the ability of the *bba52* mutants to transmit back from infected ticks to naive

mice. Separate groups of nymphs that were naturally infected with wild-type or mutant isolates were allowed to feed on naive C3H mice (9 animals/group) and were collected as partially fed (for 48 and 60 h) or fully engorged ticks.

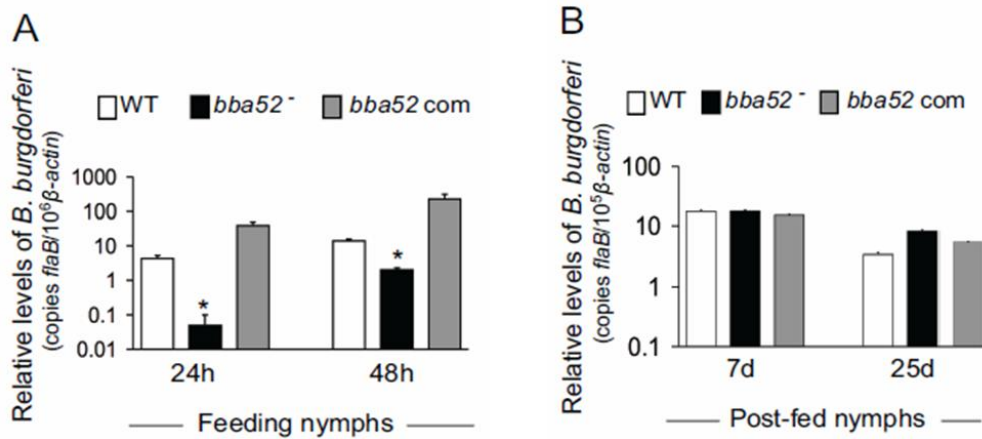


Figure 9. Impaired ability of *bba52* mutant *B. burgdorferi* to be transit between murine hosts and ticks.

(A) *B. burgdorferi* burdens in ticks during acquisition from infected mice. Mice were infected with *B. burgdorferi* (3 mice/group), and after 2 weeks of infection, naive *Ixodes scapularis* larvae or nymphs (25 ticks/mouse) were allowed to feed on mice. *B. burgdorferi* burdens in ticks were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses at the indicated time intervals after feeding, by measuring the no. of copies of the *B. burgdorferi flaB* RNA and normalizing against tick  $\beta$ -actin RNA. Bars denote the mean  $\pm$  standard error (SE) of 2 independent infection experiments. Differences in the spirochete burdens in ticks infected with *bba52*<sup>-</sup> and those with the *bba52*<sup>-</sup> complemented (*bba52* com) isolates or wild-type (WT) spirochetes were significant both at 24 h (\* $P < .002$ ) and 48 h (\* $P < .02$ ). (B) *B. burgdorferi* burdens in postfed ticks. Nymphs were allowed to engorge on infected mice, as shown in panel A, and *B. burgdorferi* burdens in postfed ticks were analyzed by qRT-PCR analyses at the indicated time intervals, by measuring the number of copies of *B. burgdorferi flaB* RNA and normalizing against tick  $\beta$ -actin RNA. Bars denote the mean  $\pm$  SE of 2 independent infection experiments. Similar burdens of *bba52*<sup>-</sup>, WT, and *bba52* com isolates were evident at day 7 or day 25 ( $P > .05$ ).

Spirochete burdens in ticks were assessed by qRT-PCR and confocal

immunofluorescence analyses. After 7 days of feeding, mouse infection was assessed by culture analysis of the heart and spleen samples and by qRT-PCR analysis of skin, heart,

and bladder tissues. Results indicated that burdens of wild-type and *bba52* mutants were similar in fed tick gut; however, the *bba52* mutant was highly impaired in its ability to migrate to salivary glands (FIGURE 12) and transmit to mice (FIGURE 11). Both wild-type and *bba52* complemented isolates were recovered by culture analysis of murine spleen and heart samples. In contrast, the *bba52* mutant remained undetectable in all of the 6 individual mouse spleens analyzed, but it was recovered from 4 of the 6 heart samples. This observation indicates that a minor proportion of *bba52* mutants that remained untraceable in immunofluorescence and qRT-PCR analyses are still capable of transmission. Collectively, these data establish that BBA52 function is nonessential for the persistence of *B. burgdorferi* in murine hosts or ticks but that it facilitates *B. burgdorferi* transitions between hosts and vector.

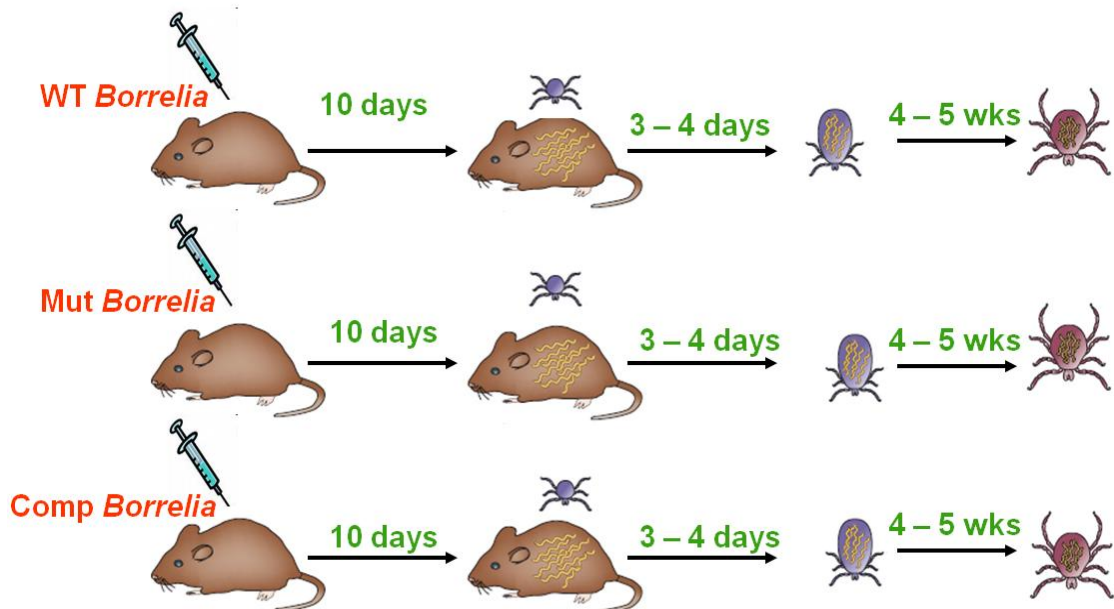


Figure 10. A schematic diagram showing major steps in generation of infected nymph after feeding naive larvae on infected mouse (modified from Rosa *et al.*, 2005).

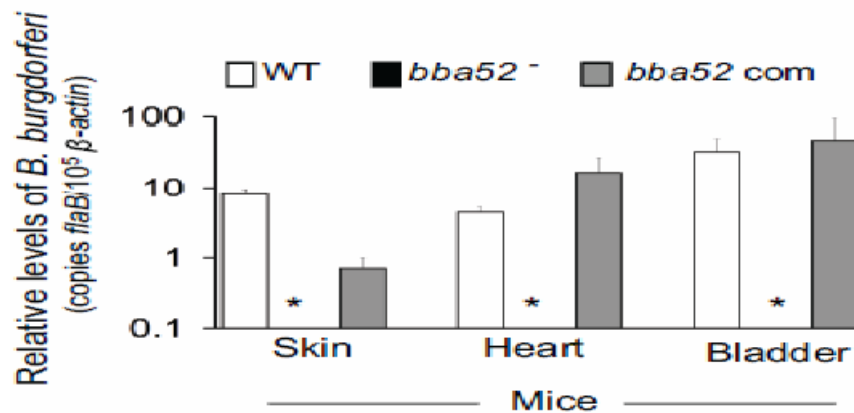


Figure 11. *B. burgdorferi* transmission from infected ticks to mice.

*B. burgdorferi*-infected nymphs were generated by feeding larvae on mice infected with WT and genetically manipulated spirochetes, as described in the text. Newly molted *B. burgdorferi*-infected nymphs were allowed to feed on naive mice (1 tick/mouse and 3 animals/group). *B. burgdorferi* burdens were assessed in the indicated murine tissues by qRT-PCR analyses performed after 1 week of tick feeding, by measuring the number of copies of *B. burgdorferi* *flaB* RNA, and they were normalized against mouse  $\beta$ -actin levels. Bars denote the mean  $\pm$ SE of 2 independent animal infection experiments. \**bba52*<sup>-</sup> was undetectable.

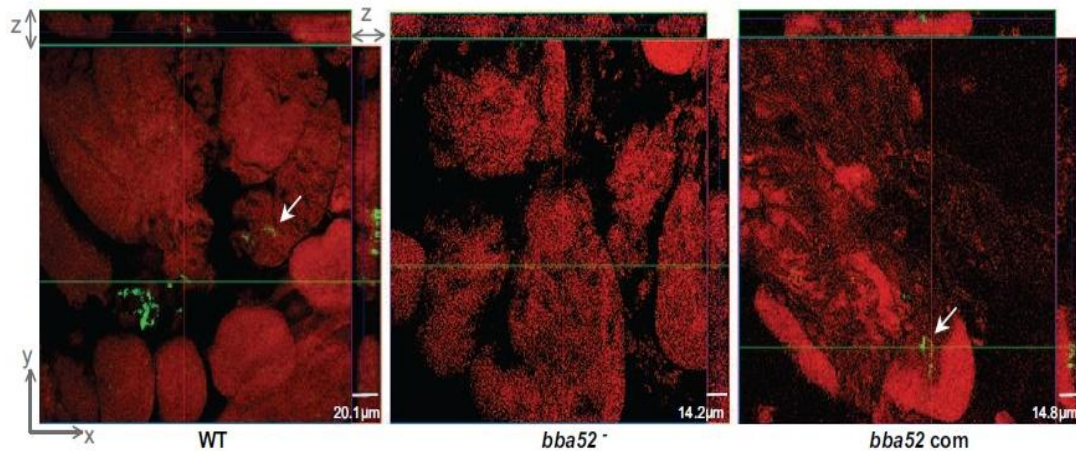


Figure 12. *B. burgdorferi* localization in infected salivary glands during transmission.

A confocal orthogonal image display of infected salivary glands in the xz-axis and yz-axis revealing the distribution of spirochetes through the full thickness of the 60 h fed salivary glands is shown. The spirochetes (arrow) were labeled with fluorescein isothiocyanate-labeled goat anti-*B. burgdorferi* antibody (green), and gland morphologic findings were revealed by labeling of acinar actin filaments with Texas Red-phalloidin (red). Although WT and *bba52* com were occasionally observed within the gland, *bba52*<sup>-</sup> was consistently undetected.



### 3.4.7 Phenotype of *bba52* mutant at late stage of mice infection when transmitted by ticks

Since my previous transmission studies suggest that low levels of *bba52* mutant were able to transmit and could be recovered by culture, I was interested in assessing *bba52* persistence for establishment of long-term infection such as 4 week after tick-borne infection. To accomplish this, I microinjected the wild type, mutant and complemented *B. burgdorferi* isolates in naïve ticks. After 5 days of microinjection, two ticks were fed on each mouse for assessment of *B. burgdorferi* transmission. At 4 week of tick transmission, each group of mouse was sacrificed and specific tissues were pooled as described in section 2.2.3. Spirochete burden was measured in the mouse as previously described. FIGURE 13 shows that the mutant *B. burgdorferi* can be detected in late stage of infection by qRT-PCR although there is significant difference in bacterial burden in skin and bladder of *bba52* mutant.

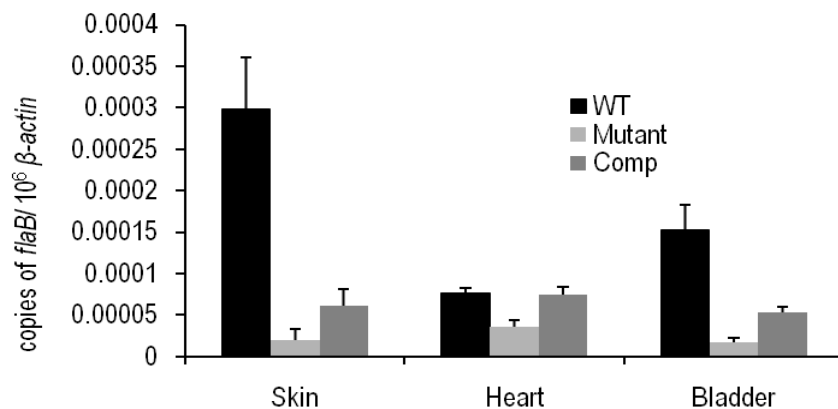


Figure 13. Spirochete load in mice at 4 week of tick transmission.

Ticks were microinjected with WT, mutant and complemented *B. burgdorferi*. After 5 days of microinjection, two ticks were fed on each mouse for assessment of *B. burgdorferi* transmission. At 4 week of tick transmission, each group of mouse was sacrificed and specific tissues were pooled for RNA extraction. Spirochete burden was measured in the mouse by qRT-PCR as copies of *flaB*/ mouse  $\beta$  *actin*. Bars denote the mean  $\pm$  SE of single infection experiment.

### 3.5 Discussion

*B. burgdorferi* has developed remarkable abilities to persist in a complex enzootic cycle involving wild rodents and arthropods (Lane et al., 1991). While a tick engorges on the host blood meal, the pathogen transits between the radically different tissue environments of mammals and arthropods. As membrane proteins, including microbial adhesins (Coburn et al., 2005), may greatly contribute to pathogen interaction with new environments, I assessed the expression of selected spirochete gene products with putative membrane-localization signals (Yang et al., 2009). I identified *bba52* as one of the genes that is selectively expressed in ticks and show that BBA52 facilitates microbial transitions between the vector and the host (Kumar et al., 2010). *bba52* gene deletion studies shows that there is no difference in growth and pathogenesis using mouse as a animal model whereas phenotype was obvious during the tick phase of *B. burgdorferi* enzootic cycle.

*B. burgdorferi* undergoes notable changes in antigenic composition as it invades and colonizes diverse tissues in arthropods or mammals (Fingerle et al., 2002; Liang et al., 2002c; Narasimhan et al., 2003; Narasimhan et al., 2002a; Schwan et al., 1995; Tilly et al., 2008; Yang et al., 2009). These changes are partly mediated by known regulatory network, involving Rrp2-RpoN/RpoS or Rrp1-Hpk1 TCS and c-di-GMP (Boardman et al., 2008; Caimano et al., 2007; Hubner et al., 2001; Rogers et al., 2009b; Xu et al., 2010; Yang et al., 2003). However, recently it has been proposed that activation of the Rrp2-RpoN-RpoS pathway occurs via the small, high-energy, phosphoryl-donor acetyl phosphate (acetyl~P), the intermediate of the Ack-Pta (acetate kinase-phosphate acetyltransferase) pathway that converts acetate to acetyl-CoA rather than by histidine

kinases (Xu et al., 2010). Microarray analyses of transcriptional alterations in cultured spirochetes identified a large number of genes that are differentially expressed, including *bba52*, which responded to physiochemical alterations including variations in temperature, the addition of blood, or growth in a dialysis membrane chamber (DMC) implanted within the murine host (Brooks et al., 2003; Caimano et al., 2007; Ojaimi et al., 2002; Tokarz et al., 2004).

BBA52 is encoded by the linear plasmid (lp) lp54, which, unlike many other *B. burgdorferi* linear or circular plasmid (cp)s, is a stable extra-chromosomal element and considered as an integral part of core spirochete genome (Terekhova et al., 2006). lp54 retains a large number of paralogous genes and shares multiple blocks of sequence homologies with cp32-based plasmids (Casjens et al., 2000b). For example, the DNA block harboring genes *bba38-bba55* in lp54 is significantly homologous to cp32-1 DNA housing *bbp1-bbp18* with unidirectional ORFs (Casjens et al., 2000b; Fraser et al., 1997a). However, lp54 contains a few unique intervening insertions, including the *bba52* locus, within these homologous (cp32-like) DNA blocks. Incidentally, other unique loci in lp54 encode for OspA/B and DbpA/B, which are differentially expressed *in vivo* and contribute to microbial persistence in arthropods (Yang et al., 2004) and mammalian hosts (Blevins et al., 2008; Shi et al., 2008), respectively. Therefore, I speculate that the occurrence of *bba52* as a unique locus in one of the few stable plasmids of *B. burgdorferi* (Stewart et al., 2005), possibly signify an important role for BBA52 in maintaining the microbial life cycle. This is supported by my findings that *bba52* is expressed *in vivo*, confined to the vector-specific microbial life cycle with highest levels of expression during tick feeding. Consistent with undetectable *bba52* expression in murine hosts,

BBA52-deficient spirochetes lack an obvious phenotypic defect in mice. In contrast, *bba52* ablation affected *B. burgdorferi* persistence in feeding ticks during microbial entry, and to greater extents, severely impeded pathogen migration to tick salivary glands and transmission to mice. Therefore, BBA52 function is highly relevant during microbial transitions between the vector and hosts, which is essential for successful maintenance of the pathogen through the complex enzootic cycle.

Identification of *B. burgdorferi* gene products important for infectivity is made largely possible by seminal discovery of borrelial genetic transformation process (Samuels, 1995) and further progress in borrelial mutagenesis (Rosa et al., 2005). However, the unusual organization of spirochete genome and lack of promoter information poses serious challenges to the genetic complementation efforts, as I encountered for *bba52*. The ORF of 14 consecutive genes upstream of *bba52* are unidirectional, encode mostly hypothetical proteins, lack a discernible promoter and possess overlapping or short intergenic regions. A majority of them display similar environmental regulatory responses, such as enhanced transcript levels in spirochetes grown at 23°C relative to 37°C (Ojaimi et al., 2002), thus indicating a potentially linked expression profile. *bba52* shares a short (40 base pairs) intergenic region with *bba51*; however, use of this intergenic sequence as a native promoter to drive *bba52* expression in *B. burgdorferi* was unsuccessful. *E. coli* was able to produce BBA52 when transformed with *bba52* ORF driven under heterologous and constitutively-active borrelial promoter, *flaB*. Noticeably, the *flaB* promoter failed to restore *bba52* expression in *B. burgdorferi*, possibly indicating that constitutive expression of *bba52* is detrimental to spirochetes. This problem is not limited to genetic manipulation of *bba52*. Use of constitutively-active heterologous

promoters or native promoters when placed in non-native cis locations or in shuttle vectors could potentially result in aberrant gene expression that can complicate phenotypic analysis. Thus, genetic complementation of differentially regulated borrelial genes remains technically challenging, however, in the current case; I was able to complement *bba52* by replacing the gene in its native cis location without apparent polar effects and fully restored wild type phenotypes. This strategy could be helpful for complementation efforts of other regulated borrelial genes, particularly those lacking discernible promoters yet are retained in an optimal borrelial locus amenable for genetic manipulation.

Although I have determined that BBA52 participates in pathogen transitions between the host and the vector, with the most prominent role during pathogen exit from ticks to mice, the function of this gene product in spirochete biology remains unknown. However, correlated with tick-specific expression of *bba52* and significant extracellular exposure of the antigen, BBA52 might be involved in pathogen interaction with vector environments. Based on the temporal upregulation of the *bba52* in feeding ticks and phenotypic analysis of *bba52* mutants *in vivo*, I propose that BBA52 facilitates spirochete transitions between murine hosts and ticks by assisting pathogen persistence in rapidly changing environments of the feeding tick gut (Sonenshine, 1993) or its migration between the host dermis and vector gut. BBA52 is likely conserved in major *B. burgdorferi* isolates as generated BBA52 antisera readily recognized the native protein in N40 and 297 isolates, and retained 65-68% amino acid identity across orthologs in *B. afzelii* and *B. garinii*. Characterization of surface-exposed microbial antigens expressed in feeding arthropods provides an opportunity to understand the pathogen interactions

with environments likely required for microbial transition between hosts and vectors, and may contribute to the development of novel transmission-blocking vaccine against vector-borne diseases.

## **Chapter 4: Characterization of BBA52 - localization, function and its potential as a transmission blocking vaccine**

### **Abstract**

BBA52, annotated as a conserved hypothetical protein of *B. burgdorferi*, is an outer membrane protein expressed exclusively in tick phase of Lyme disease enzootic cycle. My proteinase K and immunofluorescence assay (IFA) shows BBA52 as a surface-exposed protein. Phase separation of *B. burgdorferi* proteins using Triton X-114 suggests that BBA52 is amphiphilic in nature as it could be detected in both aqueous and detergent phases. Presence of BBA52 protein in detergent phase is consistent with IFA result showing BBA52 integration in the outer membrane (OM) of *B. burgdorferi*. Far western analysis using recombinant BBA52 and *B. burgdorferi* proteome, showed the existence of two BBA52 interacting partners; one is located in outer membrane and the other in protoplasmic cylinder fraction of *B. burgdorferi*. My data suggested BBA52 also interacts with a tick gut protein at ~ 35 kDa. BBA52 was assessed as a potential candidate for transmission-blocking Lyme disease vaccine. Immunization of mice with recombinant BBA52 protein conferred partial protection from Lyme disease during tick transmission indicating that BBA52 can be potentially used as a tick transmission-blocking vaccine. Characterization of surface-exposed microbial antigens expressed in feeding arthropods provides an opportunity to understand the pathogen interactions with environmental factors that are likely required for successful microbial transition and persistence in arthropod and mammalian hosts.

## 4.1 Introduction

Lyme borreliosis, caused by *B. burgdorferi*, is a vector-borne zoonosis prevalent in many parts of the globe (Piesman and Eisen, 2008a, b). While feeding on an infected reservoir host, usually wild rodents, immature *Ixodes* ticks acquire the pathogen, transstadially maintain the infection and, during a subsequent blood meal, transmit the pathogen to mammals. Selected mammalian hosts including humans manifest a wide array of clinical symptoms of Lyme borreliosis, frequently including the characteristic skin rash erythema migrans, arthritis, carditis and neurological symptoms (Steere et al., 2004). Inbred rodent hosts such as C3H mice, once infected with *B. burgdorferi*, develop some of the complications of Lyme borreliosis encountered by humans and are considered excellent animal models of Lyme borreliosis (Barthold, 1992). Since rodents constitute the natural reservoir hosts of *B. burgdorferi*, murine models are also used to unravel the molecular details of spirochete infection and tick transmission cycle.

*B. burgdorferi* houses an unique genome (Casjens et al., 2000b; Fraser et al., 1997a) that enables the microbe to persist and cycle between dramatically different hosts. Identifying spirochete antigens expressed *in vivo* and understanding their functions in pathogen biology and infectivity are necessary to develop preventive measures against Lyme borreliosis. However, such efforts face serious challenges due to the evolutionary distinctness of spirochetes from other bacterial species. Although *B. burgdorferi* encode many gene products that have significant homology to other bacterial proteins, the major fraction of the spirochete genome, including 90% of the plasmid-borne genes, encodes for proteins with little resemblance to known virulence factors or proteins. Thus, mechanisms of pathogen persistence and host interaction are likely different from more



well-studied bacterial pathogens (Casjens et al., 2000b). The majority of spirochetal proteins are annotated as ‘hypothetical proteins’; however, as many of them are indeed transcribed, at least *in vitro* (Brooks et al., 2003; Ojaimi et al., 2002; Revel et al., 2002b), they likely represent proteins with unidentified functions related to microbial persistence in a complex enzootic cycle. Microarray and RT-PCR studies have revealed differential expression of a large number of borrelial genes with unique genetic regulatory networks, possibly relevant to microbial survival in a wide variety of tissue environments that spirochetes encounter *in vivo* (Brooks et al., 2003; Caimano et al., 2007; Fisher et al., 2005; Liang et al., 2002a; Narasimhan et al., 2003; Ojaimi et al., 2002; Revel et al., 2002b; Rogers et al., 2009b; Tokarz et al., 2004). Despite identification of a handful of antigens critical for maintenance of a spirochete infectious cycle (Rosa et al., 2005), the function of most borrelial antigens remains elusive.

*B. burgdorferi* persists *in vivo* for months to years, yet efficiently transits between new hosts or vectors during relatively short episodes of tick feeding (de Silva et al., 2009). As ticks attach to the host and take a blood meal, *B. burgdorferi* migrate between evolutionarily distant species. Notably, only a small fraction of pathogens from an infected host or vector actually transmit, possibly ones that are accessible to the host-vector interface. For example, *B. burgdorferi* colonizes multiple internal organs in a host, however, during acquisition by ticks, spirochetes proximal to the dermal feeding lesion are the most likely to migrate to the vector. Similarly, when infected ticks engorge on a mammal, spirochetes multiply exponentially in the gut (de Silva and Fikrig, 1995); however, only a minor fraction of *B. burgdorferi* migrate to the host dermis, possibly only those with the ability to exit the feeding gut and invade the salivary glands (Piesman

et al., 2001). As antigenically and genetically-diverse populations of *B. burgdorferi* exist *in vivo* (Ohnishi et al., 2001), spirochetes that are maintained in an enzootic infection cycle might display favorable genetic regulation, for example ones regulated by sigma 54 (Fisher et al., 2005), that facilitate vector-host transmission. The mechanisms of spirochete transfer between host and vector, by active locomotion (chemotaxis) or by passive transfer (diffusion) through host body fluids or tick saliva, are unknown. Although the *B. burgdorferi* genome (Casjens et al., 2000b; Fraser et al., 1997a) harbor bacterial orthologs associated with spirochete locomotion (Li et al., 2000) and chemotaxis (Motaleb et al., 2007; Motaleb et al., 2005), and certain organs, such as tick salivary gland lysates contains chemoattractant for spirochete migration (Shih et al., 2002), antigenic determinants and detailed mechanisms associated with the complex transmission process of spirochetes remains a subject of further investigation. Previous genetic study against novel protein BBA52, which is exclusively expressed by *B. burgdorferi* in vector, showed that *bba52* facilitates transmission of *B. burgdorferi* from ticks and mammals. The identification of spirochete antigen that assist pathogen transmission between ticks and mammals, and understanding their biological function are important for the development of novel transmission-blocking approaches to intervene in Lyme borreliosis.

## **4.2 Materials and Methods**

### **4.2.1 Analysis of surface exposure of BBA52 and characterization of BBA52 antibodies**

BBA52 antibodies bind to the surface of intact unfixed *B. burgdorferi*.

Spirochetes were immobilized on glass slides and probed with BBA52 or control (GST) antibodies. Antibody against known surface outer surface protein A (OspA) and subsurface Ip6.6 (BBA62) spirochete proteins were used as controls. Spirochete loading and antibody labeling was assessed using propidium iodide (PI) and Alexa-488 tagged secondary antibodies, respectively. Images were acquired using a 40×objective lens of a Zeiss confocal microscope. A borreliacidal activity of BBA52 antibodies *in vitro* was analyzed. Spirochetes were incubated with either normal rabbit sera (NRS), rabbit OspA antibodies (OspA), serum collected from 15-day infected mice or BBA52 antibodies. The sensitivity of spirochetes to the bactericidal effect of the antibodies was assessed by a re-growth assay after 48 h of antibody incubation and presented as mean ± SEM of viable spirochete number (cells/ml). The numbers of viable spirochetes were significantly reduced in the samples exposed to the OspA or *B. burgdorferi* antibodies, compared to spirochetes that received no treatment or were incubated with BBA52 or normal serum ( $P < 0.002$ ).

#### **4.2.2 Proteinase K accessibility assay**

Proteinase K accessibility assays was performed as described before (Coleman et al., 2008). Briefly, *B. burgdorferi* ( $2 \times 10^8$ ) were gently washed three times in 1 ml of PBS (pH 7.4) and collected by centrifugation at  $4,000 \times g$  for 4 min. Washed spirochetes were then gently resuspended in 1 ml of PBS and split into two equal 500  $\mu$ l volumes. One aliquot received 200 mg of Proteinase K (PK) (Sigma), while the other aliquot received an equal volume of PBS without PK. Both aliquots were incubated for 1 h at room temperature before the addition of 10  $\mu$ l of phenylmethylsulfonylfluoride (PMSF)

(Sigma) to stop PK activity. Spirochete suspensions were subsequently pelleted by centrifugation at 10,000×g for 10 min and resuspended in PBS for immunoblot analysis using antibodies against FlaB (1:200), OspA (1:200) and BBA52 (1:15000).

#### **4.2.3 Purification of outer membrane vesicles and immunoblotting**

Isolation of outer membrane vesicles (OMV) of *B. burgdorferi* was performed as described (Skare et al., 1995). Briefly,  $1 \times 10^{11}$  *B. burgdorferi* cells were washed in phosphate-buffered saline pH 7.4 (PBS) supplemented with 0.1% BSA. The cells were resuspended in ice-cold 25 mM citrate buffer pH 3.2 containing 0.1% BSA and incubated on a rocker at room temperature for 2 h. OMV were released from whole cells and were isolated from protoplasmic cylinder (PC) using sucrose density gradient centrifugation. For localization of BBA52, immunoblotting was performed using equal amounts (0.3µg/lane) of OMV and PC, and probed with either BBA52 (1:5000), FlaB (1:200), or OspA (1:200) antibodies.

#### **4.2.4 Phase separation of integral membrane proteins in Triton X-114 solution**

The protein samples were prepared as described before (Bordier, 1981). Briefly,  $1 \times 10^9$  spirochetes grown in BSK medium were spun and washed 3 times with PBS. The pellet obtained was resuspended in 800 µl of PBS and sonicated four times with 20 sec burst each. Thereafter, 200 µl of 10% Triton X-114 was added to it and was rocked overnight at 4°C. The insoluble debris was removed by centrifugation at 13000×g for 15 min at 4°C and placed at 37°C water bath for 10 min. The suspension was centrifuged for 10 min at 13000× g at room temperature to separate detergent and aqueous phase.

The detergent phase and aqueous phase was washed as follows. The detergent phase (50µl) was mixed to 1 ml of original buffer at 0°C, rewarmed at 37°C and spun in a microcentrifuge as before. Later, the final aqueous phase and detergent phase obtained after four rounds of wash was precipitated with 10 volumes of chilled acetone. The aqueous and detergent phase was mixed with equal volume 2×Laemmli buffer (BioRad) and heat denatured at 95°C for 10 min. Immunoblot was performed with aqueous and detergent phase proteins using BBA52 and BBA74 antibodies. BBA74 antibodies were used as a control which is known to be present in aqueous phase (Mulay et al., 2007).

#### **4.2.5 Osmotic shock assay**

Osmotic shock assay was performed as detailed (Elias et al., 2000) with minor modifications. Briefly, stationary-phase *B. burgdorferi* cultures were incubated for 40 min at 34°C in the presence or absence of 1 M NaCl in BSK medium. Thereafter, aliquots were inoculated in fresh BSK medium and were allowed to re-grow for 48 h at 34°C. The number of viable spirochetes was counted using Petroff-Hausser cell counter under dark field microscopy.

#### **4.2.6 Oxidative stress assay**

For determination of spirochete susceptibility to oxidative stress (Boylan et al., 2008), *B. burgdorferi* ( $10^7$  cells/ml) grown in BSK medium was treated with hydrogen peroxide at concentrations of 1-10 mM. In a parallel control, the hydrogen peroxide was replaced with equal volumes of nuclease-free water. After incubation for 24 h at 34°C, number of motile spirochetes was counted with a Petroff-Hausser cell counter using dark field microscopy.

#### **4.2.7 Detergent treatment assay**

The outer membrane integrity of the spirochetes was assessed using a detergent treatment assay as described before (Slavik, 1982), with the following modifications. Spirochetes in BSK medium ( $10^7$  cells/ml) were incubated in the absence or presence of SDS (2 mg/ml). Following 4 h incubation at 34°C, cells were pelleted by centrifugation at 12000 rpm for 10 min. Five  $\mu$ l of the supernatant was diluted in 100  $\mu$ l of PBS. DNA was extracted from this mixture using phenol:chloroform extraction and analyzed by quantitative PCR (Coleman et al., 2008) to measure the amount of cellular DNA released in the supernatant.

#### **4.2.8 Confocal immunofluorescence microscopy**

Confocal immunofluorescence of tick gut and salivary glands were performed using LSM-510 laser scanning microscope (Zeiss) as detailed earlier (Pal et al., 2004a; Pal et al., 2004b). Samples for each time point of analysis were dissected from a minimum of 5 ticks and whole organs were scanned at 1  $\mu$ m (gut) and 0.6  $\mu$ m (salivary gland) intervals through the full tissue thickness. Spirochetes were detected using FITC-labeled anti-*B. burgdorferi* goat IgG (KPL), whereas tick gut and salivary glands were labeled with propidium iodide (Sigma) and Texas Red-phalloidin (Invitrogen), respectively.

#### **4.2.9 Immunization of mouse with recombinant BBA52 and tick transmission**

Recombinant BBA52 was produced in baculovirus expression system as described before in section 3.2.2.4. A group of 5 mice were immunized with rBBA52 or PBS in Freund's complete adjuvant (FCA). Antigen was prepared by syringe

emulsification of the adjuvant and the recombinant protein. Each mouse was injected intradermally with 10 µg of recombinant BBA52 purified protein. After 10 days of primary immunization, mice were given first booster doze of 10 µg of protein per mice in incomplete Freund's adjuvant (IFA). After about 7 days, serum was collected from the mice to check the antibody development by performing immunoblot against *B. burgdorferi* lysate. Mice were given the second booster after day 10 of the first booster. Later, after 5 days of second booster, wild type *B. burgdorferi* infected ticks (2 ticks/mouse) were infested on mice immunized with PBS and rBBA52 antigen. Repleted ticks fallen after complete feeding was collected to check by qRT-PCR, the infectivity of ticks with the spirochete. After 10 days of tick feeding, mice were sacrificed and specific tissues were pooled together on group basis to extract RNA as described in section 3.2.1. Bacterial load was assessed in each tissue by qRT-PCR. Three independent experiments were done and the average values of three qRT-PCR results were statistically analyzed.

#### **4.2.10 Passive transfer of BBA52 antibodies and *in vivo* transmission-blocking**

A microinjection procedure was used for the passive transfer of antibodies into the infected tick gut as previously described (Pal et al., 2004b). Naturally-infected nymphs were generated by allowing larvae to engorge on *B. burgdorferi*-infected mice and molt to nymphs. Two groups of nymphs (10 ticks/group) were injected with equal amounts of rabbit BBA52 antibody or normal rabbit antibody. To assess effects of antibody-treatment on spirochete persistence in unfed ticks, one group of injected nymphs (5 ticks/group) kept in the unfed condition for 5 days and spirochete burden were determined by qRT-PCR and confocal immunofluorescence. To assess the effects of BBA52 antibodies on spirochete transmission from ticks to mice, single antibody-

injected ticks were fed on groups of two naïve mice and *B. burgdorferi* burden in the repleted ticks were determined by qRT-PCR analysis. At day 7 following tick feeding, all the mice were sacrificed, and the tissues were isolated for the assessment of spirochete burden by qRT-PCR and culture analysis (Yang et al., 2009). Two independent experiments were performed and the average of the results obtained was statistically analyzed.

#### **4.2.11 Infection of mice during active or passive immunization**

Groups of immunized mice after 5 days of second booster were anaesthetized by intraperitoneal injection of ketamine and xylazine. While mice remained unconscious, two infected ticks per mouse were allowed to feed on each mouse, which were removed as repleted ticks. For the passive immunization studies, I injected peptide antibodies into infected ticks via microinjection procedure (Pal et al., 2004b). After 5 h of microinjection of BBA52 antibody in ticks, they were allowed to feed on naïve mouse until repleted ticks were recovered from each mouse. At day 10 of tick feeding, mice were sacrificed to collect tissues for quantifying spirochete load in heart, joint and bladder as described in section 3.2.1. Spirochete load was determined by quantifying copies of *flaB* per mouse  $\beta$ -actin.

#### **4.2.12 Far-western assay**

Far-western, also known as gel-overlay assay was performed to identify possible interacting partner of BBA52 in the *B. burgdorferi* or the tick gut. *B. burgdorferi* or 48 h fed tick gut was resolved in a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% of skimmed milk in TBS-T (0.05%) for



1 h and then 5-10 µg of rBBA52 which has a 6×His tag at N-terminus was overlaid on the nitrocellulose membrane in 5% skimmed milk, overnight at 4°C. Separate controls were also processed in the absence of BBA52 protein. Nitrocellulose membrane was washed 3 times with PBS-T (0.05%) and then was probed with anti-BBA52 antibody, followed by HRPO conjugated secondary antibody or directly anti His-HRPO conjugate.

#### **4.2.13 Enzyme-linked immunosorbent assay and confocal microscopy to assess protein binding in tick gut extract**

Recombinant BBA52 was expressed as a fusion protein with GST, as previously described (Zhang et al., 2009). Guts from flat Nymphal ticks were microdissected in PBS and homogenized on ice with Kontes micro homogenizer. About ten tick gut were dissected and homogenized in 1 ml of PBS. Each well was coated with 100 µl of tick gut extract (TGE) and incubated overnight at 4°C. On an average, 100 µl of TGE contains 0.5 µg of ticks gut protein determined by Bradford assay. For control, 100 µl per well of PBS was added. The plates were tightly covered with cellophane to avoid evaporation. Plates were then washed three times with PBS-T (0.05%). The TGE coated and control wells were blocked with 200 µl of 5% BSA for 2 h at 37°C. Plates were then incubated with 100 µl of rBBA52 protein at a concentration of 1ng/µl at 37°C for 1 h. As a control, plates were also incubated with equal quantity of GST protein. Plates were then washed three times with PBS-T to remove non specific binding of BBA52 protein. Wells were then probed with 1:1000 dilution of anti-BBA52 in 100 µl solution per well for 1 h at 37°C. After washing three times with PBS-T, plates were probed with anti-mouse IgG conjugated with HRPO for 1 h at 37°C. All wells were washed three times with PBS-T and then binding was detected with TMB microwell peroxidase substrate

(KPL Gaithersburg, MD) for color development. The OD was measured at 450 nm wavelength, after 15 min of color development and adding of stop solution. The assessment of protein binding by confocal microscopy was performed as previously described (Pal et al., 2004b). About 10 tick guts were dissected from unfed nymphal ticks in 100 µl of PBS. The tissue was cut into pieces and placed on sialylated glass slides (PGC Scientific, Gaithersburg, MD) to increase adhesion efficiency. Samples were incubated with PBS-T with 5% goat serum for 30 min at room temperature after washing three times with PBS. Recombinant BBA52 protein was overlaid at a concentration of 50 µg per ml for 1 h at room temperature. As a control, recombinant BBA62 was overlaid on it, at similar concentration. After three times wash, tissues were probed with anti-BBA52 serum at 1:1000 dilutions. Secondary antibody against mice IgG conjugated with Alexa 488 was used to observe binding of BBA52 protein with tick gut. Samples were subsequently stained with propidium iodide (50 µl of a 10 µg per ml solution) for 5 min at room temperature and then washed three times with PBS-T, and mounted in glycerol for examination. The tissues were viewed under Zeiss LSM 510 scanning laser confocal microscope.

#### **4.2.14 Treatment of tick gut with trypsin**

TGE was prepared as described above followed by trypsinization of the tick gut to partially understand the biochemical character of BBA52 receptor as described before (Pal et al., 2000). Equal aliquots of tick gut extract suspended in PBS were incubated with trypsin (Sigma) for 1 h at 37°C at 10 µg /ml concentration in the presence or

absence of 20 µg/ml of soyabean trypsin inhibitor (sigma). After 1 h of enzymatic digestion of tick gut protein, the TGE and the enzyme treated samples were coated on microtiter plates and incubated with rBBA52 as described above in ELISA to find the BBA52 interaction.

#### **4.2.15 Two dimensional (2D) gel electrophoresis**

*B. burgdorferi* or tick gut protein was prepared in sample buffer as recommended by the manufacturing company (GE healthcare). The IPG strip (GE healthcare) of pH 3-10 was rehydrated overnight in 125 µl volume of rehydration buffer. Protein loading was performed on the IPG strips as per the instructions provided by the manufacturer (Ettan IPGphor cup loading manifold manual, GE healthcare) and was run on Ettan IPGphor II electrophoresis equipment under following condition.

S1	Stp	300V	0:30h
S2	Grd	1000V	0:30h
S3	Grd	5000V	1:20h
S4	Stp	5000V	0:20h

Where 'S' stand for step; 'Stp' for step "N" hold; 'Grd' for gradient.

The IPG strip was run for total 2 h and 40 min under fixed and gradient voltage to increase the band sharpness. After completion of electrophoresis, the IPG strip was equilibrated in SDS equilibration buffer for 15 min. Thereafter, protein was further separated on the second dimensional SDS-PAGE.

### **4.3 Results**

#### **4.3.1 BBA52 is a surface exposed outer membrane protein that undergoes possible oligomerization**

To obtain further information on potential function of BBA52 in spirochete biology, I assessed whether the antigen is present on the microbial OM and exposed to the extracellular surface. To accomplish this, OM vesicle and protoplasmic cylinder (PC) fractions were separated from cultured spirochetes and subjected to immunoblot analyses using antibodies against BBA52, OspA and FlaB. While FlaB was mostly undetectable in the OM, both BBA52 and OspA were readily detected in the OM (FIGURE 14A). Both immunofluorescence labeling of unfixed spirochetes (FIGURE 16A), and Proteinase K digestion of *B. burgdorferi* surface proteins (FIGURE 14B), suggested that BBA52 is exposed on the extracellular surface of the spirochetes. Although *bba52* expression is responsive to environmental changes *in vitro* (Brooks et al., 2003; Ojaimi et al., 2002; Tokarz et al., 2004) and is differentially expressed in ticks, the *in vitro* growth kinetics of wild type and *bba52* mutants did not differ. When spirochetes are exposed to varied temperatures (23°C, 34°C and 37°C) (FIGURE 17), high salt concentration (FIGURE 18) and hydrogen peroxide (FIGURE 19), *bba52* deletion did not interfere with *B. burgdorferi* survival, suggesting that BBA52 function may not be associated with spirochete adaptation to temperature and oxidative or osmotic stress *in vitro*. Although BBA52 is localized on the OM, its deletion did not affect membrane integrity against detergent-mediated cell lysis (FIGURE 20). My phase separation experiment using Triton X-114 show that BBA52 can be detected in both aqueous phase (A) and detergent phase (D), providing evidence of being amphiphilic in nature and portion of being integrated in the *B. burgdorferi* membrane (FIGURE 14C). Another property of BBA52 is that the complete ORF contains 2 cysteine residues that have the tendency of forming intermolecular disulfide bond formation. This is evident when the *B. burgdorferi* lysate

is resolved in SDS-PAGE without adding  $\beta$ -mercaptoethanol and then processed for immunoblot analysis using BBA52 antibody. Results revealed, multiple bands at gel positions that correspond to approximate molecular weight of monomeric, dimeric, trimeric and hexameric forms of BBA52 (FIGURE 15A). The same feature of BBA52 homo-oligomerization is also reflected by the baculovirally-produced recombinant BBA52 and other strains of *B. burgdorferi* (FIGURE 15C). In the absence of  $\beta$ -mercaptoethanol, each band corresponds to BBA52 monomer, dimer, Trimer, hexamer; appears as doublet (FIGURE 15A and C). To prove BBA52 undergoes post translational modification, *B. burgdorferi* or the protoplasmic cylinder fractions were treated with N-Ethylmaleimide (NEM) to prevent any disulfide bonding during protein preparation. There was no effect in migration of BBA52 protein as detected by immunoblot on NEM treated *B. burgdorferi* lysate (FIGURE 15B and D). The ability to form strong disulfide bond in BBA52 was compared with known OspC protein (FIGURE 15E), which conveys BBA52 has higher tendency to form homooligomer than OspC.

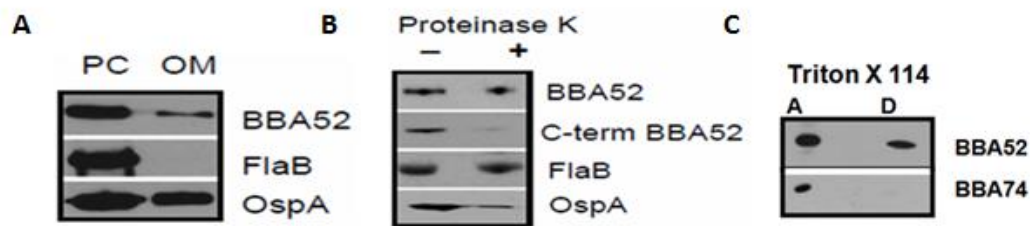


Figure 14. BBA52 is localized in the outer membrane and exposed to the microbial surface.

(A) BBA52 is distributed in the outer membrane of cultured spirochetes. *B. burgdorferi* protoplasmic cylinders (PC) and outer membranes (OM) were separated by sucrose density gradient centrifugation and equal amounts of protein from two sub-cellular fractions were separated by SDS-PAGE, and immunoblotted with BBA52, OspA and FlaB antiserum. (B) BBA52 is highly sensitive to proteinase K-mediated degradation of *B. burgdorferi* surface proteins. Viable spirochetes were incubated with (+) or without (-) proteinase K for the removal of protease-sensitive surface proteins and processed for immunoblot analysis using BBA52 antibodies. *B. burgdorferi* OspA and FlaB antibodies were utilized as controls for surface exposed and sub-surface proteins, respectively. (C) Phase partitioning assay using Triton X-114. Phase separation using Triton X-114 shows that BBA52 is located in both detergent phase (D) and aqueous phase (A) by immunoblot. A known aqueous phase protein BBA74 is used as a control.

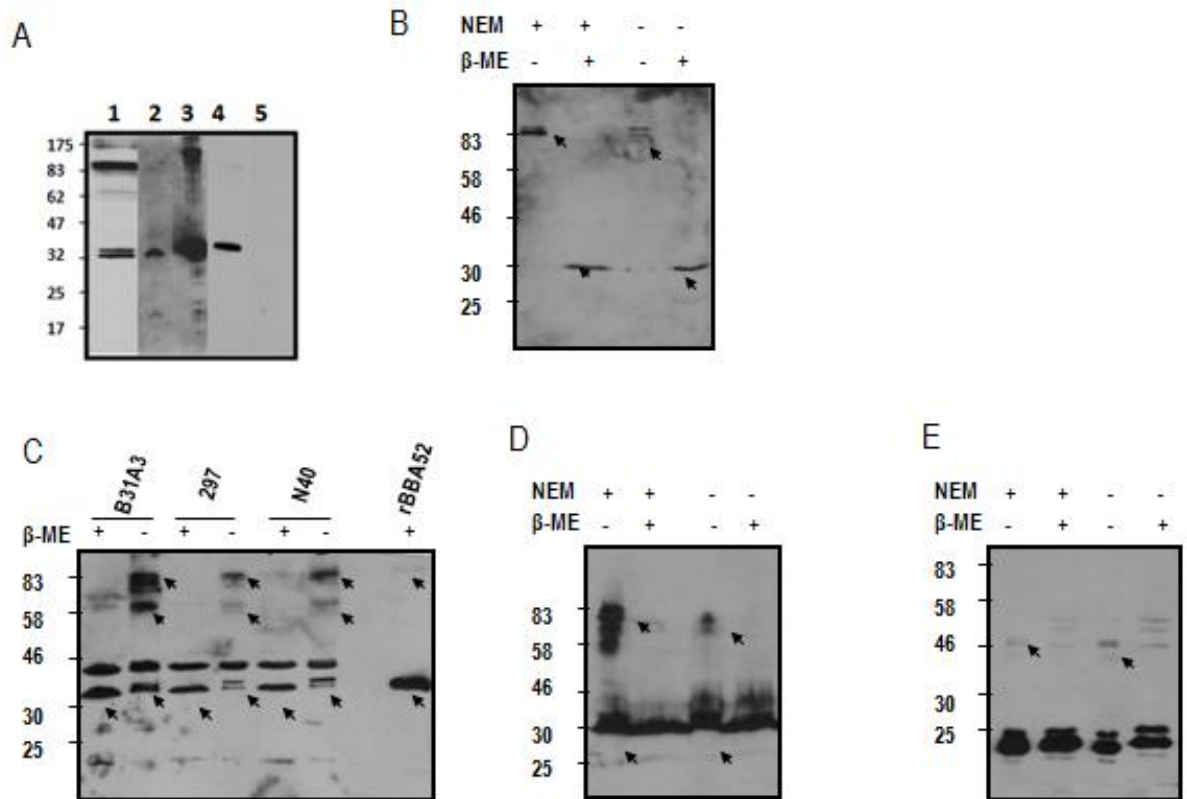


Figure 15. BBA52 exists as oligomeric protein.

(A) Immunoblot showing oligomerization of native and recombinant BBA52 protein. Lane 1 and 2 shows resolution of spirochetes in the presence or absence of  $\beta$ -mercaptoethanol. Under nondenatured condition, BBA52 forms dimer, trimer and hexamer whereas in denatured condition it exists as a monomeric protein of 33 kDa. Similar pattern is observed after resolving recombinant protein without (lane 3) and with  $\beta$ -mercaptoethanol (lane 4). Lane 5 is BBA52 mutant lysate to show the specificity of immunoblot. (B) Immunoblot of N-Ethylmaleimide (NEM) and  $\beta$ -mercaptoethanol ( $\beta$ -ME) treated *B. burgdorferi* lysate. Log phase grown *B. burgdorferi* were lysed in the presence or absence of 0.2 mg of NEM per 75  $\mu$ g of *B. burgdorferi* lysate and then was mixed with equal quantity of with or without  $\beta$ -ME containing SDS loading buffer. The lysates in different combination of NEM and  $\beta$ -ME treated samples were immunoblotted using BBA52 antibody. Arrow denotes the oligomerization tendency of BBA52. Treatment with NEM did not bring any change in migration pattern of BBA52 protein whereas in the absence of  $\beta$ -ME, BBA52 exists as oligomeric protein. (C) Different strains of *B. burgdorferi* BBA52 exists as oligomeric protein. *B. burgdorferi* strains, B31A3, 297 and N40 lysates, prepared in the presence or absence of  $\beta$ -ME were immunoblotted using BBA52 antibody. All the three strains follows similar pattern of BBA52 oligomerization, marked by arrow. (D) NEM treatment to protoplasmic cylinder (PC) fraction of *B. burgdorferi*. Protoplasmic cylinder fraction was isolated from WT *B. burgdorferi* to analyze the location where disulfide modification takes place. PC fractions of *B. burgdorferi* in the presence or absence of NEM and  $\beta$ -ME were immunoblotted with BBA52 antibody. Arrows show that the oligomerization occur only in the absence of  $\beta$ -ME. (E). Immunoblot using OspC antibody on NEM and  $\beta$ -ME treated *B. burgdorferi* lysate. Arrows show that oligomerization of OspC, a known disulfide bond forming protein, is not inhibited in the presence or absence of NEM, whereas it is inhibited in the presence of  $\beta$ -ME.

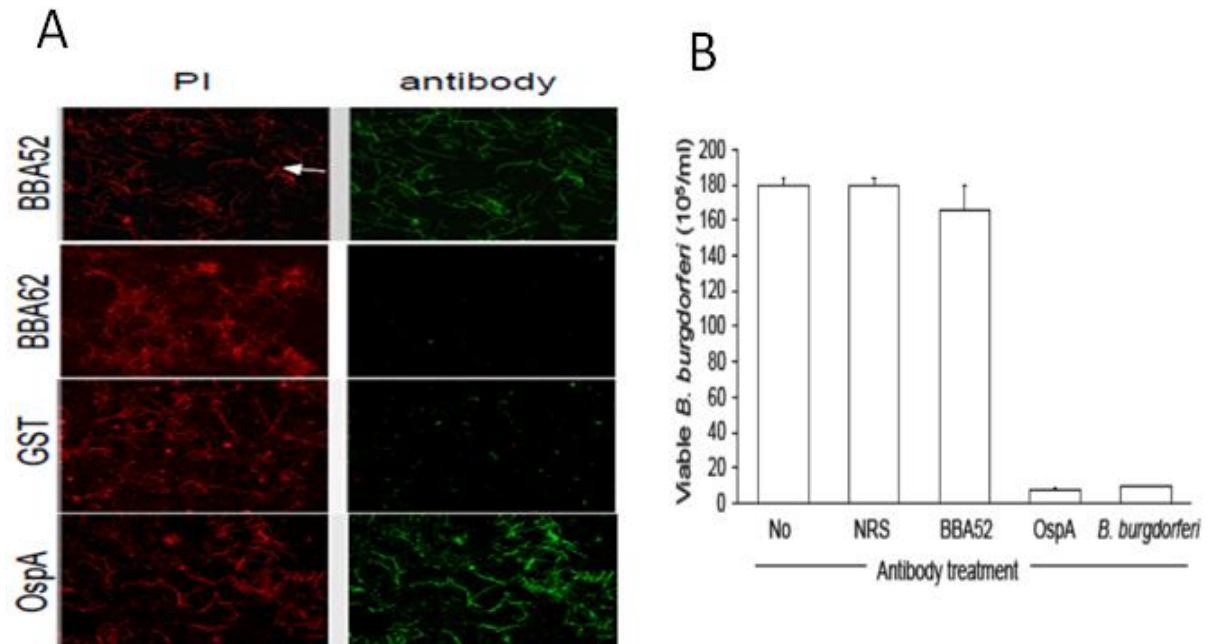


Figure 16. Characterization of BBA52 antibodies.

(A) BBA52 antibodies bind to the surface of intact unfixed *B. burgdorferi* (arrow). Spirochetes were immobilized on glass slides and probed with BBA52 or control (GST) antibodies. Antibody against known surface (OspA) and subsurface (BBA62) spirochete proteins were used as controls. Spirochete loading and antibody labeling was assessed using propidium iodide (PI) and Alexa-488 tagged secondary antibodies, respectively. Images were acquired using a 40x objective lens of a Zeiss confocal microscope. (B) Borreliacidal activities of BBA52 antibodies *in vitro*. Spirochetes were incubated with either normal rabbit sera (NRS), rabbit OspA antibodies (OspA), serum collected from 15-day infected mice or BBA52 antibodies. The sensitivity of spirochetes to the bactericidal effects of the antibodies was assessed by a re-growth assay after 48 h of antibody incubation and presented as mean  $\pm$  SEM of viable spirochete number (cells/ml). The numbers of viable spirochetes were significantly reduced in the samples exposed to the OspA or *B. burgdorferi* antibodies, compared to spirochetes that received no treatment or were incubated with BBA52 or normal serum ( $P < 0.002$ ).

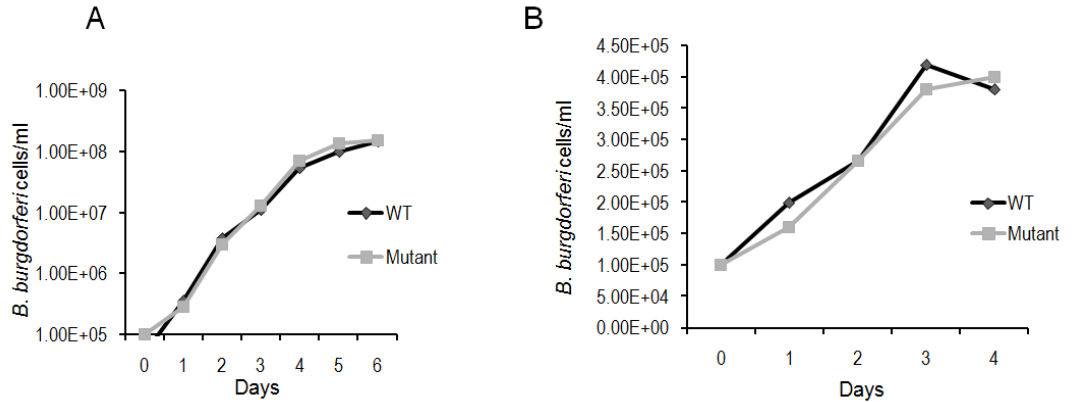


Figure 17. *In vitro* growth kinetics of *bba52* mutant and WT *B. burgdorferi* at 37°C and 25°C.

(A) Spirochetes were grown at 37°C in BSK medium, starting from  $10^5$  *B. burgdorferi* per ml and were counted until day 6 using Petroff-Hausser counting chamber. (B). Spirochetes were grown at 25°C in BSK medium, starting from  $10^5$  *B. burgdorferi* per ml and was counted until day 4. The values are shown as average of two counts from single experiment.

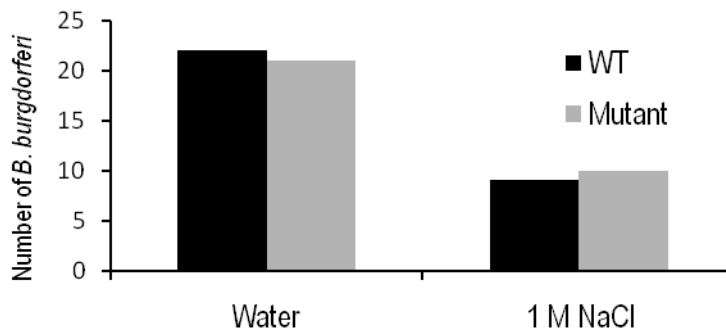


Figure 18. Osmotic stress using 1 M Sodium chloride on *bba52* mutant *B. burgdorferi* culture.

Stationary phase *B. burgdorferi* of WT and *bba52* mutant cultures were incubated with 1 M NaCl or equal amount of sterile water for 40 min at 34°C. After incubation 40 min of osmotic stress, equal volume of spirochetes were recultured for 48 h at 37°C and were counted in 1:10 dilution under dark field microscopy. There was no effect on giving osmotic stress to *bba52* mutant. The values are shown on single experimental analysis.



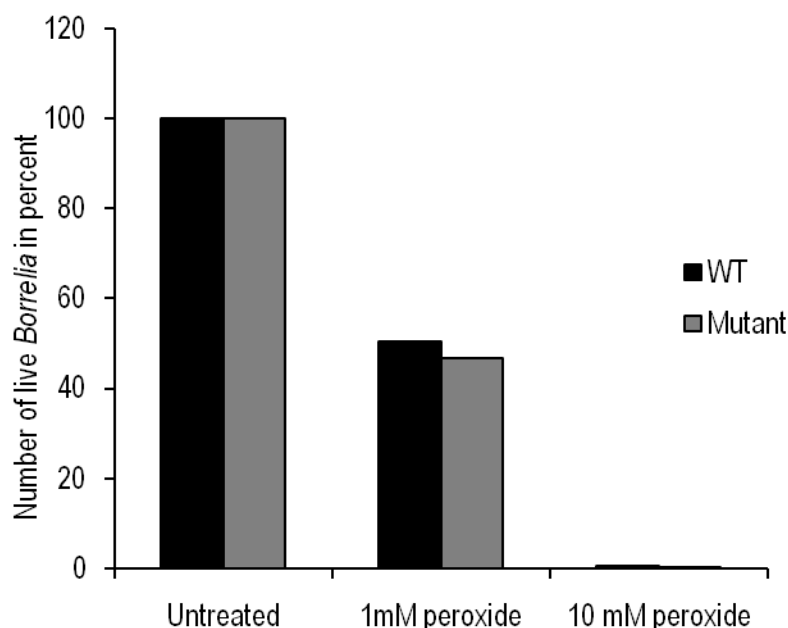


Figure 19. Effects of Oxidative stress on *bba52* mutant *B. burgdorferi* using different concentration of Hydrogen per oxide.

*B. burgdorferi* were grown in BSK in the presence of 1 mM and 10 mM Hydrogen per oxide for 24 h at 34°C. After incubation in the presence or absence of Hydrogen peroxide, equal volume of the culture was re-grown in fresh BSK medium for 24 h and the number of live *B. burgdorferi* was counted twice from single experiment. Number of live *B. burgdorferi* is represented in terms of the percentage of the untreated samples. The values are shown as average of two counts from single experiment.

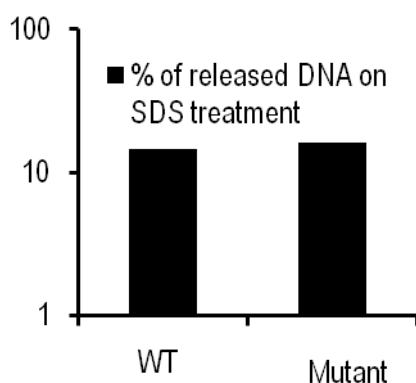


Figure 20. Determination of outer membrane integrity of *bba52* mutant *B. burgdorferi* using Sodium dodecyl sulfate (SDS).

Spirochetes in BSK medium ( $10^7$  cells/ml) were incubated in presence of SDS (2 mg/ml) for 4 h at 34°C. As a standard, WT spirochetes was incubated with 10 mg/ml SDS considering as a hundred percent destruction of outer membrane. DNA was extracted from equal aliquots of supernatants of each samples and analyzed by qPCR as percentage of released DNA in terms of standard. There was no effect on the integrity of outer membrane in the absence of BBA52 on spirochetes. The values are represented on single experimental analysis.

### 4.3.2 BBA52 is a potential target for transmission-blocking Lyme disease vaccine

As BBA52 is exposed on the microbial surface and primarily produced in ticks, I explored whether BBA52 antibodies, despite lacking borreliacidal properties (FIGURE 21B & 16B) could interfere with BBA52 function *in vivo* and influence the transmission of *B. burgdorferi* from the feeding ticks to a naïve murine host. To mimic the natural transmission process, *B. burgdorferi*-infected nymphs was generated by allowing larvae to acquire spirochetes from infected mice and then molt into nymphs. A microinjection procedure (Pal et al., 2004b) was used to deliver similar amounts of normal rabbit antibodies or BBA52 antibodies that recognized the extracellular part of the antigen (FIGURE 14B) into the tick gut. Following infection, one group of ticks remained in the unfed condition for 5 days, while a parallel group was allowed to feed on naïve C3H mice for 72 h. The spirochete burden in ticks were determined by confocal immunofluorescence (FIGURE 21A) and qRT-PCR (FIGURE 21B), which indicated that BBA52 antibody did not affect persistence of *B. burgdorferi* in unfed ticks. Strikingly, qRT-PCR analysis of mouse infection following one week of tick engorgement indicated that, unlike the control antibody, BBA52 antibody treatment effectively blocked the transmission of spirochetes from ticks to mice (FIGURE 21C). The culture analyses corroborated with these data by the observation that while *B. burgdorferi* was readily recovered from murine skin, spleen and heart tissues in control antibody-treated groups, while the mice fed by BBA52 antibody-treated ticks remained culture negative at all tissue locations.

I next assessed whether BBA52 antibodies generated in mice via active immunization could interfere with BBA52 function in the feeding vector and influence the transmission of *B. burgdorferi* from ticks to naïve hosts. To accomplish this, separate groups of C3H mice (5

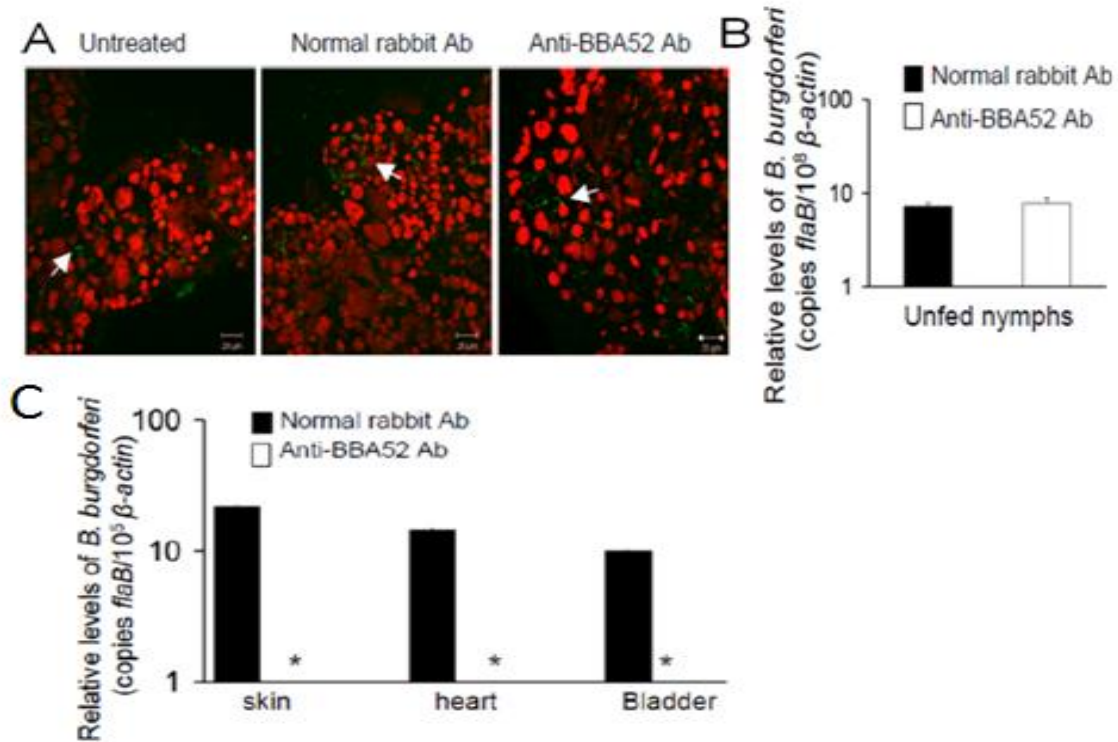


Figure 21. Non-borreliaicidal BBA52 antibody does not interfere with microbial persistence in feeding ticks but blocks pathogen transmission from ticks to mice.

(A) BBA52 antibodies failed to interfere with the spirochete persistence in unfed ticks. Naturally-infected nymphal ticks were microinjected with antibodies against BBA52 (Anti-BBA52 Ab) or control antibody (Normal rabbit Ab) and spirochete distribution in the gut of unfed ticks were analyzed at 5 days after injection. The spirochetes (arrow) were labeled with FITC-labeled goat anti-*B. burgdorferi* antibody (shown in green), and the nuclei of the gut cells were stained with propidium iodide (shown in red). Images were obtained using a confocal immunofluorescence microscope and presented as merged image for clarity. (B) Quantitative representation of the data shown in Figure 21A. *B. burgdorferi* burden in ticks were assessed by qRT-PCR analysis by measuring copies of the *B. burgdorferi flaB* RNA and normalize against tick  $\beta$ -actin levels. Bars represent the mean  $\pm$  SEM of four qRT-PCR analyses derived from two independent infection experiments. Differences in the spirochete burden in unfed ticks passively transferred with BBA52 antibody and those with the control were insignificant ( $P > 0.05$ ). (C) BBA52 antibodies blocked transmission of *B. burgdorferi* from ticks to mice. Naturally infected nymphal ticks were microinjected with antibodies as described above and placed on naïve mice 48 h after injection. Ticks were allowed to fully engorge on mice and the transmission of *B. burgdorferi* was assessed by measuring copies of the *B. burgdorferi flaB* gene in the indicated murine tissues 7 days after tick feeding. Amounts of mouse  $\beta$ -actin were determined in each sample and used to normalize the quantities of *B. burgdorferi flaB*. Bars represent the mean  $\pm$  SEM of relative tissue levels of *B. burgdorferi* from two independent animal experiments. \* Spirochetes were undetectable in Anti-BBA52 Ab group.

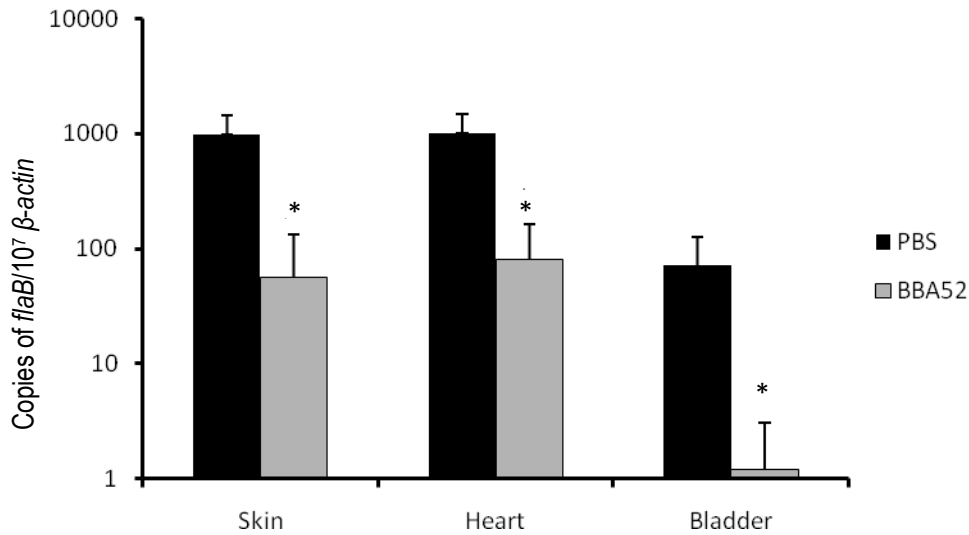


Figure 22. Active immunization of mice with recombinant BBA52 significantly interfere with spirochete transmission from infected ticks.

A group of 5 mice were immunized with PBS or recombinant BBA52. At day 5 of second boosting, two naturally infected ticks with *Borrelia burgdorferi* were infested on each mouse until complete feeding. At day 10 of tick transmission mice were sacrificed and tissue samples were collected to generate cDNA and analyze the spirochete load in each tissue by qRT-PCR as copies of *flaB*/ $10^7 \beta$ -actin. Three independent experiments were performed and the average values were analyzed statistically. Bars represent the mean  $\pm$  SEM of relative tissue levels of *B. burgdorferi* from three independent animal experiments. Asterisk denotes the  $P < 0.05$ .

animals/group) were actively immunized with either recombinant BBA52, purified as full-length protein or PBS (control) mixed with similar volume of adjuvant. ELISA and immunoblotting performed after the final boosting indicated that immunized mice had developed a strong antibody titer that specifically recognized recombinant and native BBA52. Ten days after the final immunization, mice were parasitized by *B. burgdorferi*-infected nymphs (2 ticks/mouse). The spirochete burdens in engorged ticks were determined by qRT-PCR, which indicated that BBA52 antibody did not affect persistence of *B. burgdorferi* in feeding ticks. Strikingly, qRT-PCR analysis of mouse infection following one week of tick engorgement indicated that, unlike the control, BBA52 immunization effectively blocked the transmission of spirochetes from ticks to mice

(FIGURE 22). However, in spite of significantly impaired transmission, spirochetes were still recovered by culture analyses of BBA52-immunized infected murine tissues.

These data establish that BBA52 facilitates *B. burgdorferi* transition from feeding ticks to the murine hosts and potentially serve as an antigenic target to interfere with the *B. burgdorferi* transmission.

#### **4.3.3 Interaction of recombinant BBA52 protein with *B. burgdorferi* proteins**

BBA52 is a unique and highly conserved protein, in *B. burgdorferi sensu stricto*, expressed only in *B. burgdorferi*, in reference to the current data base availability. As BBA52 display no similarities with known proteins, identification of molecular function of BBA52 in spirochete biology remains as a challenging preposition. I next assessed if BBA52 is involved in protein-protein interaction. A gel overlay assay using rBBA52 as a bait and the wild type and BBA52 mutant lysates as prey protein were performed that showed two possible interacting partners of recombinant BBA52 (FIGURE 24). One of the BBA52-interacting protein has the same molecular weight as BBA52 (33 kDa) and the second partner is above 25 kDa in molecular weight (FIGURE 24 A & C). To localize the interacting partner, outer membrane vesicle and protoplasmic cylinder of WT and mutant *B. burgdorferi* was extracted, and resolved on SDS-PAGE and transferred on nitrocellulose membrane as prey proteins, followed by overlay with rBBA52 as a bait protein to analyze by far western technique. It was observed that the 25 kDa interacting partner was localized in the protoplasmic cylinder content whereas the 33 kDa partner was localized in both outer membrane vesicle and the protoplasmic cylinder (FIGURE 23). This finding was further confirmed by performing far-western analysis using the

wild type *B. burgdorferi* lysate and rBBA52 protein. The bound protein was detected using anti-His IgG HRPO conjugate (FIGURE 24A). To identify the interacting partners, I performed two dimensional (2D) gel electrophoresis. The wild type *B. burgdorferi* lysate was resolved using IPG strip ranging from pH 3-10 in triplicates, on Ettan IPG phor as described in section 4.2.15. After separation of individual *B. burgdorferi* proteins based on their isoelectric point, the strip was transferred carefully to resolve by the second dimensional SDS-PAGE. One of the triplicate gels was stained with Coomassie stain and the other two gels was transferred on nitrocellulose membranes to detect the interacting partners by far-western, using with and without recombinant BBA52 overlay (FIGURE 24C). The spot where BBA52 interacting partner was found by far-western was excised from the SDS-PAGE gel after Coomassie staining, protein were eluted from the gel, digested with trypsin and processed for protein identification by Liquid-chromatography (LC)-mass spectrophotometry (MS) (FIGURE 24B). To further validate the mass spectrophotometric data, another independent pull down assay was performed using 6×His tag rBBA52 bound to Probond Nickel column resins (Invitrogen). Outer membrane vesicles (OMV) and protoplasmic cylinder (PC) fractions from BBA52 mutant was purified from 1 liter culture of BSK, as described in section 4.2.3. The OMV and PC fractions were suspended with the rBBA52 bound to Probond Nickel column resin separately and incubated overnight at 4°C on a slow rocking platform. After overnight shaking, the unbound proteins were removed by several washing with PBS. The bound rBBA52 protein with the *B. burgdorferi* interacting partners was eluted by SDS-PAGE sample buffer (Bio-Rad) at 95°C for 10 min. Elute obtained was resolved on SDS-PAGE, stained with Coomassie and the protein bands at 33 kDa and 25 kDa were excised

from the gel and processed for protein identification using LC- MS/MS analysis. Common proteins identified by MS analysis in two independent experiments were analyzed for their expression profile from *in vitro* and *in vivo* (mouse and tick) transcriptome of *B. burgdorferi* (FIGURE 25). The MS identified spirochete genes *bbi36*, *bbi38*, *bbi39* and *bbj41* were to be the closest match in terms of molecular weight and expression pattern with *bba52*. The interaction of BBA52 with 33 kDa and 25 kDa protein remains a subject of further investigation, which might shed light to the role of BBA52 and interacting proteins in the biology and infectivity of *B. burgdorferi*.

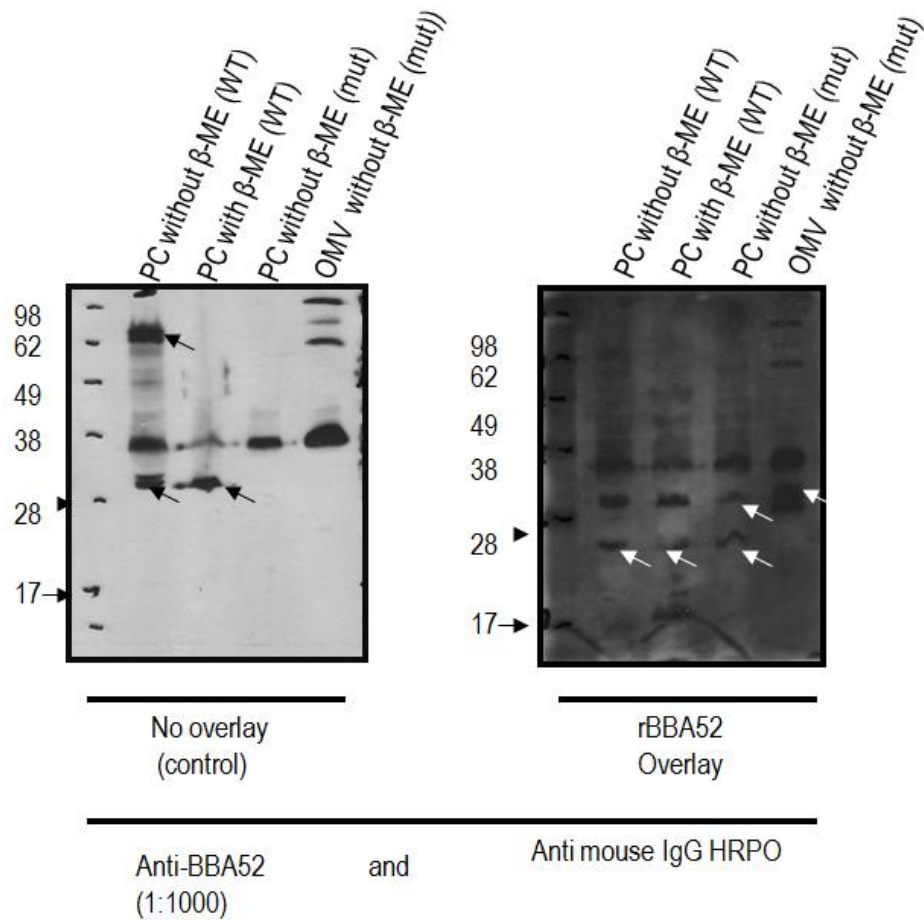


Figure 23. BBA52 interacts with *B. burgdorferi* proteins.

Outer membrane vesicles (OMV) and protoplasmic cylinder (PC) were separated from WT and BBA52 mutant *B. burgdorferi*. Each sample was heated with or without  $\beta$ -mercaptoethanol and was resolved on SDS-PAGE and then transferred to nitrocellulose membranes, blocked with 5% milk. Membranes were incubated overnight in the absence or presence of 5-10  $\mu$ g of rBBA52. Each membrane was then probed with anti BBA52 followed by anti-mouse HRPO. Two interacting partners in the OMV and PC fraction binds BBA52 are indicated by arrows.



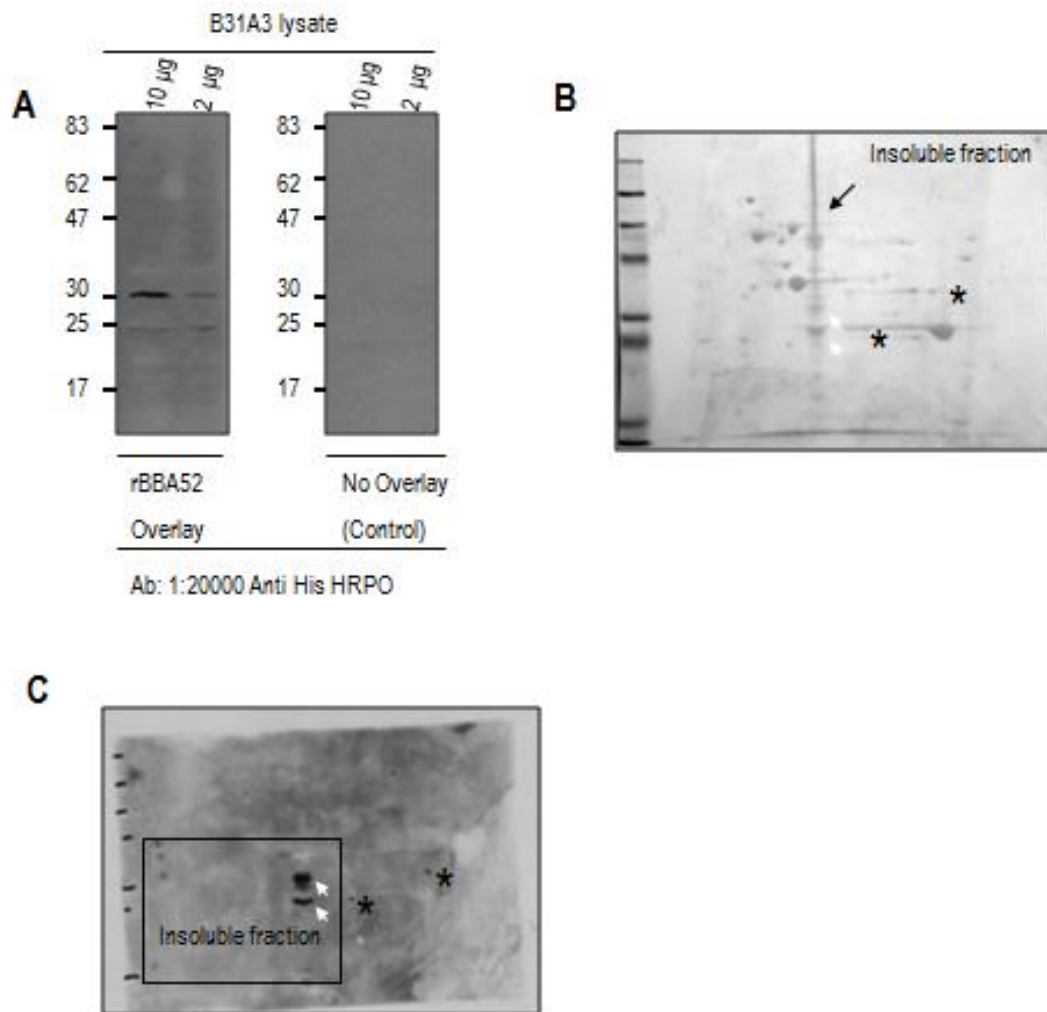


Figure 24. Identification of BBA52 interacting proteins by far-western assay involving one and two-dimensional SDS-PAGE gels.

(A) Far-western analysis was performed using *B. burgdorferi* lysates resolved using one-dimensional SDS-PAGE and overlaying with rBBA52. The membrane was probed with anti-His HRPO conjugate to detect rBBA52 containing amino terminal His tag. Two binding partners were detected at 33 kDa and 25 kDa molecular weight. (B) Coomassie stained 2D gel of wild type *B. burgdorferi* lysates. Some insoluble fractions of the protein failed to separate in the one dimension, as marked by arrows. (C) Far-western performed on the 2D gel transferred nitrocellulose membrane using rBBA52 as overlay protein. The membrane was probed with anti-His HRPO conjugate. Two spots are visible, as marked by asterisks were the possible BBA52 interacting partners. The white arrows indicate BBA52 interacting proteins from insoluble fraction.

**Table 3:** Oligonucleotide primers used in the current study

ORF	Putative Function or Gene Symbol	Paralog Family	Sequence (5' → 3': Forward, Reverse)
BBI36/138/139/J41	antigen, P35, putative	54	GTGAAAAAGAAGCCGAAAAGT, TAGTAGATAAAGAGGATGGGAATA
BB0057	gap		CAGATCCTAAAACCTTGCACATCT, CTGATGAAAAAACACCTGTTGACT
BB0141	membrane fusion protein; lp		TATGAAAGTTAAAAAGGGAATCTTGAG, GGACTTTTCAAATATACAGAACCAGG
BB0754	ABC transporter, binding protein	13	TCATTATCCAAGCAAAGGTAATGTAA, ATGCTTATTACTCTGTCAATTTCTTTT
BB0334	OppD	13	TCCGTAACAAGTATGGCTATTTTAAA, AAGTTGAGTTGATATTCTTAAAAATGG
BB0418	HP		ACCCAAGTTTAAAAATTCCAGTCA, AAGCAATAGGAGTAAAGCTTACTGTG
BB0176	AAA, putative		GAAATGATAGATGCTATTTTAATGGGT, AGTGCCTGTAGCACTTTTATAAACC
BBI36	antigen, P35, putative		ATACTATTAAGCTTAACGTTATTACAGCAATATT, TGCTGAATTTTCTGAATTGGTG
BBI39	Putative surface protein		ACTTAACGTTATTACAGCAATATTAECTTCAA, TCTGAATTGGTGTGATTGCTTG

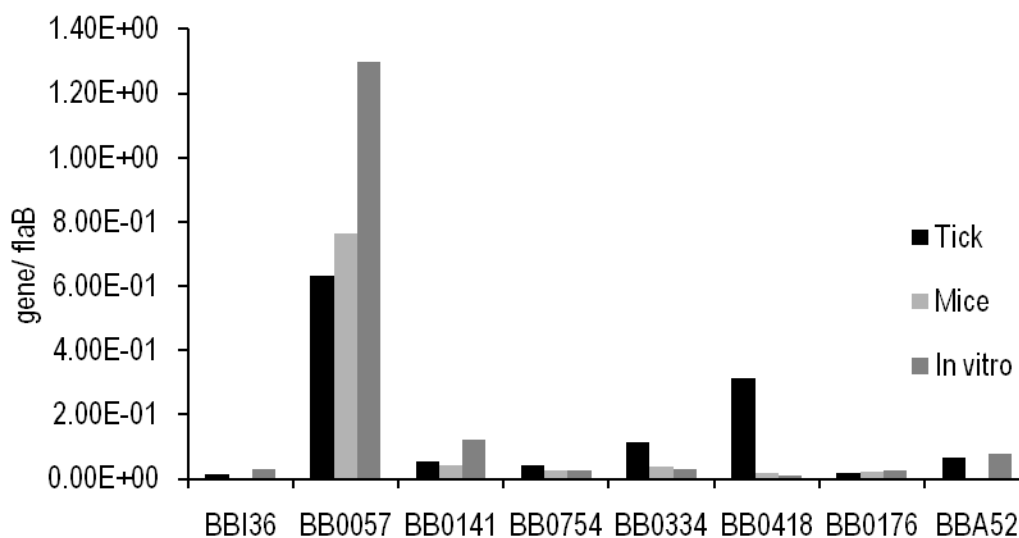


Figure 25. Expression analysis of the BBA52 interacting borrelial gene products.

Total RNA was isolated from cultured spirochetes; from pooled *B. burgdorferi*-infected nymphs collected at 24, 48, and 96 h of feeding on naive mice; and from murine skin after 2 weeks of *B. burgdorferi* infection. Total RNA was converted to complementary DNA for measuring-specific transcripts by qRT-PCR and normalized against *flaB* Ct values.

#### 4.3.4 Interaction of BBA52 with *Ixodes scapularis* tick gut extract

Since, BBA52 is surface localized *B. burgdorferi* protein and expressed during migration in ticks, I proceeded to investigate for its interacting partners in tick gut. ELISA was performed after coating the tick gut extract in a microtiter plate as described in section 4.2.13. The binding was measured using TMB microwell peroxidase substrate (KPL Gaithersburg, MD) for color development. The OD was measured at 450 nm after adding stop solution at 15 min of color development. The binding study showed that there was specific binding of rBBA52 protein with the tick gut extract compared to the control protein glutathione s-transferase (FIGURE 26A). The result was further

supported by visualizing the interaction of BBA52 with the tick gut using confocal microscopy. The rBBA52 protein binding was specific as the control protein rBBA62 which is known to be sub-surface localized protein in *B. burgdorferi*, doesn't show any interaction with the tick gut (FIGURE 26B). To screen the nature of BBA52 receptor in the tick

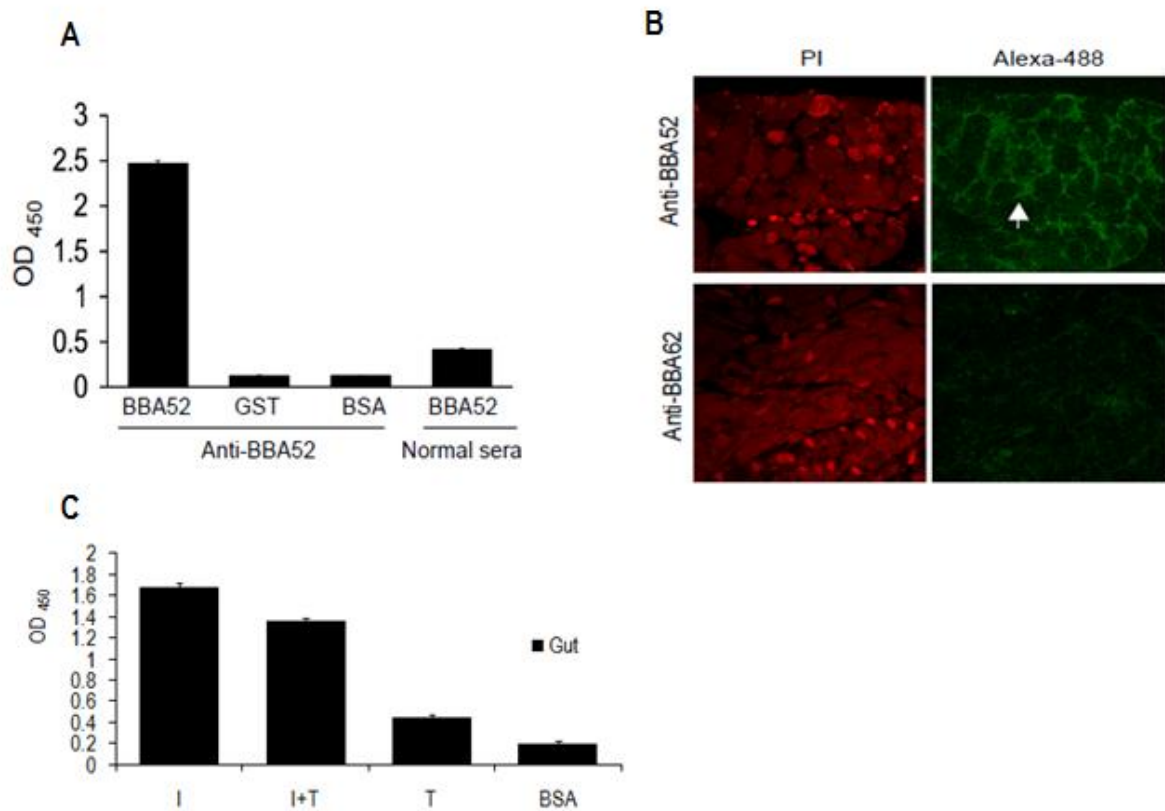


Figure 26. Interaction of rBBA52 with tick gut.

(A) ELISA showing interaction of rBBA52 to tick gut lysate. Tick gut extract (TGE) was coated overnight in a microtiter well plate, which was then overlaid with rBBA52 protein or GST and BSA as control. The interaction with BBA52 and tick gut protein was probed using anti BBA52 antibody as described in materials and methods. It shows rBBA52 interacts strongly with tick gut. (B). Confocal analysis of rBBA52 interaction with tick gut. The interaction was visualized by using anti-mouse IgG tagged with Alexa 488. For control BBA62 was overlaid on it which doesn't show any specific interaction unlike BBA52. Tick gut nucleic acid was stained with Propidium Iodide (PI), to visualize the gut tissue. (C) Interaction study of tick gut extract with rBBA52 after trypsin (T) treatment. Equal aliquots of Tick gut extract suspended in PBS were incubated with trypsin (Sigma) for 1 h at 37°C at 10 µg /ml in the presence or absence of 20 µg of soyabean trypsin inhibitor (I) (Sigma)/ml. After 1 h of enzymatic digestion of tick gut protein, the TGE and the enzyme treated samples were coated on microtiter plates and then were incubated with rBBA52 as described for ELISA.

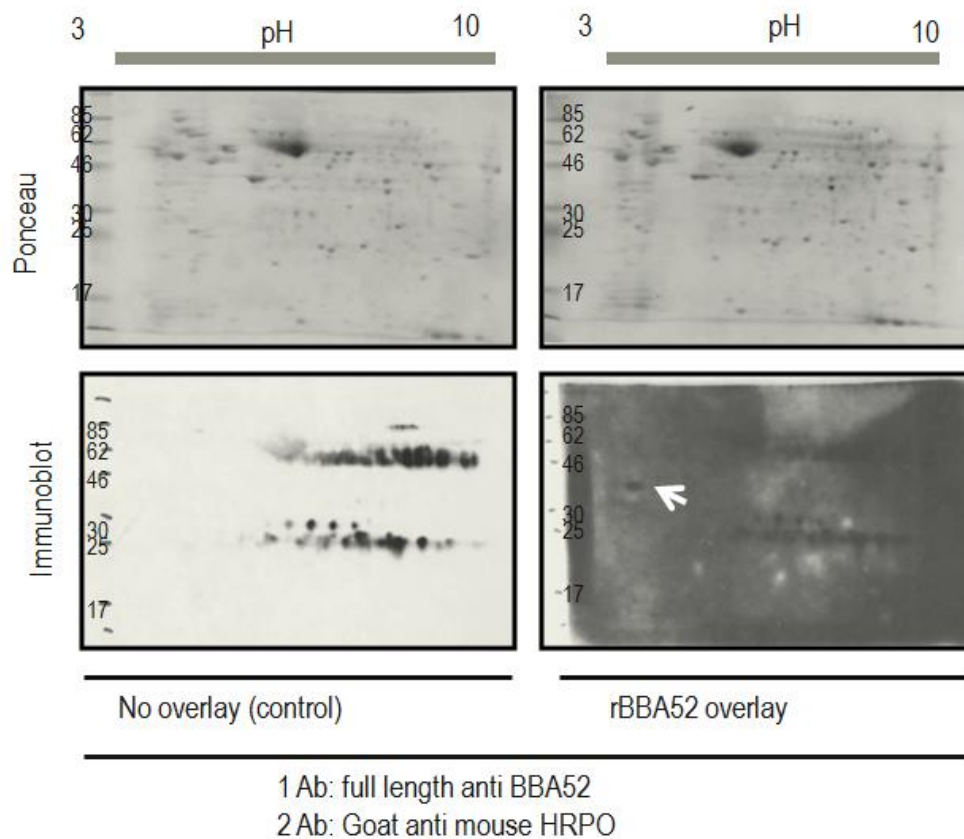


Figure 27. Far western analysis for identification of recombinant BBA52 receptor in the tick gut.

Tick gut proteins were resolved using SDS-PAGE gels and transferred to nitrocellulose membrane. The membrane was blocked with 5% of skimmed milk and incubated with 5-10  $\mu\text{g}$  of recombinant BBA52. Parallel control lanes were incubated in the buffer without recombinant BBA52. The membrane was finally washed with PBS-Tween 20 (0.05%) and then was probed with anti-BBA52 and followed by secondary HRPO conjugated antibody. The arrow denotes the possible BBA52 receptor in tick gut.

gut, tick gut extract were trypsinized (T) in the presence or absence of soyabean trypsin inhibitor (I) (Both from Sigma). The samples were coated in a microtiter plate and the interaction of BBA52 was investigated as described in section 4.2.14. Trypsinization experiment provided the evidence that BBA52 receptor in tick gut is protein in nature,

since there was a reduction of interaction in the wells which were coated with trypsinized tick gut extract whereas gut extract containing soyabean trypsin inhibitor did not influence the BBA52 interaction with its tick receptor (FIGURE 26C). Far-western analysis using the 48 h uninfected fed tick gut and rBBA52 as overlay protein, showed ~ 35 kDa interacting partner in the tick gut (FIGURE 27). From my findings, BBA52 appears to be sandwiched between the *B. burgdorferi* outer membrane and a tick gut protein. Characterizing the interacting partner will be an interesting subject for future investigation.

#### **4.4 Discussion**

*B. burgdorferi* is unique among the pathogenic spirochetes by requiring obligate blood-feeding arthropods for their transmission and maintenance in susceptible vertebrate host populations (Canale-Parola, 1984). *B. burgdorferi* does not readily infect most tick species, suggesting that the interactions between *I. scapularis* and *B. burgdorferi* are highly specific, and limited studies have shed important light on the complex interactions between *B. burgdorferi* and ticks (de Silva AM, 2009; Fikrig and Narasimhan, 2006; Munderloh and Kurtti, 1995; Tsao, 2009). I identified *bba52* as one of the genes that is selectively expressed in ticks and showed that BBA52 is a surface exposed protein, which can specifically interact to an uncharacterized-tick gut protein. Analysis of surface exposure of BBA52 by subcellular fractionation of OM, PK assay, and immunofluorescence studies provides solid evidences that BBA52 is localized in the OM and exposed to the surface. The phase partition of *B. burgdorferi* proteins in solution of

Triton X-114 showed BBA52 to be present in both aqueous and detergent phase, suggesting BBA52 to be amphiphilic in nature and partly integrated to membrane. Antibodies against BBA52 partially but significantly blocked *B. burgdorferi* transmission from ticks to mammals, indicating that the antigen might contribute to development of a transmission-blocking Lyme disease vaccine.

Although I have determined that BBA52 participates in pathogen transitions between the host and the vector, with the most prominent role during pathogen exit from ticks to mice, the function of this gene product in spirochete biology remains unknown. No phenotypic effects of *bba52* deletion were evident *in vitro*, when mutants were exposed to varied temperature, osmotic and oxidative shock or detergents. Therefore, BBA52 is not likely to be involved in spirochete growth, stress response or membrane integrity, at least *in vitro*. Attempts for generation of recombinant BBA52 protein in bacterial cells seems to exert toxic effect, as *E. coli* was unable to grow properly when BBA52 expression was induced. I successfully produced the recombinant BBA52 in insect cells using baculovirus expression system. I also show that BBA52 protein in *B. burgdorferi* appeared to occur as homooligomers possibly through inter- and intramolecular disulfide bond formation. I have detected several oligomeric forms of BBA52 which includes monomer, dimer, trimer and hexamer that appear to be resolved in SDS-PAGE gel as doublets of close proximity. The doublet form of native BBA52 could be due to difference in the protein configuration. This pattern of oligomerization of *B. burgdorferi* protein is very rarely reported, except for OspC (Kumaran et al., 2001) and CRASP1 proteins (Cordes et al., 2005). However, this is puzzling as *B. burgdorferi* genome lacks orthologs of bacterial enzymes involved in the formation of disulfide bond

(Dutton et al., 2008). Disulfide bonds in proteins play an important protective role for microbes, as it may function as a reversible switch to regulate protein activity (Ladenstein and Ren, 2008). However, I did not find any difference in the ability of *bba52* mutant and parental isolates for growth under different Hydrogen peroxide concentrations. Moreover, native BBA52 in borrelial cells is likely to be subjected to disulfide modification as treatment with N-Ethylmaleimide, which prevents artificial disulfide bond formation in lysed cells or solubilized proteins, also demonstrated oligomerization property of native BBA52. It appears that BBA52 is more stable as a trimer compared to dimeric and hexameric forms.

My data suggest that correlated with tick-specific expression of *bba52* and significant extracellular exposure of the antigen, BBA52 might be involved in pathogen interaction with vector environment. In the trypsin digested tick gut, BBA52 failed to show interaction with the tick gut receptor, showing possible existence of protein receptor for BBA52. In agreement with genetic studies, BBA52 antibody interfered with the transmission of spirochetes to naïve mice. BBA52 antibody did not show borreliacidal activities *in vitro* or cause significant changes in the feeding behavior of ticks, gut morphology or distribution of spirochetes in unfed ticks. This suggests that the effects of the BBA52 antibody on spirochete transmission were not likely due to the bactericidal action of the antibody or indirect effects on tick biology, but potentially, through interfering with BBA52 interaction with the vector environment. So, I attempted to study its function by following an indirect approach. Since, BBA52 is an OM protein and expressed only in vector-specific spirochete life cycle, it could interact with both tick and other borrelial proteins. I identified two possible *B. burgdorferi* proteins that



interacted with BBA52. Although precise identity of these proteins remains significant future efforts, I preliminarily identified one of these BBA52-interacting proteins as BBI36, BBI38, BBI39, or BBJ41. Based on protein sequence analyses, these display 91-99% homology. Specifically, BBI36 and BBI38 & BBI39 and BBJ41 have 99% similarity. Analyses of their gene expression *in vivo* show that *bbi39* has the similar expression pattern like BBA52 while *bbi36* is not expressed. The primers for *bbi36* and *bbi39* were designed against the non consensus nucleotide sequence for qRT-PCR. Thus, I ruled out the possibility of BBI36 as interacting partner of BBA52 but I cannot differentiate the expression pattern of *bbi39* and *bbj41* due to 99.5% sequence homology. Since, this protein also turned out to be a hypothetical protein; I have not been able to predict the function of BBA52. Further investigation is required to study the interaction of BBA52 with BBI39 to draw any conclusion.

Similar to borrelial protein, I identified a tick protein of 35 kDa also interact with BBA52. As tick proteome is remarkably diverse, I was unable to identify the BBA52 receptor. Future studies using yeast two hybrid assay or phase display assays could be executed to identify the BBA52 receptor in ticks. Characterization of surface-exposed microbial antigens expressed in feeding arthropods provides an opportunity to understand the pathogen interactions with its environments likely required for microbial transition between hosts and vectors, and may contribute to development of novel transmission-blocking vaccine against vector-borne diseases.

## Chapter 5: Conclusions

*B. burgdorferi* has evolved a remarkable capability for persistence in nature through a distinctive but diverse range of organisms, such as in *I. scapularis* ticks and wild rodents. Spirochete genes that are temporally induced in particular host or vector locations could be functionally important for *B. burgdorferi* persistence and transmission through the natural enzootic cycle. My data show that selected *B. burgdorferi* genes are variably expressed *in vivo*, some of which are highly transcribed in feeding ticks during transmission. The expression pattern of many of genes *bb0323*, *bba52*, *bba62*, *bba74*, *bb0262*, *bb0769*, *bb0353*, *bb0213*, *bbg01*, and *bbe31* showed preferential upregulation of expression in ticks, whereas *bb0028*, *bb0108*, *bb0319*, *bb0381*, *bbb27*, *bb123*, *bbn39*, *bb0258*, *bb0298*, *bba73*, *bbj23*, *bbj27*, *bbm38*, *bbn26*, *bbn38* and *bb0735* were preferentially upregulated in mouse. A selective assessment of the microbial transcriptome, limited to putative membrane proteins, reveals that *bba52* expression is only detectable in ticks and upregulated during pathogen transmission. *bba52* deletion did not affect murine inflammation or long-term pathogen persistence in mice or ticks, but did impair transitions between hosts and vector, a defect that could be rescued when native *bba52* expression was genetically complemented. I show that the antigen, which lacks homology to known proteins, is localized in the outer membrane, exposed extracellularly via the carboxyl terminus and potentially involved in pathogen-vector interaction. Interference with BBA52 function, both through active immunization of mice and passive antibody transfer to ticks, impaired pathogen transmission from ticks to naïve hosts. These studies establish that BBA52 facilitates vector-host transitions and as such, is a potential antigenic target for a transmission-blocking vaccine.

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