ABSTRACT

Title of Document: **NOVEL SURFACE PROTEINS IN THE PATHOGENESIS AND DIAGNOSIS OF LYME DISEASE**

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Lyme disease is caused by an infection with the spirochete *Borrelia burgdorferi*. Transmitted between mammal reservoirs by the bite of an *Ixodes* tick, the pathogen exists in a complex life cycle that requires long-term persistence in arthropod and mammal hosts. The mechanisms responsible for persistence and the pathogenesis of Lyme disease are not well understood, but may involve interactions between bacterial surface proteins and the host. Previous experiments have shown that differential gene expression of surface proteins assists the pathogen in adaptation and persistence in a new host. Most *B. burgdorferi* surface proteins have no homology to known proteins, making the identification of virulence factors difficult. Gene expression analyses can be used to identify potentially important gene products for further study, based on the conditions under which they are expressed.

To this end, the *B. burgdorferi in vivo* transcriptome of selected potential surface proteins was analyzed to identify promising targets for further study. Based on these analyses and other observations from the literature, the lipoproteins BbCRASP-2 and

BBK07 were selected for further characterization. My hypothesis is that these proteins are important for *B. burgdorferi* virulence and persistence in the murine host. The surface exposure of each protein was assessed, as well as a detailed transcriptional profile of each gene. Using specific antibody-mediated interference and gene inactivation, I show that neither BbCRASP-2 nor BBK07 is essential for infectivity or pathogenesis in the murine model of Lyme disease. My results also indicate that BBK07 is a novel immunodominant antigen of *B. burgdorferi* that could be used as a serodiagnostic marker for human Lyme disease. Using a peptide library, the most immunodominant epitopes of BBK07 were identified, and shown to improve the diagnostic accuracy over that of the full-length recombinant BBK07. Finally, I show that BBK07-based diagnosis was sensitive even in the early stages of Lyme disease, and that the addition of BBK07 epitopes to current serodiagnostic assays could improve their sensitivity.

NOVEL SURFACE PROTEINS IN THE PATHOGENESIS AND DIAGNOSIS OF LYME DISEASE

By

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Dedication

I dedicate this dissertation to my grandfather, Charles George Coleman, and my father, Steven Charles Coleman. You taught and modeled for me the initiative, thoughtfulness and faithfulness required of a scientist, a man, and a child of God.

And to my son, Jonathan Adam Coleman: I hope that I can one day provide an equally excellent example for you.

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Table of Contents

List of Abbreviations

µg	Microgram
µl	Microliter
°C	Degrees Celsius
bp	Base pair
BSA	Bovine serum albumin
BSK	Barbouer Stoener Kelly medium
cDNA	Complementary DNA
ml	Milliliter
ELISA	Enzyme-Linked Immunosorbent Assay
GST	Glutathione S-tranferase
HRPO	Horse raddish peroxidase
kDa	Kilodalton
LB	Luria Bertani medium
LD	Lyme disease
LB	Lyme borreliosis
MCS	Multiple cloning site
mm	Millimeter
mRNA	Messenger RNA
ORF	Open reading frame
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline + 0.05% Tween-20
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
rpm	Revolution per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline + 0.05% Tween-20

List of Tables

List of Figures

Chapter 1 - Introduction

1.1 History and distribution of Lyme disease

Lyme disease (LD), also known as Lyme borreliosis, is a potentially debilitating bacterial infection transmitted by ticks belonging to the *Ixodes scapularis* complex. It is named for the city in which it was first recognized, Old Lyme, Connecticut. Due to the unusual expanding skin lesion in patients and the tight geographic clustering of children suffering from arthritis, doctors at the Yale-New Haven Hospital identified LD as a distinct clinical entity in 1975 (Steere et al., 1977a). The causative agent of LD, the spirochete *Borrelia burgdorferi*, was cultured from infected ticks in 1982, and the same organism was isolated later from patients with LD (Burgdorfer et al., 1982; Steere et al., 1983). Symptoms resembling LD were previously reported in Europe (Afzelius, 1921; Bannwarth, 1941), and later studies showed that mouse specimens collected in the United States in 1894 contained *B. burgdorferi* DNA (Marshall, Telford et al. 1994). Recent phylogeographical analyses have suggested that *B. burgdorferi* was present in North America before the first European settlements (Hoen et al., 2009). As the tools of molecular biology and understanding of the disease has progressed, the wide geographic range of Lyme disease has become apparent, and it is now recognized as the most prevalent vector-borne disease in the United States, Europe, and parts of Asia (Steere et al., 2004).

The number of reported cases of LD continues to increase since the identification of the causative agent in the 1980's (Bacon et al., 2008). According to the Centers for Disease Control and Prevention (CDC), more than 20,000 new cases are reported annually in the United States, and in some counties the incidence is more than 500 cases per 100,000 individuals (Bacon et al., 2008). The actual number of cases may be higher due to misdiagnoses because of the variability of clinical symptoms (Steere et al., 2004). Most cases in the United States occur in the northeast, mid-Atlantic and north-central regions (Bacon et al., 2008). The forests of these regions are frequently inhabited by the *Ixodes* tick, the arthropod vector of *B. burgdorferi* (Dennis et al., 1998). It is thought that the recent expansion of LD is due to the expansion of the *Ixodes* tick into areas of farmland that have reverted to forests since the mid-20th century (Chen et al., 2005). LD remains an emerging infectious disease in many parts of the world (Piesman and Eisen, 2008), and a continued human health threat to suburban and rural populations in the Northeast and Midwest United States.

1.2 Clinical manifestations

LD is a multi-system disorder that affects a variety of organs (Nadelman and Wormser, 1998). Patients often experience a pathognomic skin rash at the start of the infection known as erythema migrans (Steere and Glickstein, 2004). Another commonly observed symptom of disease is acute Lyme arthritis, a painful inflammation of the joint that occurs in 60% of untreated patients (Steere and Glickstein, 2004). An infection of the heart causes Lyme carditis in 10% of untreated patients, which is a swelling of the

heart and associated tissues caused by the infiltration of neutrophils and macrophages (Duray, 1989). The complications of carditis, while rare, can be severe, including complete atrioventricular block, acute myopericarditis, cardiomyopathy, and cardiomegaly (Steere et al., 1980; Lelovas et al., 2008). Similarly, infection within the central nervous system can result in a complex neurological disorder called neuroborreliosis, reported in 3% of patients, that can be difficult to distinguish from more common disorders, leading some to conclude that it is underreported (Batinac et al., 2006).

Antibiotic treatment of LD is often successful in the early stages of disease (Steere and Glickstein, 2004). When diagnosis is missed or delayed symptoms can become chronic, and in some cases resistant to antibiotic treatment (Steere and Glickstein, 2004). Sensitive and specific diagnosis is a limiting factor in the improvement of LD treatment. Only tick bites can expose patients to *B. burgdorferi* infection, but they can go unnoticed. Up to 30% of patients do not display the erythema migrans rash indicative of LD, and instead require laboratory diagnosis to detect *B. burgdorferi* infection (Aguero-Rosenfeld et al., 2005). The CDC currently recommends two-tiered serological testing for these patients (CDC, 1995). If the patient's serum is positive or equivocal in the first, more sensitive test, then it is tested in a more specific test to confirm the diagnosis (CDC, 1995). The format of the first test is commonly an enzyme-linked immunosorbent assay (ELISA), while the second test is usually an immunoblotting assay (Aguero-Rosenfeld et al., 2005). While many antigens have been tested as serodiagnostic markers to detect *B. burgdorferi* antibodies in patients, to date

none has demonstrated the sensitivity and specificity required to replace two-tiered serological testing (Aguero-Rosenfeld et al., 2005). Research to improve diagnostic testing is ongoing, and the identification and characterization of new *B. burgdorferi* antigens is a central focus of LD research.

1.3 *Borrelia burgdorferi*

Lyme borreliosis is caused by many species of spirochetes from the genus *Borrelia*, broadly referred to as *Borrelia burgdorferi* sensu lato (Wang et al., 1997). *B. burgdorferi* sensu stricto, hereafter referred to as *B. burgdorferi*, constituting several well-characterized and closely-related isolates, is the only *B. burgdorferi* sensu lato species known to cause disease in North America, while *Borrelia garinii* and *Borrelia afzelii* cause Lyme borreliosis in Europe and Asia. In nature, *B. burgdorferi* exists primarily in rodent populations, transmitted between mammalians hosts by *Ixodes* ticks (Lane et al., 1991). When an infected tick bites a rodent or other incidental hosts, including humans, *B. burgdorferi* is deposited in the skin. The pathogen then disseminates throughout the host, preferentially colonizing the joint, heart, and skin tissues as well as central nervous system (Steere et al., 2004). Wild mice do not appear to develop disease during *B. burgdorferi* infection, but some incidental hosts and laboratory mice mount a vigorous immune response against the pathogen, causing the symptoms of LD (Wooten and Weis, 2001).

1.3.1 Genome

B. burgdorferi is an obligate parasite, highly evolved to exist in a specific ecological niche. As such, its genome is not closely related to any other bacterium outside of the *Borrelia* genus (Fraser et al., 1997a). The genome is highly segmented, containing a linear chromosome, as well as a large number of both linear and circular plasmid DNA elements (Casjens et al., 2000). The largest replicon in the *B. burgdorferi* is referred to as the chromosome, but several of the plasmids appear to harbor essential genes, blurring the line between chromosomal and extrachromosomal elements (Labandeira-Rey and Skare, 2001; Byram et al., 2004; Jewett et al., 2007). *B. burgdorferi* carries a relatively small genome of less than 2 million base pairs, which is less than half the genome size of *Escherichia coli* (Fraser et al., 1997a). The small genome size is possible, in part, by host-provided nutrition that negates the need for many biosynthetic pathways. *B. burgdorferi* lacks the ability to synthesize de novo all amino acids, nucleosides, and lipids, and relies on the host to provide these and other nutrients (Fraser et al., 1997a). While encoding relatively few biosynthetic enzymes, *B. burgdorferi* likely encodes for a sophisticated array of membrane transport systems that assist in acquisition of metabolites from the remarkably diverse host and vector environments. For example, compared to other bacteria, *B. burgdorferi* retains the largest percentage of phosphotransferase system permeases and encodes a large repertoire of peptide transporters (Saier and Paulsen, 2000). Additionally, the borrelial outer membrane contains many differentially regulated outer surface proteins, presumably necessary for interactions with the pathogen's vertebrate and arthropod hosts (Casjens et

al., 2000). Overall, the genomic structure shows *B. burgdorferi* to be a highly adapted bacterial pathogen.

The chromosome is highly conserved among the spirochetes of *B. burgdorferi* sensu lato (Terekhova et al., 2006). It is unique amongst bacteria, as it exists as a covalently closed linear DNA molecule, with each end containing hairpin telomeres (Casjens et al., 1997). Genes that encode proteins appear to cover 93% of the chromosome's sequence, 846 in all, with less than 11 psuedogenes among them (Fraser et al., 1997a). Many chromosomal genes are clustered by function, and are transcribed as operons. These operons include genes that are responsible for basic cellular functions, including DNA, RNA and protein synthesis, glycolysis, and cell wall synthesis (Fraser et al., 1997a). The tightly packed, highly organized, and essential nature of many of the genes present on the chromosome indicate that the chromosome may be an evolutionarily stable replicon (Lynch, 2006). *B. burgdorferi* relies on its chromosome to provide the bacterium with a stable set of the basic genes required for cell replication. The chromosome's stability stands in stark contrast to the plasmids of *B. burgdorferi*, relative hotbeds of recombination and evolution.

B. burgdorferi contains as many as 23 plasmids, more than any other known bacterium. There are at least 12 linear and 9 circular plasmids identified in the sequenced B31 M1 isolate (Fraser et al., 1997a), maintained at very low copy numbers, in the range of 1 or 2 copies per cell (Hinnebusch and Barbour, 1992). While the chromosome is highly conserved among *B. burgdorferi* sensu lato species, both plasmid content and

sequence varies widely among isolates (Terekhova et al., 2006). Plasmids show increased genetic rearrangement when compared to the chromosome, as there are numerous paralogous sequences, putative psuedogenes, and noncoding DNA sequences (Fraser et al., 1997a; Casjens et al., 2000). Bioinformatic analyses have shown that numerous recombination events have occurred among the plasmids, for reasons that are not yet understood (Casjens et al., 2000). Recombination events could result in gene duplication, allowing mutations to accumulate on genes due to reduced selective pressure. Only about 6 percent of genes located on plasmids have homologues outside the *Borrelia* genus. Due to plasmid loss during *B. burgdorferi* replication, several studies have linked certain plasmids to mouse and tick infectivity (Schwan et al., 1988; Xu et al., 1996; Purser and Norris, 2000; Stewart et al., 2005). In addition, purported virulence factors such as VlsE and OspC are located on plasmids (Zhang et al., 1997; Pal et al., 2004b). *B. burgdorferi* encodes a vast array of surface lipoproteins on plasmids, an estimated 17% of plasmid genes (Fraser et al., 1997a; Casjens et al., 2000). It appears that the more stable *B. burgdorferi* chromosome encodes most housekeeping functions, while the rapidly evolving plasmids are primarily responsible for host-pathogen and vector-pathogen interactions.

1.3.2 Proteome

The structure of *B. burgdorferi* is highly adapted to survive and replicate in both the tick vector and vertebrate host. The pathogen is a spirochete: a long, flat wave shaped bacterium with a distinctive diderm ultrastructure composed of an outer membrane

surrounding a peptidoglycan-covered inner membrane (Barbour and Hayes, 1986; Charon et al., 2009). Periplasmic flagella attach to each pole of the protoplasmic cylinder and wrap around the cylinder in a ribbon, imparting the flat wave morphology essential for motility (Motaleb et al., 2000; Charon et al., 2009). Rotation of the flagella results in a wave propagated along the axis of the cell, propelling the spirochete forward (Goldstein et al., 1994). This unique motility has been shown to be an order of magnitude faster than a neutrophil, the fastest cell in the human body, and allows movement through high viscosity fluids and penetration deep into tissues (Malawista and de Boisfleury Chevance, 2008).

The outer surface lacks lipopolysaccharide, unlike Gram-negative bacteria, and has a relatively low density of transmembrane proteins (Barbour and Hayes, 1986; Takayama et al., 1987). *B. burgdorferi* boasts an unusually large array of lipoproteins, many of which are known to be exposed on the bacterial surface (Haake, 2000). These proteins may serve to stabilize the outer membrane, in addition to other functional roles (Xu et al., 2008). Since research thus far has not been able to demonstrate major toxic molecules secreted by *B. burgdorferi*, it is thought that surface lipoproteins have a major contribution to spirochete persistence, interacting with a variety host molecules to facilitate dissemination, tissue colonization, and immune evasion (Zhang et al., 1997; Kraiczy et al., 2001; Coburn et al., 2005). Some lipoproteins have also been shown to be differentially expressed during the life cycle of the pathogen. These observations have provided clues as to the functional roles of several proteins, aiding in the identification of

several promising vaccine candidates (Howe et al., 1986; Fuchs et al., 1992; Hanson et al., 1998).

Surface lipoproteins are thought to be essential in several stages of the *B. burgdorferi* life cycle through interactions between the bacterial and host cell surfaces. The outer surface protein (Osp) family has been studied extensively for their contribution to virulence and persistence. OspA, a surface exposed lipoprotein and a major constituent of the outer surface, has been demonstrated to mediate attachment to and escape from the tick gut, among other roles, such as tissue dissemination via plasminogen interaction and immune evasion in tick blood meal (Fuchs et al., 1994; Coleman et al., 1997; Pal et al., 2004a; Battisti et al., 2008). Another lipoprotein, OspC, has been shown to facilitate spirochete invasion of tick salivary glands (Pal et al., 2004b; Fingerle et al., 2007) and establish murine infection (Grimm et al., 2004; Stewart et al., 2006; Tilly et al., 2007). OspC also binds the tick salivary protein Salp15, an immune suppressor, to blunt the initial host immune response against *B. burgdorferi* (Ramamoorthi et al., 2005). A substantial portion of the linear plasmid lp28-1 encodes the essential lipoprotein VlsE and its fifteen *vls* recombination cassettes (Casjens et al., 2000). During mammalian infection the sequence of the *vlsE* gene is altered by continual recombination with portions from randomly selected cassettes (Zhang et al., 1997; Coutte et al., 2009). By altering the sequence of this surface antigen, it is thought that *B. burgdorferi* continually generates an ineffective humoral response, thereby evading adaptive immunity (Zhang et al., 1997). Likewise, the surface protein Lmp1 has been shown to be essential in immune evasion, as *B. burgdorferi* isolates deficient in Lmp1 survive in immunodeficient, but not

in immunocompetent mice (Yang et al., 2009). A variety of lipoproteins cover the surface of *B. burgdorferi*, performing functions essential to the life cycle of the spirochete.

1.3.3 Gene expression

B. burgdorferi survives and proliferates in a number of diverse host environments. The unfed tick, the feeding tick, and the mammalian host each pose a new set of challenges to survival, including the host immune response, the available nutrients, and physiochemical parameters such as temperature and pH (Seshu et al., 2004; Clifton et al., 2006; Hyde et al., 2007). *B. burgdorferi* uses differential gene expression to adapt to these environments. Microarray analyses have indicated that global gene expression changes occur *in vitro* when *B. burgdorferi* are exposed to conditions that mimic different stages of the pathogen's life cycle (Ojaimi et al., 2002; Revel et al., 2002; Brooks et al., 2003; Tokarz et al., 2004). This has allowed researchers to more directly study gene regulation in the bacterium, albeit under artificial conditions. It is thought that the alternative sigma factors RpoS and RpoN are involved in the large gene expression changes that occur during the transition between arthropod and mammal hosts (Elias et al., 2000; Hubner et al., 2001). Few predicted transcriptional regulator proteins are encoded in the *B. burgdorferi* genome, including just two two-component systems (Fraser et al., 1997a). However, research has shown that the pathogen is responsive to a variety of unconventional stimuli, including the host immune response (Fikrig et al., 2009). The importance of gene regulation combined with the many *B. burgdorferi*

proteins with no known function suggest that novel gene regulation mechanisms could be at work. While the mechanisms of gene regulation are not fully understood, the diverse microenvironments in which *B. burgdorferi* thrives highlights the importance of gene regulation in the life cycle of the pathogen.

1.3.4 Genetic manipulation

Tools for the manipulation of *B. burgdorferi* at the DNA level began to appear with the seminal development of selectable markers and *B. burgdorferi* transformation in 1994 (Samuels et al., 1994). New DNA can be introduced to *B. burgdorferi* via shuttle vectors or allelic exchange. Shuttle vectors contain an origin of replication similar to that of *B. burgdorferi* plasmids, and can therefore be stably maintained in a *B. burgdorferi* isolate (Sartakova et al., 2000). Site-directed mutagenesis of *B. burgdorferi* has also been accomplished through the use of recombinant DNA transformation. Recombination events can be targeted by flanking a selectable marker on both sides with DNA sequences up- and downstream of the intended site. During replication, a *B. burgdorferi* cell may use the recombinant DNA as a template and introduce new DNA into the genome of the cell via homologous recombination (Rosa et al., 2005). Some recent studies have utilized transposon-mediated mutagenesis, useful in genome-wide screening, but this technique is not yet widely used in the mutagenesis of infectious isolates (Stewart et al., 2004; Botkin et al., 2006).

Shuttle vectors have been used in many experiments (Rosa et al., 2005), including the expression of foreign genes (Sartakova et al., 2000), restoration of a wild type copy of an inactivated gene (Hubner et al., 2001), overexpression of a particular gene (Strother et al., 2007), or the elimination of entire plasmids by introducing an incompatible origin of replication (Eggers et al., 2002). Inserting DNA via allelic exchange is another powerful tool, as it can provide an opportunity to modify or delete existing DNA sequences. Homologous recombination has been used to inactivate genes (Yang et al., 2004), restore wild type copies of a gene (Revel et al., 2005), and insert inducible promoters to allow control of gene expression (Lenhart and Akins, 2010). These studies are essential for the elucidation of individual gene function and will lead to a greater understanding of *B. burgdorferi* biology. Overall, recently developed tools allow the genetic manipulation of *B. burgdorferi*, and may eventually map the essential genes for *B. burgdorferi* persistence and the pathogenesis of LD.

1.4 Experimental mouse model

Several animal models of LD have been developed, including the use of mice, rats, hamsters, guinea pigs, rabbits and nonhuman primates (Philipp and Johnson, 1994). *B. burgdorferi* infection of C3H HeN mice constitutes a widely used experimental model for understanding LD pathogenesis. It has been shown that all tested mice are susceptible to *B. burgdorferi* infection, but many develop only mild symptoms (Donahue et al., 1987; Barthold et al., 1990). C3H mice have a mutation in Toll-like receptor 4, and are used because of the severe and repeatable disease caused by *B. burgdorferi* infection (Barthold

et al., 1990). These symptoms can mimic those seen in human patients, whether the infection begins via tick bite or syringe inoculation of *in vitro*-grown *B. burgdorferi*. Within the first week, the mice experience a mild bacteraemia with subsequent tissue colonization. The clinical complications of arthritis, and carditis are apparent within a few weeks and spontaneously resolved after several months (Barthold et al., 1990). Tibiotarsal joint diameter is an effective means to quantify arthritis, and histochemical staining of joint and heart sections can be used to show the extend of inflammation (Pal et al., 2004b). Quantitative polymerase chain reaction using infected mouse tissues is often used to measure the pathogen burden in particular tissues (Germer et al., 1999; Piesman et al., 2001). Studies in mice have drastically enhanced our knowledge of *B. burgdorferi*, and were crucial in the development of the only Food and Drug Administration-approved human vaccine, as well as some diagnostic assays for LD (de Silva et al., 1996; Liang et al., 1999).

1.5 Research objectives

The main goal of this dissertation research is to better understand the pathogenesis and prevention of *B. burgdorferi* infection through the characterization of important surface proteins of unknown function. Many of the unique abilities of the pathogen are likely imparted by functional surface proteins. Although many such proteins are predicted by genomic sequence analysis, the roles of most surface proteins remain a mystery. I believe that the techniques of molecular biology can be used to identify and study several promising surface proteins and their potential contributions to

virulence and bacterial persistence. Previous studies using cultured spirochetes have contributed much to our understanding of *B. burgdorferi*, but the microenvironments encountered by the pathogen *in vivo* are likely too complicated to be accurately reproduced *in vitro*. Direct analyses of the *in vivo* transcriptome of *B. burgdorferi* will identify possible surface proteins that are transcribed during mammalian infection. My major goals were to study the contribution of these surface proteins to microbial persistence and virulence. To accomplish this, I used gene deletion and specific antibody-mediated interference in the laboratory model of LD. Finally, the diagnostic utility of these proteins was assessed. My overall hypothesis is that these proteins are important molecular tools of *B. burgdorferi*, and that their study will enhance our understanding of the pathogen and could lead to new therapeutic or diagnostic applications.

Chapter 2 – *In vivo* gene expression analysis

Abstract

Borrelia burgdorferi, the bacterial pathogen of Lyme disease, differentially expresses select genes *in vivo*, likely contributing to microbial persistence and disease. The complex life cycle in which *B. burgdorferi* persists requires continuous adaptation to the changing environments of the arthropod vector and the mammal reservoir. Studying the genes expressed in each environment can give an indication of the role of a particular gene product during the life cycle of *B. burgdorferi*. Research on *B. burgdorferi* has failed thus far to identify major toxic molecules secreted by the pathogen. This led me to theorize that important interactions that contribute to the development of LD may occur between host molecules and the proteins expressed on the *B. burgdorferi* cell surface. To this end, I used a quantitative RT-PCR approach to compare a select portion spirochete transcriptomes in a variety of infected mouse tissues. The transcription levels of genes encoding potential surface exposed proteins were assessed to identify genes that are expressed or induced during mammalian infection. My results show that only a portion of these genes are transcribed at detectable levels, and that while many of these genes are transcribed at consistent levels, some genes are selectively induced in a tissue-specific manner.

2.1 Introduction

Lyme disease (LD), caused by *Borrelia burgdorferi* sensu lato, is the most prevalent tick-borne human disease in the United States, Europe and many parts of Asia (Steere et al., 2004). Once the pathogen is deposited in the mammalian dermis by feeding *Ixodes* ticks, it establishes a localized infection at the bite site, then disseminates to distant cutaneous sites and various internal organs, including the spleen, bladder, joints, heart and central nervous system (Barthold et al., 1990; Barthold et al., 1991; Steere et al., 2004). While *B. burgdorferi* persists in several tissue locations in mammals, only a limited set of organs, most frequently the joints and the heart, experience robust host inflammatory responses resulting in clinical complications, such as Lyme arthritis and carditis. Antibiotic treatment is usually, but not always, successful, and some patients develop a form of antibiotic-resistant arthritis that is thought to be unrelated to persistent infection (Radolf, 2005).

The *B. burgdorferi* transcriptome undergoes dynamic changes during the complex enzootic cycle of the spirochetes (Liang et al., 2002; Narasimhan et al., 2002; Pal et al., 2008a; Pal et al., 2008b). *B. burgdorferi* grown in laboratory medium or within host-implanted dialysis membrane chambers readily responds to altered environments, adapting to changes in temperature, pH, nutrients, and host immune responses (Schwan et al., 1995; Carroll et al., 1999; Narasimhan et al., 2002; Ojaimi et al., 2002; Revel et al., 2002; Brooks et al., 2003; Tokarz et al., 2004; Fisher et al., 2005). A significant fraction

of the *B. burgdorferi* genome (8.6%), or 150 genes, could be differentially expressed *in vitro* in response to physiochemical alterations in growth conditions, and a major proportion of these genes (46%) encode proteins with predicted export signals (Revel et al., 2002). However, while all *B. burgdorferi* lipoproteins have outer membrane export signals, some are retained in the periplasm by sequence-specific signals (Schulze and Zückert, 2006).

The clinical complications of LD are primarily triggered by *B. burgdorferi*-induced host inflammatory responses (Nadelman and Wormser, 1998; Steere and Glickstein, 2004; Connolly and Benach, 2005). Although spirochetes colonize a wide variety of host tissues, the inflammatory response that results in pathology is observed in a limited set of host organs, most commonly in one or both mouse ankles or human knees and the heart. The diversity of host niches likely influences spirochete gene expression. While microbial antigens that are expressed at higher levels in a tissue-specific manner may assist in *B. burgdorferi* persistence in local environments, antigens, especially those exposed on the microbial surface, could directly participate in host-pathogen interactions contributing to the genesis of organ-specific pathogenesis. Therefore, I assessed the expression levels of a selected set of *B. burgdorferi* genes in diverse murine tissues because of their putative membrane localization. The characterization of microbial ligands that are differentially expressed during the pathogen's life cycle is important for the identification of novel vaccine targets and the prevention of the multi-system disorders caused by *B. burgdorferi*.

2.2 Materials and methods

The identity and oligonucleotide primer sequences for the quantitative RT-PCR analysis of *B. burgdorferi* genes are indicated in Table 1. The *B. burgdorferi* target genes (Fraser et al., 1997a; Casjens et al., 2000) were selected based on their predicted localization on the spirochete membrane according to the database annotation (www.tigr.org) and PSORT *in silico* analysis (Nakai and Horton, 2007). Groups of mice (5 animals/group) were infected with *B. burgdorferi* (10^5 spirochetes/mouse), and samples of skin, heart, tibiotarsal joint and bladder were collected and frozen in liquid nitrogen at one-week intervals between 1 and 4 weeks of infection. Total RNA was extracted from tissue samples using the TRIzol reagent (Invitrogen). To reduce traces of contaminating DNA, samples were further digested with RNase-free DNaseI (Qiagen), purified using the RNeasy kit (Qiagen) and reverse transcribed to cDNA using the AffinityScript cDNA synthesis kit (Stratagene).

The relative levels of *B. burgdorferi* cDNA in each sample was assessed by quantitative PCR (qPCR), and DNA contamination in each sample was measured using an equal volume of purified RNA as a template. Samples from each time point were pooled by tissue type, and final pools of skin, heart, joints and bladder were used in the qPCR analysis. The primers used for qPCR reaction were designed using OligoPerfect Primer design software (Invitrogen) based on the *B. burgdorferi* B31 M1 genomic sequence (Fraser et al., 1997a; Casjens et al., 2000). All PCR primer pairs had a similar

annealing temperature (60ºC) and spanned 100-300 base pairs of each of the target *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 one case, where paralogous genes displayed extreme DNA homology, the same set of primers was assigned for the detection of both genes as indicated in Table 1.

To generate reliable *in vivo* gene expression data and to further ensure specific amplification of *B. burgdorferi* cDNA in murine tissue samples, the qPCR amplification in each well was followed by melt-curve analysis, and wells showing non-specific amplification were discarded from data analysis. The amplification cycle consisted of initial denaturation at 95°C for 5 min followed by 45 cycles each at 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec and final melt curve analysis: 55°C for 30 sec, increase 0.5°C per cycle to 95°C. The amplification was performed in an iQ5 real-time thermal cycler (Bio-Rad) using SYBR Green Supermix (Bio-Rad). For expression screening of *B. burgdorferi* genes, I simultaneously assayed 8 candidate genes in each 96-well PCR plate using duplicate wells of template cDNA (skin, heart, joint and bladder samples) with parallel positive (*B. burgdorferi* genomic DNA) and negative (no template) controls. Transcript levels of individual genes were assessed in spirochetes grown *in vitro* in BSK medium (10^7 cells/ml) and in each of the murine samples, calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), normalized against *flaB* transcripts and presented as fold increase in gene expression. Two independent mouse experiments used the same parameters of gene expression analysis to ensure the reproducibility of the assay.

2.3 Results

B. burgdorferi persists in diverse tissue environments of the mammalian host. To identify *B. burgdorferi* genes that are expressed at high levels *in vivo*, particularly in a tissue-specific manner, I employed a sensitive quantitative RT-PCR (qRT-PCR) approach to compare spirochete transcriptomes in multiple murine tissues and *in vitro*. A total of 92 spirochete genes were selected for expression analysis, based on their putative association with the spirochete membrane as determined by the database annotation and *in silico* analyses for extracellular exposure (Table 1). Two groups of C3H/HeN mice (5 animals/group) were challenged with *B. burgdorferi* (10^5 cells/mouse) and skin, joints, heart and bladder tissue were collected following 1, 2, 3 and 4 weeks of infection. Total RNA was isolated, and corresponding tissues from the indicated time points were combined into four separate pools of skin, joint, heart and bladder samples. qRT-PCR analysis was performed using gene-specific primers. Analysis of qRT-PCR data revealed that 44 *B. burgdorferi* genes (out of 92 assessed) were not transcribed at detectable levels *in vivo*. The remaining 48 genes displayed variable expression across different tissues, presented as fold increase in transcript levels relative to *flaB,* together with corresponding *in vitro* expression levels (Fig. 1). *B. burgdorferi bb0210,* annotated as surface-located membrane protein 1 (*lmp1*), which encodes an exported protein with type I signal peptide with unknown function (Fraser et al., 1997a), displayed the most dramatic differential expression in murine tissues, with the highest level of expression found in the heart (Fig. 1).

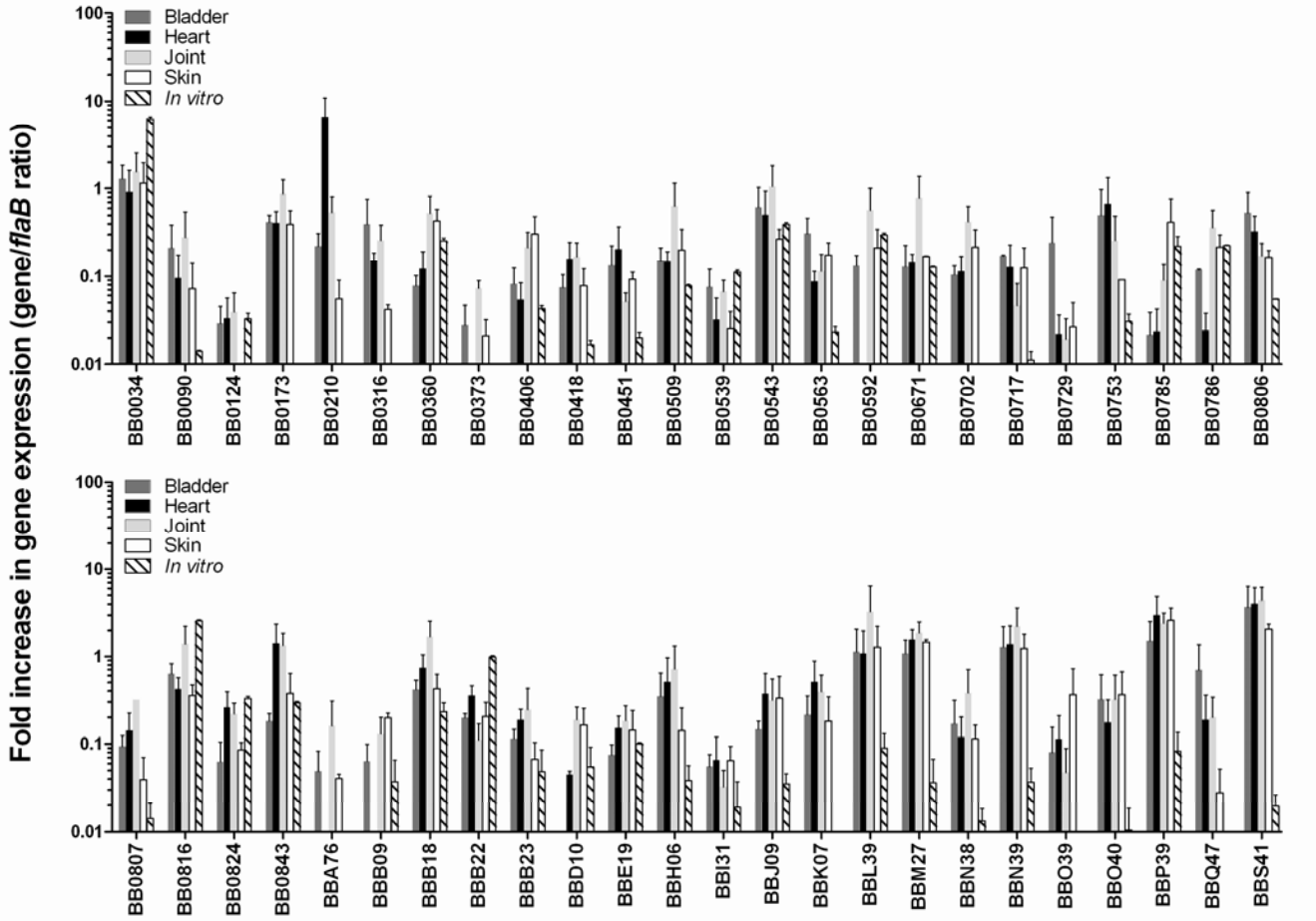

Figure 1. Relative expression of selected *B. burgdorferi* genes. Total RNA was isolated from *B. burgdorferi* grown *in vitro*, and multiple tissues of infected mice. RNA samples were pooled by tissue type and gene-specific transcripts were measured using qRT-PCR. Fold increase in the expression of individual genes in each sample was calculated based on the $2^{-\Delta\Delta Ct}$ method, normalized against *flaB* values. Bars represent the mean ± SEM from four qPCR analyses of two independent infection experiments.

Table 1. Oligonucleotide primers used in Chapter 2.

ORF	Putative Function or Gene Symbol	Sequence (5′ → 3′: Forward, Reverse)
BB0006	CHP	TTGTTTGGGCAGTTCTTTCC, TCTTTTCCCCTGCATTTTTG
BB0017	CHP	GGATTTGGCAATCAAAAAGC, CCAAGCACCTTTTCCGTCTA
BB0034	HP	GGGATGAGTGCTGGAGAAAA, TCCTTGAGCAAAGGAGCCTA
BB0037	plsC	GGGAAATCATATTGCGGCTA, TCGGGGAAAATTCCAATAGA
BB0051	CHP	TTTGGAAAATCTGGGCTTTTT, ACCACGGATTTTGTTGCTTC
BB0073	HP	TCAAACGATTTCATGCTTTCA, TTGATTTTGGGGTTTTTATCA
BB0090	V-type ATPase	CAGCAATAGGTTCTGCGTTG, AGCAATAGCAAACCCACCAC
BB0124	HP	TTTTGTTGCGTTTTTAAACATATTG, CGACCCCAAAATAGGAAACA
BB0157	HP	TAACCAGCGCCCTTGAAATA, TCCAATTATTTGCTGATTGCTTT
BB0164	Na+/Ca+ Exchange	AGACATTGCATTTGGCAACA, AAATGCCCCTACCAAAAACC
BB0165	HP	CACGCCCTTTGGAATAAAAA, TCAACGGGTTGCATCTCATA
BB0170	HP	AAACCGATTCCTGGAGAGGT, TGGAAAATTCCCTTGACCTG
BB0173	HP	TCGCTTTACGGCAATTTCAACTC, ACAATGTCAGCACCAGCACTAAGA
BB0193	LP	CGAAGACCTTGAATACTTGGAAA, AAATCAGGCTCTTTAAGGGTGA
BB0202	hemolysin	AAAAGGGGAAATCGGGAATA, TGATATTGCAAGTGCGCTGT
BB0210	lmp1	AATGACGCTCAGGCCTCTAA, TTGTTGGCGTGATTTTGTGT
BB0213	LP	TTTGCTTGTTCTTGCGACAT, TCCTTTCCAAGGCTAGAATCA
BB0234	CHP	GGCAACGGTTCAAGTGAATTA, TGAAAAATCCCCCAACAAAA
BB0303	mraY	AGGGCTGATTTTGCAGCTTA, AAAAACACCCGCAAGAATTG
BB0309	HP	GGCGAATTTACATCAAATGCT, GAGCCTGAGGCATTTCAATC
BB0311	CHP	TACGCCTATTTCTCCACATTCT, ATTCAAAAACAACGTCAACTCC
BB0316	CHP	TTGGGCGAGAAAATAAGCAGT, GCAAATAGCAAGCCAACCA
BB0360	HP	CAAAGCCTTTACAGAGCAGGA, GGCCATGAAAAATTCAGGTG
BB0361	ylxH-2	CTGGCAAACGAAGGAAAAAG, AAAAGCTGTTCCTGCTCCAA
BB0373	HP	GGGTGAAATATTGTGAGGCTCT, TGCTTTCAAGCAAATCAAAAGA
BB0378	HP	GGATGATCAAGCATATTTGAAGC,

		ACCCAATCACGCGAATCTAC
BB0398	LP	TCAAAGCTGGGATCAAAAGG, ACACGCCTATCCAAGTCCA
BB0406	HP	CGGATTGGTCAAAAACATCC, GTTGGCCCTACTGCAGGATA
BB0412	HP	GCCAAAACCACGGAATTCTT, GATTGGGAGAAAGGCATAAGG
BB0418	HP	AAAAACAAAGGCTGGGGAAG, CCAGTCCGGCTGTGTTACTT
BB0451	chromate transport	TTAAATTTCGGAGGCGGTAA, GCCCAGGGGTTATTCTTGAT
BB0452	HP	CCGCTCAACAACAAAATGAA, CCTCCGCCAATTGTAAGTGT
BB0460	LP	GATGCAAAAATTCCTTTTATGG, TTTTGAGTTTTCGGGGTTTG
BB0473	CHP	TTTGTCAGCGCTTTCACTTG, CTCCCCTTTTACACCCAACA
BB0475	LP	CTTGCATGAAAACAAGCACAA, TTGCCAATGAATTTGCATTTA
BB0509	HP	TTGCTGATGGTGCAATGAAT, TCGAATCCGCTACCCATTTA
BB0527	CHP	GGAACTGCTTGCACCATTTT, TTGGAGTGCTAATGGGGAAT
BB0532	HP	GCTCAACATTGGGCCTTAAA, TTTGAACATTGAATCGTGAGAA
BB0539	CHP	ATGGGCTTATGGGGAATCAT, GCTACAACCGCCATTCTGTT
BB0543	HP	GTTCGGATGGAACTGCAAAT, CCATTGTTCCCAGTTCTGCT
BB0563	HP	GGCAGTAAGTCAGGCTCAGG, TCCTGCGAATACTTCCCATC
BB0584	CHP	TCTGCCTTTTTCATGGCTCT, CAAGTTTGATTGCGTCCTCA
BB0592	HP	TTGCCAGAATCGGTACTTGTC, ATGGCGGTAGCTACAACAGG
BB0628	CHP, LP	CCCAATGTCTGCCAAAGAAT, ATCCATCTCAAACCGCAGTG
BB0638	nhaC-2	TATCCGGTGCTTTTTCTGCT, ATGGAAGGGGGATGTTTACC
BB0671	cheX	GCATGCCAAGGGTTTTGTAT, CCATCAGGCAAAGAAAAAGG
BB0674	HP	AGGCCTACCTTTCCCAAAGA, TATTGTTTCATGGGCGACAA
BB0702	kdtB	GCGGGTTTATTGTTGATTATGC, TTCCTTTACAAAATCCGACCTT
BB0708	HP	GTTCGTGTTTTTGGCTGGTT, CCAGAAAGAAAAATTTTAAAAACAA
BB0717	CHP	ATTTCCAGCGCATTTTTAGG, AAGCATATTTTTGGGCATGAA
BB0729	gltP	TGCTGCAACCATACCCATTA, GTTCATTGCTGAGAGCACCA
BB0746	oppC-2	TGGAACAATCATTGGCATGT, TTTGCTTGCTCCCATAGCTT
BB0753	MSP	TGTTGGTACTGCCAGCACTT, ATCAAATTCGCCCATGAAAA

BB0759	HP	ATAATCCCAGGGGTTTCTGG, TTGAGGTTAACATTCCTGTTGC
BB0766	colicin V production	GCCTGAGATTGCCTCAAGAC, GTGCATTAAAGGGGCGTAAA
BB0785	sporulation protein G	GCATATGTTGCAGTTACTTTTGA, TCAAGATCGGCTGGATTTTC
BB0786	ctc	GGGACGTCGACAAGTGGTAA, CCTTACCTTGCCCGTAAACA
BB0806	HP, LP	CATTAGGGGTTTTGCGGATT, TCAGCATTACCGGGAACATT
BB0807	CHP	CCACTTGGATCTATTGGCAGA, CAAAACCCTTAAGCCCAGTG
BB0816	HP	TGCTCATTGCAGAGAAATGC, CCTGCCTGGTTGAAACATCT
BB0824	HP	CCAGAGCAGATCATGGGATT, CCCAATTAATGGGAAAAGCA
BB0843	CHP	TTGGTGGATGCAAGAAATGA, AACCTTTCACAAACGCATCC
BBA74	oms28	GCTGTTTCTGTTGCTGGTGA, ATCTCTTGCGCCTTGAGCTA
BBA76	thy1	CATTGGAGCAGGTGGTTTTT, TTCCTCTCTTGCCAAGCTGT
BBB04	celB	ACCTTACGGTGGAGATGCTG, TTTAAAACAGCAGGCGGAAC
BBB09	HP, LP	CGCTCCCTCTGAAACTTACG, TCACAGGAGGCTCCATTTTT
BBB18	guaA	GGCCTACAATTCCATCCAGA, CTGTGCCACCAGAAAGTCCT
BBB22	HP	CAGTGGCAGCAAAAGGAAAT, TGTATGCGGTTACGGTTGAA
BBB23	CHP	CAACAAAAGGTGGCATGTTG, TTTTCCACCCTCAGCAATTC
BBD10	HP, LP	TTTGAGGCTAAAGGAGAGTTGG, CATTTCCCAAGGCAACAAAT
BBD15	HP, family 85, LP	CAAGATAGATGGGGCTTCTCA, TTTGAAACATTAGATACTCTGGAAGAA
BBE08	HP, LP	TTATTTTTGTGTTGATAAGTTCTTGC, GCATAAAAATACCTTTGCTTCTTTTT
BBE19	plasmid partition	AAAGGAGGTGTTGGCAAAAG, GGTTGATGCTTGTGGATCG
BBG02	CHP, LP	CACTTGGCAAACTTGGAACA, AGGGCATTTCTCTGCCTTTT
BBH06	HP	TGCCAGACATGTTGCTGATT, CCCCCTCAAGTTCTACAGCA
BBI26	drug transport	GGACTTTGCCACATGCTGTA, GTCAATCGTGCCAGTGCTAA
BBI31	CHP	TATATTGGTGGTGGCGCAGT, GTGAGGCTATGTGGGATGCT
BBJ09	OspD, LP	AATGAAGGCGCAAATTCAAA, TTGCCTCATTTGATGCATTT
BBK48	P37, LP	CGCCGATCAGGTTATAGACAA, CATATCGGGTTGCATGTCTG
BBK07	HP, LP	CCTATTTCAAGGGCGTGAGC, TATGGCCATTGCTGCATTCT
BBL39	ErpA8, LP	GGGACATTCGGCTACATTCT,

		CCCCCGCACTGTTATTAATTT
BBM27/P27	Rev, LP	AAATGATTCTTCTGGAGGCAAA, TTGGCCGCTAATTTATCCTG
BBN38	ErpA, LP	GTTTAAACGCTGGGGGACAT, ACGCAATATGTTCAGCACCA
BBN39	erpB2, LP	GTGCTGTTTTTGCGCTGATA, TCGCCCTGCATTAATTCTTC
BBO39	ErpL, LP	GCAAAGAAAAAGGGGGAGAG, TCACTGCCGCCATTAGAATA
BBO40	ErpM, LP	GCAGGGCGATGATCCTAATA, GCTTTTGACTTTGCTTCTCCA
BBP38	ErpA, LP	TGATGAGCAAAGCAATGGAG, GAATGTCCCCCAGCGTTTA
BBQ05	antigen, P35, LP	GACACCCCGCAATCTAAAAA, AAATGCCTCCGAATCTGTTG
BBQ46	HP	GATTAAAAATGTAATTTATATTTTACC, CTATCCTACAATCCAAATTTTG
BBQ47	ErpX, LP	GCAAGATTGATGCAACTGGT, TTTTTGCCAATTCATCTGCT
BBS41	OspG, LP	CTTGCAAGATTGATGCGAGT, GGGTGTGTTATCGTGGGAAC
BBS42	bapA	TTGGACGCTCTTGAGGCTAT, GCAAATCAGCCAAAATTTGTT

Abbreviations: CHP, conserved hypothetical protein; HP, hypothetical protein; LP, lipoprotein; IMP, inner membrane protein; MSP, membrane-spanning protein. Designations of the open reading frame (ORF), gene functions and symbols are according to the annotations in the database (www.tigr.org).

2.4 Discussion

My overall goal is a better understanding of the *Borrelia burgdorferi* infection that causes LD. SYBR Green qPCR has been used previously to study *B. burgdorferi* gene expression in infected mouse and tick tissues (Morrison et al., 1998). In order to more efficiently target genes that encode surface-exposed proteins, I used a combination of database annotation and *in silico* analyses to select genes with possible surface localization (Nakai and Horton, 2007). The study of gene expression at the level of transcription is by no means a perfect tool to assess the role of a gene-product in microbial pathogenesis. For example, some genes associated with *B. burgdorferi* motility, an essential trait for infection and dissemination, appear to be constitutively expressed, and thus not necessarily carry specialized function to adapt spirochetes in specific host tissues. However, if the pathogen has evolved to spend the energy required to preferentially upregulate a specific gene in a certain situation; it stands to reason that the protein expressed could play an important role in bacterial persistence.

Of the screened genes, two were selected as promising for future study, based on additional evidence from the literature. The first, *bbh06*, also known as *Borrelia burgdorferi* Complement Regulator-Acquiring Surface Protein 2 (*BbCRASP-2*), was among the transcriptionally active genes. Previous studies have implicated BbCRASP-2 in immune evasion by enhancing resistance to complement-mediated lysis (Hartmann et al., 2006; Rogers and Marconi, 2007). The second, *bbk07*, also appeared to be

transcribed *in vivo*. While previous work had demonstrated the immunogenicity of BBK07, the expression profile and functional role of the gene were unknown (Barbour et al., 2008). The evidence from previous research, combined with the measured transcriptional profile led me to begin detailed studies of both genes for their contribution to persistence and genesis of disease. As both are potential surface proteins, I therefore also evaluated the use of each as novel serodiagnostic markers for LD.

In conclusion, these gene expression analyses of potential surface proteins have identified more than 40 genes that are transcribed during murine infection, including some that are induced in a tissue-specific manner. These data also map the effects of tissue microenvironments on transcription, as each gene was assessed by tissue type. The further characterization of microbial ligands that are differentially expressed during the pathogen's life cycle will improve our understanding of this clinically important pathogen.

A portion of this chapter has been published previously:

Yang X, Coleman AS, Anguita J, Pal U (2009) A Chromosomally Encoded Virulence Factor Protects the Lyme Disease Pathogen against Host-Adaptive Immunity. PLoS Pathog 5(3): e1000326. doi:10.1371/journal.ppat.1000326

Chapter 3: The redundant role of BbCRASP-2 in infectivity and complement resistance

Abstract

Borrelia burgdorferi, the pathogen of Lyme disease, cycles in nature through *Ixodes* ticks and mammalian hosts. At least five Complement Regulator-Acquiring Surface Proteins (BbCRASPs) are produced by *B. burgdorferi*, which are thought to assist spirochetes in host immune evasion. Recent studies established that BbCRASP-2 is preferentially expressed in mammals, and elicits a robust antibody response in infected hosts, including humans. I show that *BbCRASP-2* is ubiquitously expressed in diverse murine tissues, but not in ticks, reinforcing a role of BbCRASP-2 in conferring *B. burgdorferi* defense against persistent host immune threats, such as complement. BbCRASP-2 immunization, however, fails to protect mice from *B. burgdorferi* infection and does not modify disease, as reflected by the development of arthritis. An infectious *BbCRASP-2* mutant was generated, therefore, to examine the precise role of the gene product in spirochete infectivity. Similar to the wild type *B. burgdorferi, BbCRASP-2* mutants remain insensitive to serum complement-mediated killing *in vitro*, retain full murine infectivity, and induce arthritis. Quantitative RT-PCR assessment indicates that survivability of BbCRASP-2-deficient *B. burgdorferi* is not due to altered expression of other *BbCRASP*s. Together, these results suggest that the function of a selectively expressed spirochete gene, *BbCRASP-2,* is dispensable for *B. burgdorferi* infectivity in the murine host.

3.1 Introduction

Borrelia burgdorferi is the causative agent of Lyme disease (LD), the most prevalent vector-borne disease in the United States and Europe (Orloski et al., 2000; Steere et al., 2004). In nature, *B. burgdorferi* cycles between rodent reservoirs and *Ixodes scapularis* ticks. This complex enzootic life cycle requires successful colonization and coordinated transmission between strikingly different host and vector environments. It is thought that differential gene expression plays an important role in allowing the spirochete to navigate the transitions between hosts and in establishing persistent infection (de Silva and Fikrig, 1997; Schwan and Piesman, 2002; Rosa, 2005; Neelakanta et al., 2007). Due to the availability of excellent murine models of Lyme disease, *B. burgdorferi* gene expression through the tick-rodent transmission cycle can be examined in the laboratory (Barthold et al., 1990; Schaible et al., 1991; Simon et al., 1991; Barthold, 1992; Barthold et al., 1993). These efforts may provide important clues for understanding functions of microbial gene products that support *B. burgdorferi* persistence in nature (de Silva and Fikrig, 1997; Schwan and Piesman, 2002; Grimm et al., 2004; Rosa, 2005; Neelakanta et al., 2007; Pal et al., 2008b).

The genes encoding the Complement Regulator-Acquiring Surface Proteins (BbCRASPs) of *B. burgdorferi* are differentially expressed in the pathogen life cycle (Hefty et al., 2001; Bykowski et al., 2007a). As many as five BbCRASPs were identified that bind host proteins of the factor H (FH) family, and possibly contribute to the

spirochete defense against host complement-mediating killing (Alitalo et al., 2001; McDowell et al., 2003; Hartmann et al., 2006). *BbCRASP-1* (also known as *cspA* or *bba68*) and *BbCRASP-2* (also called *cspZ* or *bbh06*), located on linear plasmids lp54 and lp28-3 respectively, share little sequence homology with other *BbCRASP* sequences. In contrast, *BbCRASP-3*, *-4* and *-5* have similar sequences and belong to the *erp* paralog family and are known as *erpP* (*bbn38*), *erpC* and *erpA* (*bbp38* and *bbl39*), respectively. Collectively these *erp* genes are also known as *ospE*, and are encoded on multiple cp32 plasmids (Fraser et al., 1997a; Casjens et al., 2000; Kraiczy et al., 2004; Rogers and Marconi, 2007). The gene *erpC* (located on cp32-2) and one of the three *erpA* genes (located on cp32-5) currently lack TIGR database annotations, as the sequenced B31 M1 isolate lost these plasmids. Of all the *BbCRASP* genes, *BbCRASP-2* is the only gene without paralogous family members, and is therefore unique in *B. burgdorferi* (Hartmann et al., 2006).

Evasion of host complement is especially important for *B. burgdorferi*, as it establishes an extracellular and disseminated infection in many tissue environments where the complement system is readily available through host vasculature or body fluids. The complement system includes soluble membrane binding proteins which, upon contact with foreign cells, become activated, and are then capable of direct chemical lysis via membrane disruption (Medzhitov and Janeway, 2000; Medzhitov and Janeway, 2002). Specific regulatory proteins, such as FH family proteins, protect the host from self-inflicted damage by preventing unwarranted complement activation. Pathogens such as *Candida albicans*, *Neisseria meningiditis*, and *Streptococcus pneumonia* have been

shown to bind host FH, and that FH binding in *N. gonorrhoeae* and *B. burgdorferi* provides protection against complement killing *in vitro* (Ram et al., 1998; Ram et al., 1999; Jarva et al., 2002; Meri et al., 2002; Brooks et al., 2005).

BbCRASPs have been identified according to their ability to bind proteins of the FH family, although individual BbCRASPs vary in their affinities for particular FH family proteins. For example, only BbCRASP-1 and -2 preferentially bind factor H-like protein (FHL-1), while BbCRASP-3, -4 and -5 selectively bind factor H-related protein (FHR-1) (Hartmann et al., 2006; Haupt et al., 2007; Haupt et al., 2008). BbCRASPs also vary in their interaction with uncharacterized serum proteins (Hovis et al., 2006; Bykowski et al., 2007a). Though the binding affinities and the expression profiles of the BbCRASPs have been studied, the independent role of each BbCRASP in *B. burgdorferi* infectivity is not clear. Recently studies using a non-infectious mutant demonstrated that the loss of BbCRASP-1 sensitized the *B. burgdorferi* to complement-mediated lysis in human serum, an effect that can be rescued with gene complementation (Brooks et al., 2005). While there is some disagreement as to the expression of BbCRASP-1 during mammalian infection, RT-PCR analysis indicate that it is only expressed transiently at the tick bite site and in ticks (Bykowski et al., 2007a), but not expressed in mice (McDowell et al., 2006). BbCRASP-1 therefore, may not play an essential role in mammalian infection (McDowell et al., 2006), but could be important in spirochete survival in feeding ticks. Although the above set of studies suggest an important role for BbCRASPs in spirochete immune evasion, the precise role of individual BbCRASPs, or their orchestrated role in the *B. burgdorferi* infection cycle is not clear, largely because

infectious BbCRASP-deficient *B. burgdorferi* have not yet been successfully generated (Bykowski et al., 2007a).

BbCRASP-2 is expressed by *B. burgdorferi* during murine infection (Bykowski et al., 2007a; Bykowski et al., 2007b), and infected hosts, including human patients, readily generate BbCRASP-2-specific antibodies (Hartmann et al., 2006; Kraiczy et al., 2008). This protein is conserved among *B. burgdorferi* isolates (Rogers and Marconi, 2007), reported to be localized on the spirochete surface (Hartmann et al., 2006) and has recently been suggested as a possible target for a second generation Lyme disease vaccine (Hartmann et al., 2006; Kraiczy et al., 2008). The previous studies also suggest a possible functional role for BbCRASP-2 in immune evasion and pathogen survival (Hartmann et al., 2006; Rogers and Marconi, 2007). In order to test this hypothesis, I sought to determine whether BbCRASP-2 is consistently produced in diverse murine tissues throughout the infection, and whether BbCRASP-2 immunization could provide host immunity and influence disease outcome. To explore the precise role of BbCRASP-2 in *B. burgdorferi* infectivity of a mammalian host, I assessed how targeted deletion of *BbCRASP-2* in an infectious isolate influences *B. burgdorferi* infection in the murine model of LD. Functional characterization of microbial ligands that are differentially expressed in the complex enzootic cycle of *B. burgdorferi* is critical to understanding the adaptive strategies of a pathogen that has evolved to persist in diverse tissue environments resulting in multi-system disorders.

3.2 Materials and Methods

Bacteria, Mice and Ticks. *Borrelia burgdorferi* infectious isolate A3 (Elias et al., 2002), a clonal derivative of B31 M1, was used throughout the study. Female C3H/HeN mice between 4 and 6 weeks old purchased from the National Cancer Institute. Mice were inoculated with a single subcutaneous injection of 10^5 spirochetes per mouse. All animal procedures were in compliance with the guidelines set by the Institutional Animal Care and Use Committee. The ticks used in this study belong to a colony that is reared and maintained in the laboratory as described (Pal et al., 2004a).

PCR. Mice were sacrificed following infection, and the heart, tibiotarsal joint, and skin samples were removed and frozen in liquid nitrogen. *B. burgdorferi*-infected ticks were isolated by allowing ticks to feed on an infected murine host as described (Pal et al., 2004a). RNA was extracted using TRIzol reagent (Invitrogen) and further treated with DNase I (Invitrogen), and finally purified using the RNeasy kit (Qiagen). RNA was used as a template for reverse-transcriptase polymerase chain reaction (RT-PCR) using the AffinityScript cDNA synthesis kit (Stratagene). The primers used for PCR reactions are indicated in Table 2. Quantitative PCR analysis was performed using iQ SybrGreen Supermix (BioRad) as previously described (Pal et al., 2008b). For quantitative analysis of gene expression, the target transcripts were normalized to the number of *flaB* transcripts, whereas for quantitative measurement of *B. burgdorferi* burden in infected

tissues, *flaB* transcripts were normalized to mouse or tick *β-actin* levels. All quantitative PCR results were checked for specificity by melting curve analysis.

Production of recombinant BbCRASP-2 protein and BbCRASP-2 antibodies. Recombinant BbCRASP-2 protein was produced in *E. coli* using the pET303/CT-His Champion vector (Invitrogen) using specific primers (Table 2). Recombinant BbCRASP-2 was fused with a C-terminal 6-histidine tag for purification, and lacked the peptides encoding the lipidation signal. Polyclonal antibodies against recombinant BbCRASP-2 were generated in mice as described earlier (Pal et al., 2004a; Pal et al., 2008a). Briefly, recombinant BbCRASP-2 (10 μg/animal) was emulsified in complete Freund's adjuvant (Sigma) and injected into groups of 10 mice. The animals were boosted twice at 10 days intervals with the same dose of antigen in incomplete Freund's adjuvant (Sigma) and the sera were collected two weeks following the second boost. Titer and specificity of the serum was tested by ELISA and immunoblot blot as described previously (Pal et al., 2004b).

Proteinase K accessibility assay. Proteinase K accessibility assay were performed as described (Brooks et al., 2006). Briefly, *B. burgdorferi* (2×10^8) were gently washed three times in 1 ml of PBS (pH 7.4) and collected by centrifugation at 4,000 x g for 4 min. Washed spirochetes were then gently resuspended in 1 ml of PBS and split into two equal 500 μl volumes. One aliquot received 200 μg of proteinase K (PK) (Sigma Chemical Co.) while the other aliquot received an equal volume of PBS without PK. Both aliquots were incubated for 1 h at room temperature before addition of 10 μl of

phenylmethylsulfonylfluoride (Sigma Chemical Co.) to stop PK activity. Spirochete suspensions were subsequently pelleted by centrifugation at 10,000 x g for 10 min and resuspended in PBS for immunoblot analysis using antibodies against BbCRASP-2, FlaB, or OspA.

Active immunization and infection studies. Groups of mice (6 animals/group) were immunized with adjuvant containing either recombinant BbCRASP-2, or adjuvant containing the same volume of phosphate buffered saline (PBS) in similar fashion as describe in above paragraph. Ten days after the final boost, mice were infected with a subcutaneous injection of *B. burgdorferi* (10^5 spirochetes/mouse). Mice were sacrificed after 7, 12, 15 and 30 days of infection. Heart, tibiotarsal joint, and skin samples were collected and frozen in liquid nitrogen. RNA was isolated from infected tissues and *B. burgdorferi* burden was measured using quantitative PCR. *B. burgdorferi*-infected mice were examined for swelling and histology of the tibiotarsal joints as detailed (Bolz et al., 2004; Wang et al., 2005; Pal et al., 2008b).

Generation and phenotypic analysis of *BbCRASP-2* mutant. BbCRASP-2-deficient *B. burgdorferi* was generated by homologous recombination replacing the entire open reading frame of the *BbCRASP-2* gene with a kanamycin resistance cassette (Pal et al., 2004b; Yang et al., 2004; Li et al., 2007b; Pal et al., 2008a; Pal et al., 2008b) using primers as indicated in Table 2. DNA fragments flanking the *BbCRASP-2* open reading frame on the 5′ and 3′ sides were PCR-amplified and inserted into the plasmid pXLF10601. This plasmid was sequenced to confirm identity and electroporated into

wild-type *B. burgdorferi* A3, and transformants were selected for growth in the presence of kanamycin. PCR analysis was used to confirm the intended recombination event using primers P5-P12. Immunoblotting analysis using BbCRASP-2 antibodies was performed to confirm the loss of BbCRASP-2. The plasmid profile of the mutant *B. burgdorferi* was assessed to confirm no loss of wild type plasmids (Yang et al., 2004; Pal et al., 2008a; Pal et al., 2008b).

The serum sensitivity of the *BbCRASP-2* mutant was measured using a published procedure (Alitalo et al., 2001). Briefly, triplicate samples of wild type *B. burgdorferi* or isogenic BbCRASP-2 and BbCRASP-1 mutant (Brooks et al., 2005) were seeded into 1 ml tubes at a final concentration of 5 x 10^6 bacteria per ml. Spirochetes were subsequently incubated for different times with 50% normal human serum or 50% heat-inactivated human serum. At 0, 1, 4, and 16 hours after the addition of serum, samples were extracted and spirochete viability was examined using dark-field microscopy. Normal human serum (Sigma) collected from healthy, anonymous donors with no reactivity to *B. burgdorferi* after chemiluminescent immunoblot analyses were used in the assay.

To examine the phenotype of the *BbCRASP-2* mutant *in vivo*, the mutant and wild type *B. burgdorferi* were separately inoculated into 2 groups of mice (6 animals/group, 10^5 spirochetes/mouse). A skin sample, the heart and the joints from infected mice were isolated at day 7, 12 and 18 after challenge, and *B. burgdorferi* burdens were measured by quantitative RT-PCR analysis. Before sacrifice, ear and spleen tissues from the mice

were cultured in BSK medium for the presence of viable *B. burgdorferi.* Rear ankle joints of individual mice were measured before infection and at 7, 12 and 18 days after infection until sacrifice. Acquisition of wild type and mutant *B. burgdorferi* by nymphal *I. scapularis* ticks was performed as described earlier (Pal et al., 2004a). Briefly, groups of C3H mice (6 animals/group) were infected with wild type or *BbCRASP-2* mutant *B. burgdorferi* (10^5 spirochetes/mouse). Following 12 days of infection, 25 *I. scapularis* nymphs were placed on each mouse. The ticks were forcibly detached from the mice following repletion and immediately stored in liquid nitrogen. *B. burgdorferi* burdens in each tick sample were measured as described earlier by quantitative RT-PCR analysis.

Statistical analysis. Results are expressed as the mean ± standard error of the mean (SEM). The significance of the difference between the mean values of the groups was evaluated by two-tailed Student *t* test.

3.3 Results

***BbCRASP-2* is ubiquitously expressed during murine infection.** To understand role of BbCRASP-2 in *B. burgdorferi* infectivity, I first assessed the transcript levels of *BbCRASP-2* in multiple murine tissue locations where *B. burgdorferi* persists during infection, and in various stages of infected ticks. C3H mice were infected with *B. burgdorferi,* and heart, joints and skin samples were collected at days 5, 7, 12, 16, and 24 following infection. Larval and nymphal ticks were fed on parallel groups of 15 day-infected mice (25 ticks/mouse) and fully engorged ticks were isolated following 3 days of repletion. Batches of infected fed larvae were allowed to molt and then collected as unfed nymphs. Total RNA was prepared from murine and tick samples, and subjected to quantitative RT-PCR to measure the *B. burgdorferi BbCRASP-2* transcript levels. *BbCRASP-2* is ubiquitously and consistently expressed throughout infection (Fig. 2), but was undetectable in larval or nymphal ticks.

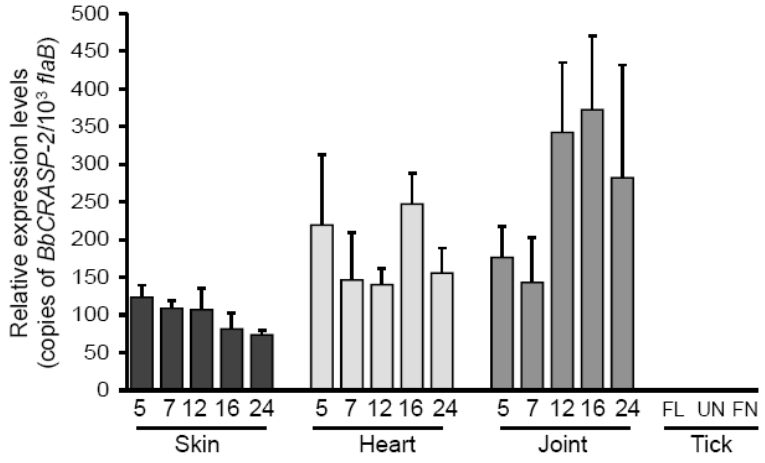

Figure 2. Ubiquitous expression of *BbCRASP-2* in infected mice.
The relative expression levels of *BbCRASP-2* in the murine host, and in representative life stages of ticks, are analyzed and presented as copies of *BbCRASP-2* transcript per 1000 copies of *flaB* transcripts. Total RNA was isolated from multiple tissues of *B. burgdorferi*-infected mice, from infected fed larva (FL) after 3 days of feeding, unfed nymphs following larval molting (UN) and fed nymphs (FN) after 3 days of feeding. *BbCRASP-2* transcripts were measured using quantitative RT-PCR. Bars represent the mean ± SEM from four qPCR analyses of two independent mouse experiments.

BbCRASP-2 immunization failed to evoke protective immunity in mice. Because BbCRASP-2 is surface-exposed (Hartmann et al., 2006), immunogenic, and expressed throughout the murine infection, I next assessed if immunization of mice using recombinant BbCRASP-2 could elicit protective immunity and influence the outcome of Lyme disease. To accomplish this, I produced recombinant BbCRASP-2 in *E. coli* and immunized the murine host with purified BbCRASP-2. Separate groups of C3H mice (6 animals/group) were immunized with either BbCRASP-2 or PBS (control) mixed with similar volume of adjuvant. Immunoblotting performed after final boosting indicated that immunized mice had developed strong antibody titer that specifically recognized recombinant and native BbCRASP-2 (Fig. 3A). Although previous immunofluorescence studies indicated that BbCRASP-2 antibodies recognized native antigen on the surface of

the intact spirochetes (Hartmann et al., 2006), the proteinase K accessibility assay indicated that BbCRASP-2 is not exposed on the spirochete surface (Fig. 3B). Ten days after the final immunization mice were needle-inoculated with *B. burgdorferi* (10^5 spirochetes/mouse). *B. burgdorferi* levels were measured by quantitative PCR from skin, heart and joint samples collected at 7, 12, 15 and 30 days after infection. Results indicated no significant difference in spirochete burden between mice immunized with BbCRASP-2 or the control at any time points (Fig. 4A). These results indicated that BbCRASP-2 immunization did not influence the ability of *B. burgdorferi* to establish infection. Development of ankle swelling (Fig. 4B) or histopathological changes in the joint tissue (data not shown) in *B. burgdorferi*-infected mice immunized with BbCRASP-2 did not differ from the control, suggesting that host BbCRASP-2 antibodies fail to influence the ability of *B. burgdorferi* to induce arthritis in the murine host.

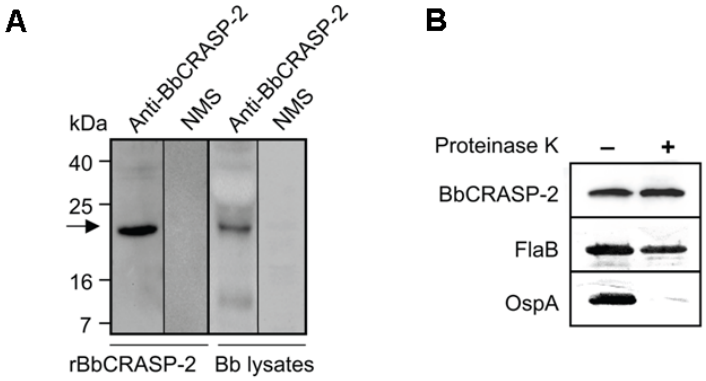

Figure 3. Characterization of BbCRASP-2 antisera and surface localization. (A) Recognition of recombinant and native BbCRASP-2 proteins by immunized murine serum, as assessed by immunoblotting. Recombinant BbCRASP-2 protein or *B. burgdorferi* lysates were probed with BbCRASP-2 antiserum or normal mouse serum (NMS). The arrow indicates the position of BbCRASP-2. Migration of protein standards is shown to the left.
(B) BbCRASP-2 is not sensitive to proteinase K-mediated degradation of *B. burgdorferi* surface proteins. Viable spirochetes were incubated with (+) or without (–) proteinase K for removal of protease sensitive surface proteins and probed with BbCRASP-2 antisera. *B. burgdorferi* OspA and FlaB antibodies were utilized as controls for surface-exposed and sub-surface proteins, respectively.

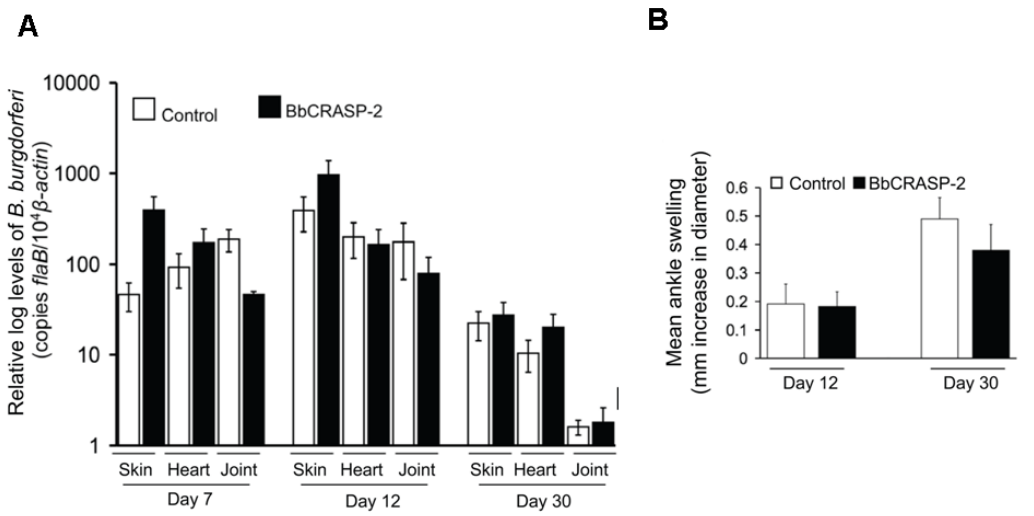

Figure 4. BbCRASP-2 immunization does not interfere with *B. burgdorferi* infectivity. (A) *B. burgdorferi* infection in rBbCRASP-2-immunized or control mice. Groups of mice were immunized with either rBbCRASP-2 or PBS (control) mixed with adjuvant, and were infected with *B. burgdorferi*. The spirochete burdens in both groups of mice were assessed by measuring copies of the *B. burgdorferi flaB* gene normalized to murine *ß-actin*. Bars represent the mean measurements ± SEM from four quantitative PCR analyses from two independent infection experiments. The burdens found in BbCRASP-2-immunized mice were not statistically significant from the control in any tissue or time point ($P > 0.05$, n = 4). (B) Severity of joint swelling in BbCRASP-2-immunized and infected mice. Groups of mice (6 animals/group) were immunized with BbCRASP-2 (black bars) or PBS (control, white bars) and infected with *B. burgdorferi*. Development of joint swelling was assessed after 12 and 30 days of spirochete challenge by measuring the largest diameter of the rear tibiotarsal joints using a digital caliper.

Generation of an infectious isolate of BbCRASP-2-deficient *B. burgdorferi.* Since BbCRASP-2 immunization did not influence spirochete infection, I created a BbCRASP-2-deficient *B. burgdorferi* to more directly assess the role of the gene product in *B. burgdorferi* survival and infectivity. An infectious isogenic mutant was created by replacing the *BbCRASP-2* open reading frame with a kanamycin resistance cassette via homologous recombination (Fig. 5A). PCR analysis was performed to ensure that the antibiotic cassette was appropriately inserted into the intended locus (Fig. 5B), and that the plasmid profile of the mutant was unchanged. Out of 4 transformed clones that grew in antibiotic-containing media, 2 clones contained the desired integration of the antibiotic cassette and retained the same set of plasmid as in the parental isolate. One of the mutant clones was chosen for further study. RT-PCR analysis showed that *BbCRASP-2* mRNA was absent in the mutant (Fig. 5C). The *BbCRASP-2* mutant spirochetes displayed a similar protein profile to that of the wild type (Fig. 5D, left panel), except that the *BbCRASP-2* mutant failed to produce BbCRASP-2 protein (Fig. 5D, upper right panel).

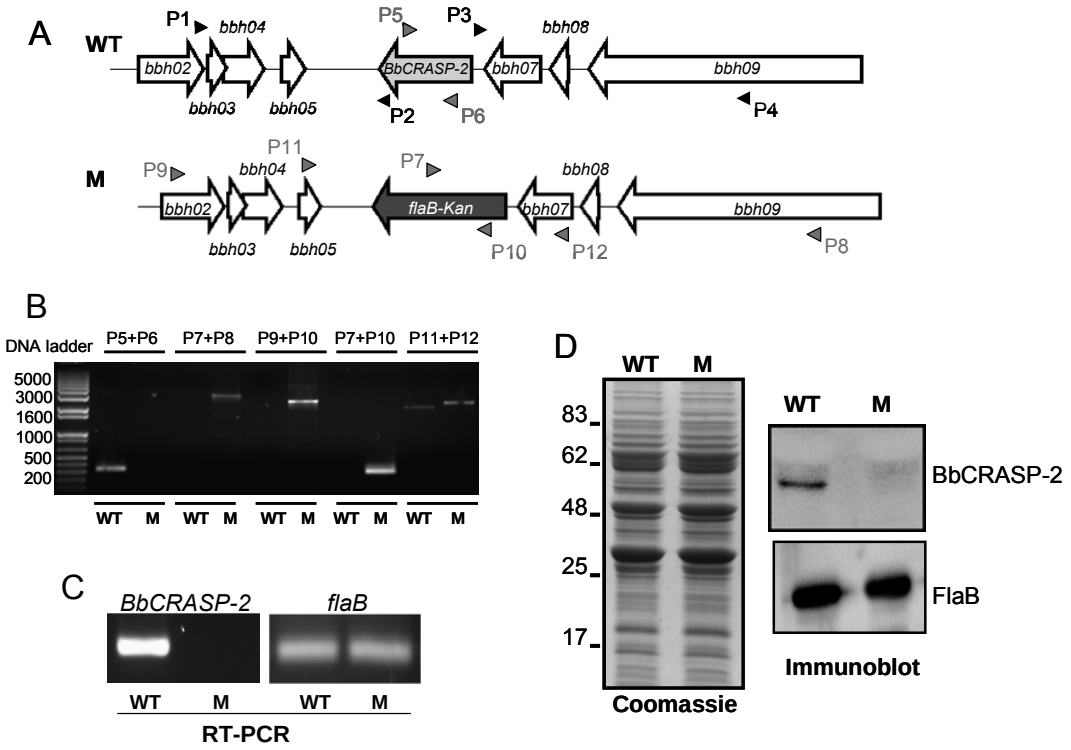

Figure 5. Construction and analysis of the *BbCRASP-2* mutant *B. burgdorferi*.
(A) Schematic drawings of the wild type isolate (WT) and the *BbCRASP-2* mutant (M) at the *BbCRASP-2* (*bbh06)* locus. Genes *bbh02-bbh09* (white box arrows) and the kanamycin-resistance cassette driven by the *B. burgdorferi flaB* promoter (*flaB-Kan*, black box arrow) are indicated. Primers P1-P4 (black arrow-heads) amplified 5′ and 3′ arms for recombination, which were then ligated to either side of the *flaB-Kan* cassette.
(B) Integration of the mutagenic construct, *flab-Kan*, in the intended genomic locus. Primers 5-12 (gray arrow-heads) were used for PCR analysis using DNA isolated from wild type or *BbCRASP-2* mutant *B. burgdorferi*.
(C) RT-PCR analysis of *BbCRASP-2* transcripts. Total RNA was isolated from either the wild type or *BbCRASP-2* mutant *B. burgdorferi* for RT-PCR amplification of regions within *BbCRASP-2* and *flaB*, and these amplicons visualized on a gel.
(D) Protein analysis of wild type (WT) or *BbCRASP-2* mutant (M) *B. burgdorferi*. Equal amounts of protein from wild type or *BbCRASP-2* mutant spirochetes were separated on an SDS-PAGE gel, and either stained with coomassie blue (left panel) or transferred onto a nitrocellulose membrane and probed with BbCRASP-2 or FlaB antibodies. Migration of protein standards is shown to the left in kDa.

BbCRASP-2 deficiency did not affected serum resistance of *B. burgdorferi in vitro.*

B. burgdorferi is known to be resistant to complement-mediated lysis in serum, and deficiency of *BbCRASP-1* has been shown to render *B. burgdorferi* highly susceptible to

serum-mediated killing *in vitro* (Brooks et al., 2005). Because *BbCRASP-2* is expressed by wild type spirochetes grown in culture, I assessed whether BbCRASP-2 deficiency affects the serum resistance of the spirochetes. Equal concentrations of wild type and *BbCRASP-2* mutant *B. burgdorferi* were separately incubated with human serum containing active complement and the serum sensitivity of each isolate was assessed. While the isogenic *BbCRASP-1* mutant were readily killed within 1 hour of serum exposure, the viability of the *BbCRASP-2* mutant did not differ significantly from that of the wild type (Fig. 6) indicating that BbCRASP-2 is not essential for *B. burgdorferi* resistance to complement-mediated lysis in serum.

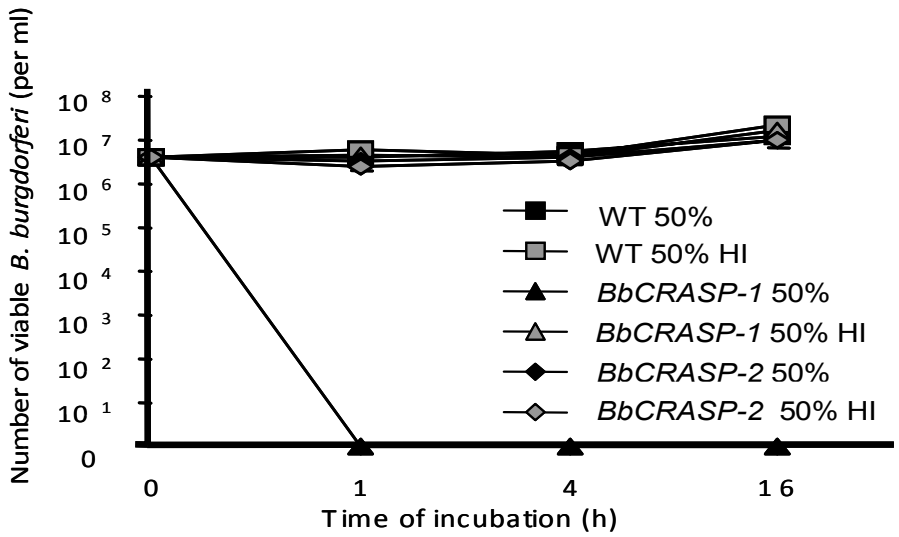

Figure 6. *BbCRASP-2* mutant is resistant to complement-mediated killing *in vitro*. Triplicate wells of spirochetes were exposed to 50% normal human serum or 50% heat-inactivated human serum (HI) and viability of *B. burgdorferi* was assessed using dark-field microscopy. An isogenic *B. burgdorferi BbCRASP-1* mutant served as a serum-sensitive positive control, which were killed within 1 hour of incubation with complement active serum as expected ($P < 0.05$, compared to corresponding HI control), while both wild type and *BbCRASP-2* remained fully resistant to serum ($P > 0.05$).

BbCRASP-2-deficient *B. burgdorferi* retain full murine infectivity. To examine whether the lack of BbCRASP-2 influences *B. burgdorferi* infectivity in a mammalian host, C3H mice were infected with wild type or *BbCRASP-2* mutant *B. burgdorferi*. Both the mutant and wild type spirochetes were readily cultured from ear and spleen tissues taken from mice 12 days after the inoculum. When nymphal ticks were allowed to feed on infected mice, *BbCRASP-2* mutant *B. burgdorferi* were able to migrate into fed ticks at a similar level to the wild type spirochetes (data not shown). Quantitative RT-PCR further showed that the *BbCRASP-2* mutant established infection in mice in comparable levels to the parental isolate. No significant differences in the burdens of *BbCRASP-2* mutant and wild type isolates were detected in murine skin, heart and joint samples isolated after 7, 12 and 18 days of infection (Fig. 7). Development of swelling in the murine joints infected with either the *BbCRASP-2* mutant or the wild type *B. burgdorferi* was also similar (data not shown). Overall, these results suggest that BbCRASP-2 is not essential for establishment of *B. burgdorferi* infection in the mouse model of Lyme disease.

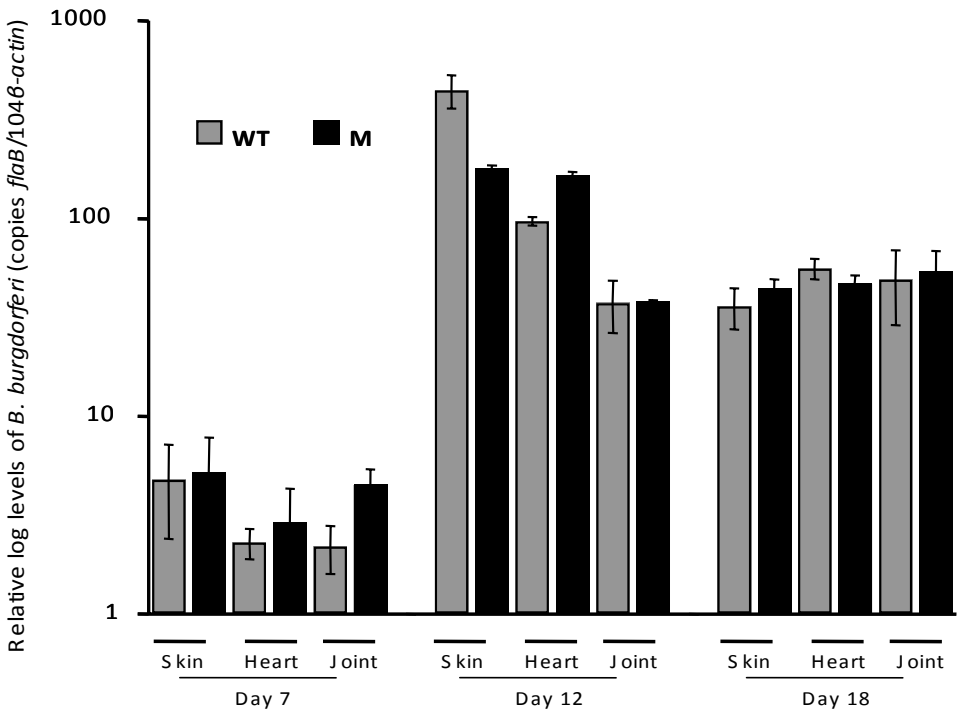

Figure 7. *BbCRASP-2* mutant *B. burgdorferi* retain full pathogenicity in the murine host.
Mice were infected with either the wild type or the *BbCRASP-2* mutant *B. burgdorferi* and spirochete burdens were analyzed as before by measuring copies of *B. burgdorferi flaB* RNA normalized to murine *ß-actin*. Bars represent the mean measurements ± SEM from four quantitative PCR measurements from two independent infection experiments. No difference between wild type and *BbCRASP-2* mutant levels was statistically significant in any tissue or time point measured ($P > 0.05$, n = 4).

Loss of BbCRASP-2 function is not compensated by augmented or new expression of other BbCRASPs. BbCRASP-2 deficiency did not affect the ability of the *BbCRASP-2* mutant to survive complement-mediated lysis *in vitro* or establish infection in a mammalian host *in vivo*. Since *B. burgdorferi* encodes multiple BbCRASPs that are capable of binding host complement regulators, I explored the possibility that the loss of *BbCRASP-2* could be compensated by altered expression of other potential *BbCRASP*

genes, such as *BbCRASP-1, -3* and *-5*. I did not examine the expression of *BbCRASP-4*, as the parental *B. burgdorferi* isolate A3(Elias et al., 2002) lacks the non-essential cp32-2 plasmid housing the gene. To examine *BbCRASP* expression, groups of 6 C3H mice were needle-inoculated with wild type or BbCRASP-2-deficient *B. burgdorferi* (10^5 spirochetes/mouse). Infected murine skin and heart samples were isolated 7, 12 and 18 days after infection, and expression of each *BbCRASP* was measured by quantitative RT-PCR. *In vitro* expression of *BbCRASP* genes was also assessed by growing wild type and mutant *B. burgdorferi* in BSK medium to various cell densities (10^6 – 10^8 spirochetes/ml) and analyzed by quantitative RT-PCR. The expression profiles of *BbCRASP-1*, *-3*, and *-5* remained unaltered in the *BbCRASP-2* mutant when compared to the wild type spirochetes, both *in vitro* and *in vivo*, such as in the murine skin and heart tissues at all time points. *BbCRASP* expression in cultured spirochetes grown *in vitro* to a density of 10^7/ml and in infected murine skin and heart tissue samples at 12 days of infection is presented (Fig. 8). These results suggest that the loss of BbCRASP-2 function is not compensated by alteration or new expression of other *BbCRASP* genes (Fig. 8).

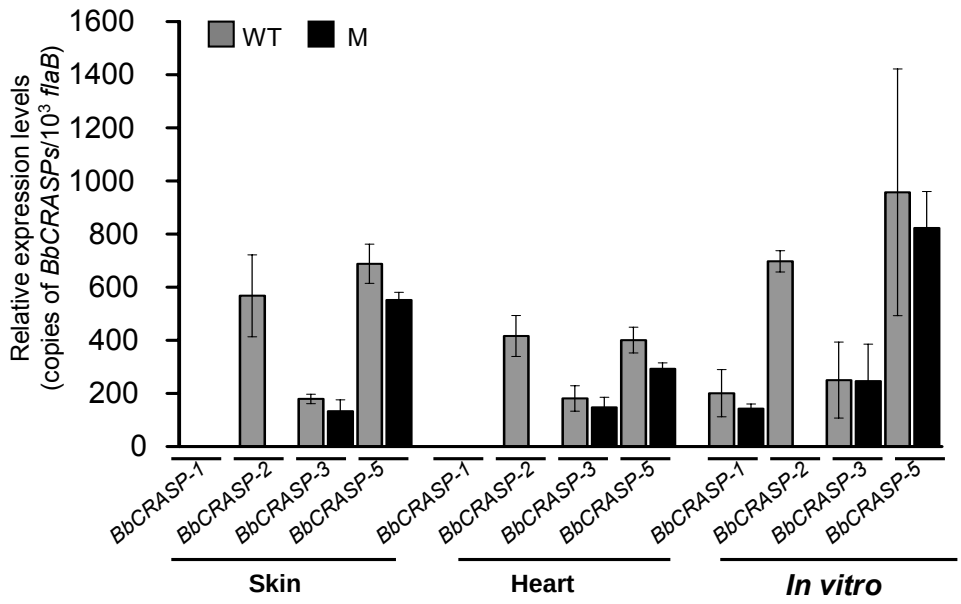

Figure 8. *BbCRASP-2* inactivation does not affect the expression of other *BbCRASP* genes.

The relative expression levels of *BbCRASP-1, -2, -3* and -5 were examined in the *BbCRASP-2* mutant and wild type spirochetes *in vitro* and *in vivo* by quantitative RT-PCR, and are represented as copies of gene per 1000 copies of *flaB* transcripts. Total RNA was isolated from *B. burgdorferi* isolates grown in culture, as well as multiple tissues of *B. burgdorferi*-infected mice following 12 days of infection. The experiments were replicated thrice, and bars represent the mean measurements ± SEM from four representative quantitative PCR measurements. Differences between *BbCRASP* transcripts in the wild type and mutant were not statistically significant ($P > 0.05$, n = 4).

Table 2. Oligonucleotide primers used in Chapter 3.

Sequence (5' to 3')	Purpose
TTCAATCAGGTAACGGCACA	Forward primer for *B. burgdorferi flaB* Quantitative RT-PCR (qRT-PCR).
GACGCTTGAGACCCTGAAAG	Reverse primer for *B. burgdorferi flaB* qRT-PCR.
AGAGGGAAATCGTGCGTGAC	Forward primer for mouse *β-actin* qRT-PCR.
CAATAGTGATGACCTGGCCGT	Reverse primer for mouse *β-actin* qRT-PCR.
TGCCAGACATGTTGCTGATT	Forward primer for *BbCRASP-2* qRT-PCR.
CCCCCTCAAGTTCTACAGCA	Reverse primer for *BbCRASP-2* qRT-PCR.
TT*TCTAGA*ATGGATGTTAGTAGATTAAATCAGA	Forward primer for producing recombinant BbCRASP-2. The italicized *XbaI* site is included for cloning.
ATA*CTCGAG*TAATAAAGTTTGCTTAATAGCT	Reverse primer for producing recombinant BbCRASP-2. The italicized *XhoI* site is included for cloning.
AGAGAGAG*GCGGCCGC*AAACAACAAAGACTCGCATATTCA	Primer P1, used to amplify and clone the 5' flanking region to generate the *BbCRASP-2* mutant. The italicized *NotI* site is included for cloning purposes.
GGA*CTGCAG*GTTTCAATCTCTCTAAACATG	Primer P2, used to amplify and clone the 5' flanking region to generate the *BbCRASP-2* mutant. The italicized *PstI* site is included for cloning purposes.
AGGA*CTCGAG*TTCATAGCCTCTAGCACAAG	Primer P3, used to amplify and clone the 3' flanking region to generate the *BbCRASP-2* mutant. The italicized *XhoI* site is included for cloning purposes.
AGG*GGTACC*ATTTTGGAGTAGGGCTAAA	Primer P4, used to amplify and clone the 3' flanking region to generate the *BbCRASP-2* mutant. The italicized *KpnI* site is included for cloning purposes.
TGCCAGACATGTTGCTGATT	Primer P5, primer used to confirm the deletion of *BbCRASP-2*.
CCCCCTCAAGTTCTACAGCA	Primer P6, used to confirm *BbCRASP-2* deletion.
GGTTGCATTCGATTCCTGTT	Primer P7, used to confirm *BbCRASP-2* deletion.
TGGAAATCCTCCATGGGAAA	Primer P8, used to confirm *BbCRASP-2* deletion.
AATTCAATATGGAGGAAGTTTTAGGGCT	Primer P9, used to confirm *BbCRASP-2* deletion.
ATTCCGACTCGTCCAACATC	Primer P10, used to confirm *BbCRASP-2* deletion.
TAAATGTATATGCTCCAGTAAACTAAACCAC	Primer P11, used to confirm *BbCRASP-2* deletion.
CCTCAGGATGTCATATTGGTAGCA	Primer P12, used to confirm *BbCRASP-2* deletion.
TCTTATTTACACACTAAACAACATTGACC	Forward primer for *B. burgdorferi bbh05* qRT-PCR.
TAAACGCACGGCGTATAAAG	Reverse primer for *B. burgdorferi bbh05* qRT-PCR.
TGATGAATTTTTGGGAATGTTTT	Forward primer for *B. burgdorferi bbh07* qRT-PCR.
TGAATTTTATACATGGGTACAGTTTTG	Reverse primer for *B. burgdorferi bbh07* qRT-PCR.
CTAAAAGCAATTGGTAAGGAACTG	Forward primer for BbCRASP-1 for qRT-PCR
TCAATAAGATCGTAAGGACCAACT	Reverse primer for BbCRASP-1 for qRT-PCR
GTTTAAACGCTGGGGGACAT	Forward primer for BbCRASP-3 for qRT-PCR.
ACGCAATATGTTCAGCACCA	Reverse primer for BbCRASP-3 for qRT-PCR.
TGATGAGCAAAGTAGTGGT	Forward primer for BbCRASP-5 for qRT-PCR.
GAATGTCCCCCAGCGTTTA	Reverse primer for BbCRASP-5 for qRT-PCR.

3.4 Discussion

B. burgdorferi express up to five BbCRASPs that are either structurally unique, such as BbCRASP-1 and -2, or closely related, BbCRASP-3, -4 and -5 (Alitalo et al., 2002; McDowell et al., 2003; Kraiczy et al., 2004; Hartmann et al., 2006). These BbCRASPs are differentially expressed and are postulated to confer defense against host-derived complements via specific interaction with FH family proteins (Alitalo et al., 2001; Hartmann et al., 2006; Hovis et al., 2006; Haupt et al., 2008). The precise role of individual BbCRASPs in the *B. burgdorferi* infection cycle, however, is currently unclear. BbCRASP-2 is specifically produced in the mammalian host including humans, and is immunogenic, and thus, is thought to be important in spirochete pathogenesis and may be useful in a future Lyme disease vaccine (Hartmann et al., 2006; Kraiczy et al., 2008). Here, I show that BbCRASP-2 is ubiquitously expressed throughout murine infection, evoking a detectable antibody response. However, BbCRASP-2 immunization fails to protect the host against *B. burgdorferi* infection or influence the genesis of disease. Targeted deletion of BbCRASP-2 did not impair the ability of the mutants to resist serum-mediated killing *in vitro*, establish infectivity *in vivo*, or the severity of disease. Deficiency of *BbCRASP-2* expression in mutants is not functionally compensated by the enhanced expression of other *BbCRASP* genes. BbCRASP-2, therefore, is not essential for *B. burgdorferi* survivability *in vitro*. Furthermore, based on the time periods covered in the present study, BbCRASP-2 function is dispensable for *B. burgdorferi* infectivity mice and in feeding ticks.

Immunization of murine hosts against specific *B. burgdorferi* antigens, such as DbpA (Cassatt et al., 1998; Hanson et al., 1998), OspC (Probert and Lefebvre, 1994) and OspA (Fikrig et al., 1990) can elicit production of borreliacidal antibodies, and thus confer protective host immunity possibly by killing spirochetes *in vivo* when administered prior to spirochete infection. In contrast, other *B. burgdorferi* antigens are also described, such as BmpA/B (Pal et al., 2008b) and Arp (Feng et al., 2000; Feng et al., 2003), that fail to protect host against *B. burgdorferi* infection, but influence pathogenesis either by reduction of the *B. burgdorferi* burden in selected tissues (Pal et al., 2008b) or by modifying the disease without affecting spirochete load (Feng et al., 2000; Feng et al., 2003). The failure of BbCRASP-2-specific host immunity to influence *B. burgdorferi* pathogenesis indicates that BbCRASP-2 antibodies lack significant neutralizing effects on spirochetes, possibly due to the lack of significant surface-exposure of the antigen. This is further confirmed by the observation that the BbCRASP-2-deficient *B. burgdorferi* displayed no phenotypic defects in their ability to infect the murine host or induce disease. Nevertheless, as *BbCRASP-2* is abundantly produced by *B. burgdorferi* throughout infection and evokes development of specific antibody, these data reinforce an earlier contention that BbCRASP-2 could be a reliable marker for the serodiagnosis of Lyme disease (Hartmann et al., 2006; Kraiczy et al., 2008).

B. burgdorferi expresses select lipoproteins (Casjens et al., 2000) in the mammalian host (Liang et al., 2002), including BbCRASP-2. Owing to its ubiquitous expression in the host, and known affinity for FH family proteins (Hartmann et al., 2006), BbCRASP-2 appears to be important for *B. burgdorferi* protection against persistent host

immune threats, such as complement system. BbCRASP-2 is conserved among infectious *B. burgdorferi* isolates, which also suggests an important role for this protein in the infectivity of *B. burgdorferi* (Rogers and Marconi, 2007). The plasmid lp28-3 that houses BbCRASP-2 is retained in most of the *B. burgdorferi* clones isolated from experimentally infected hosts (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001; McDowell et al., 2001; Iyer et al., 2003). Previous studies attempting to identify specific plasmids required for *B. burgdorferi* infectivity indicate that lp28-3 plasmid may not be strictly necessary for spirochete infectivity (McDowell et al., 2001) while other studies suggest that several plasmids, including lp28-3, in the correct combinations, may be required to mediate *B. burgdorferi* infection of the mammals (Labandeira-Rey and Skare, 2001). Nevertheless, these data conclusively show that the function of BbCRASP-2 is not essential for *B. burgdorferi* survival against serum-mediated killing *in vitro* or host infectivity. The *BbCRASP-2* mutant fails to produce both *BbCRASP-1* and *BbCRASP-2* in the murine host, yet still retains full infectivity, which suggests that complement evasion in the host, if relevant, could be mediated by BbCRASP-3 and -5, which remain fully expressed by the mutant. Alternatively, binding to certain FH family proteins may not be essential to the spirochetes, as previous studies indicate that *B. burgdorferi* are able to infect and cause disease in FH-deficient mice (Woodman et al., 2007).

In summary, I present direct evidence that *B. burgdorferi* adapts for the loss of a differentially expressed and abundant lipoprotein during mammalian infection. Past studies also identified additional *B. burgdorferi* genes encoding potential lipoproteins, such as BBA64 (Gilmore et al., 2007; Maruskova et al., 2008) or OspD (Li et al., 2007a;

Stewart et al., 2008) that display highly regulated expression *in vivo* but lack an essential role in *B. burgdorferi* persistence in tick-mouse infection cycle. Here I show that BbCRASP-2 function is also dispensable for infectivity of the murine hosts or in feeding ticks. Together, these results highlight *B. burgdorferi* as a unique pathogen that has evolved versatile adaptive strategies to survive and establish infection in a diverse array of host species, including humans.

A portion of this chapter has been published previously:

Coleman AS, Yang X, Kumar M, Zhang X, Promnares K, et al. (2008) *Borrelia burgdorferi* Complement Regulator-Acquiring Surface Protein 2 Does Not Contribute to Complement Resistance or Host Infectivity. PLoS ONE 3(8): 3010e. doi:10.1371/journal.pone.0003010

Chapter 4: The role of BBK07 in microbial virulence and biology

Abstract

The symptoms of Lyme disease are caused by infection with the spirochete pathogen *Borrelia burgdorferi*. This bacterium is able to chronically infect mammals and evade host immune responses by unknown mechanisms that remain poorly understood. As many *B. burgdorferi* proteins have no homologues outside of the species with which to compare, the roles of individual genes are often investigated through genetic manipulation and specific antibody interference studies. BBK07, a surface-exposed lipoprotein of *B. burgdorferi*, is selectively expressed during mammalian infection, and may have a function role in support of pathogen persistence and the genesis of disease. Here I show that BBK07 immunization of mice does not protect the host from *B. burgdorferi* infection or modulate the disease. The *bbk07* gene was then inactivated in an infectious isolate, showing that BBK07 plays a nonessential role in microbial infectivity and virulence in mice. Together, these results suggest that the function of BBK07 is not necessary for the pathogenesis of Lyme disease. Alternatively, it is possible that the function of BBK07 contributes to *B. burgdorferi* infectivity, but the loss of the gene product is compensated by an unknown mechanism.

4.1 Introduction

The spirochete *Borrelia burgdorferi*, pathogen responsible for Lyme disease (LD), can be transmitted by *Ixodes* ticks and can chronically infect mammals (Steere et al., 2004). Their complex life cycle has required unique adaptations, resulting in a highly specialized organism with only distant evolutionary links to other well-studied bacteria (Fraser et al., 1997a). *B. burgdorferi* contains a stable and conserved linear chromosome, housing the basic functions required for replication (Fraser et al., 1997a). In contrast, there are also around 23 plasmids of *B. burgdorferi* that show evidence of frequent recombination events. Notably, more than 90% of the 419 plasmid-encoded open reading frames having no known function (Fraser et al., 1997a; Casjens et al., 2000). Many of the unique abilities of *B. burgdorferi* could be contributed by these highly evolved plasmid-borne genes. The identification and characterization of proteins critical to successful *B. burgdorferi* infection could lead to new diagnostic, preventive and therapeutic approaches to control incidences of LD.

Previous studies have utilized mutagenesis, immunization, and repressible promoters to investigate the roles of individual *B. burgdorferi* genes in the life cycle of the bacterium(Rosa et al., 2005). Due to the large number of low-copy number plasmids harbored by *B. burgdorferi* (Hinnebusch and Barbour, 1992), studies have also taken advantage of natural mutants that have lost one or more plasmids during replication to study infectivity (Purser and Norris, 2000). One such study focused on lp36, a linear

plasmid of *B. burgdorferi* that is rarely lost during *in vitro* culture (Jewett et al., 2007). An isolate lacking lp36 was found to be severely attenuated, a phenotype that could be rescued with the addition of the BBK17 gene, which encodes a putative adenine deaminase (Jewett et al., 2007). The same degree of attenuation was not observed in an isogenic mutant lacking only the BBK17 gene, leading the authors to conclude that additional genes on lp36 might *B. burgdorferi* survival in mice (Jewett et al., 2007).

A recent study used a synthetic protein array to detect antibodies in infected sera against *B. burgdorferi* antigens (Barbour et al., 2008). Antibodies against BBK07, also located on lp36, were detected in both human and mouse sera (Barbour et al., 2008). Previous work also demonstrated that the *bbk07* gene was transcribed throughout infection, and that an amino-terminal portion of the protein was exposed on the surface of *B. burgdorferi* (Coleman and Pal, 2009). BBK07 protein was also suggested as a possible marker for the serodiagnosis of LD (Coleman and Pal, 2009). The location of the *bbk07* gene on the plasmid lp36, its expression during infection, and its surface exposure make it a promising candidate as a potentially important protein in *B. burgdorferi*. In this study I have immunized mice with a recombinant fragment of BBK07 to assess whether BBK07 antibodies interfere with spirochete persistence in the host. I then generated an isogenic mutant lacking the *bbk07* gene, and using the laboratory model of LD, more directly studied the contribution of BBK07 to bacterial persistence and severity of disease.

4.2 Materials and Methods

Bacteria and mice. The infectious *Borrelia burgdorferi* isolate A3 (Elias et al., 2002), a clonal derivative of B31 M1, was used in all experiments as wild type. Female C3H/HeN mice between 4 and 6 weeks old were purchased from the National Cancer Institute, and all animal procedures were in compliance with the guidelines set by the Institutional Animal Care and Use Committee of the University of Maryland. Mice were infected by intradermal inoculation of 10^5 spirochetes per mouse.

Generation of *bbk07* mutant *B. burgdorferi*. The BBK07-deficient *B. burgdorferi* isolate was generated by homologous recombination, using methods and plasmids described previously (Pal et al., 2004b; Yang et al., 2004; Li et al., 2007b; Pal et al., 2008a; Pal et al., 2008b). Briefly, oligonucleotide primers P1-P4 (Table 3) were used to amplify regions up- and downstream of the *bbk07* gene to serve as target DNA inserts required for allelic exchange. These fragments were inserted on the plasmid pXLF10601 into the two multiple cloning sites. A streptomycin resistance cassette (*aadA*), driving by the *B. burgdorferi flgB* promoter was inserted between the fragments (Frank et al., 2003). This plasmid was used to electroporate *B. burgdorferi* cells, with recombination mutants selected by growth in the presence of streptomycin. PCR analysis using primers P5-P11 was used to confirm the desired recombination event. Immunoblotting of wild type and mutant whole cell lysates were probed with anti-BBK07 sera, described in a previous study (Coleman and Pal, 2009). Total RNA was isolated from wild type and mutant *in vitro* grown *B. burgdorferi* pellets using TRIzol reagent (Invitrogen). RNA was

converted to cDNA using the AffinityScript Multi Temperature First Strand Synthesis kit (Stratagene). *bbk07* transcription was assessed using the primers specific for the *bbk07* gene and for the positive control gene *flaB* listed in Table 3.

Immunization experiments. Groups of mice (3 per group) were immunized with a recombinant BBK07 fragment containing the amino terminal portion of the protein and excluding the signal peptide, amino acids 18-142 (Fraser et al., 1997a). The protein was expressed using the pET302/NT-His Champion vector (Invitrogen) and purified using MagneHis nickel particles (Promega) according to the manufacturer's instructions. Recombinant protein or phosphate buffered saline was emulsified in complete Freund's adjuvant (Sigma) for the initial injection, and boosted twice emulsified in incomplete Freund's adjuvant (Sigma) at 10 day intervals. Ten days after final injection, mice were infected via intradermal needle inoculation of 10^5 spirochetes per mouse. Heart, tibiotarsal joint, and skin tissue samples were collected at the indicated time points from euthanized mice and homogenized using a mortar and pestle under liquid nitrogen. Tissue homogenates were pooled by group and total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using the AffinityScript Multi Temperature First Strand Synthesis kit (Stratagene). Quantitative RT-PCR (qRT-PCR) analysis was performed using Roche SYBR Green and iQ5 thermal cycler as described (Yang et al., 2009). Primers for mouse *β-actin* and the *B. burgdorferi* gene *flaB* (Table 3) were used to quantitate the bacterial load using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Mouse tibiotarsal joint diameters were measured weekly using precision calipers.

Mouse infection. Groups of mice (3 per group) were infected with wild type or BBK07-deficient *B. burgdorferi*. To measure pathogen load during dissemination, 100 µl of blood was taken from each anesthetized mouse by retro-orbital bleed. Mice were sacrificed and the bacterial load measured as above. Mouse tibiotarsal joint diameters were measured weekly using precision calipers.

Statistical analysis. Results are expressed as the mean ± standard error of the mean (SEM). The significance of the difference between the mean values of the groups was evaluated by two-tailed Student *t* test. Results were considered statistically significant if ($P < 0.05$).

4.3 Results

BBK07 immunization does not protect mice from *B. burgdorferi* infection. Previous studies have indicated that BBK07 protein is expressed during infection, and that it is surface exposed and highly immunogenic (Coleman and Pal, 2009). As these characteristics are ideal for effective vaccines against extracellular pathogens such as *B. burgdorferi*, I sought to evaluate possible vaccine efficacy of the BBK07 antigen. Expression and purification of a full-length recombinant BBK07 protein proved difficult in *E. coli*, as did a recombinant fragment encompassing the carboxyl-terminal half of the protein. Instead, a recombinant protein fragment encompassing the amino-terminal half of BBK07 was purified as previously described (Coleman and Pal, 2009), and was used to immunize mice. Previous research has shown that the amino-terminal half of BBK07 is surface exposed (Coleman and Pal, 2009). Immunizations were performed using Freund's adjuvant, which successfully generated an efficient antibody response that could recognize BBK07 protein in *B. burgdorferi* lysate (Coleman and Pal, 2009). Groups of mice were immunized with either BBK07 protein or buffer in an equal volume of adjuvant, and then infected intradermally with 10^5 *B. burgdorferi* per mouse. *B. burgdorferi* could be cultured from bladder tissue from all mice sacrificed at 1 week post infection. In order to measure the relative amounts of live *B. burgdorferi* in the samples, total RNA was isolated from tissue samples and the pathogen burden was measured by qRT-PCR (Fig 9). Results show that BBK07 immunization did not significantly reduce the bacterial load at any time point when compared to the control immunization. Also,

there was no significant difference in arthritis, as measured by tibiotarsal joint diameter (data not shown).

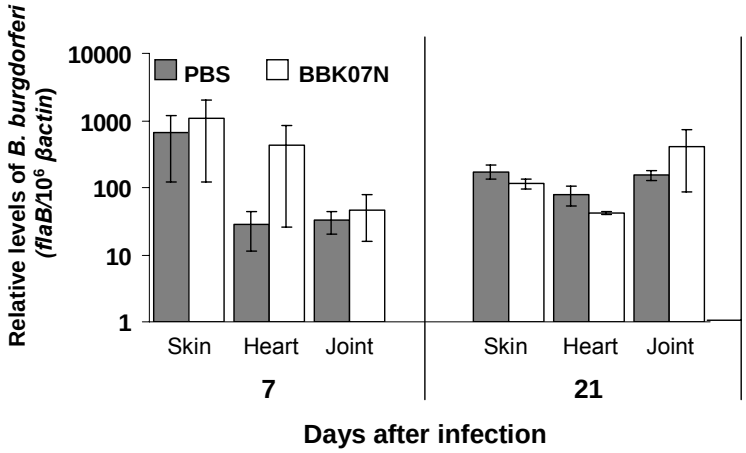

Figure 9. BBK07 immunization does not affect *B. burgdorferi* persistence in the murine host. Mice (3 animals/group) were immunized with either purified BBK07N protein or an equal volume of PBS. The immunizations were performed 3 times at 10 day intervals, first emulsified in CFA, and boosted twice in IFA. The immunized mice were infected with *B. burgdorferi* (10^5 spirochetes/mouse) 10 days after the final booster immunization. Total RNA was isolated from skin, heart, and joint tissue, and qRT-PCR was used to measure pathogen load in the tissue samples. The bars represent the mean and SEM from 4 qRT-PCR reactions from 2 independent mouse experiments.

Mutagenesis of *bbk07*. To more directly assess the role of BBK07 during infection, I inactivated the *bbk07* gene in an infectious *B. burgdorferi* isolate by homologous recombination. This method has been successfully used to inactive other genes in *B. burgdorferi* (Pal et al., 2004b; Yang et al., 2004; Li et al., 2007b; Pal et al., 2008a; Pal et al., 2008b). Briefly, a plasmid containing the *bbk07* locus and surrounding genes was constructed, with an antibiotic resistance cassette replacing a portion of the *bbk07* gene. A double-crossover event using this plasmid as a recombination template inactivated the *bbk07* gene (Fig. 10A). PCR analysis confirmed that the antibiotic cassette had indeed recombined into the intended locus (Fig. 10B), and that the isolate had lost only the nonessential plasmids lp5 and lp28-4 (data not shown) (Purser and Norris, 2000;

Labandeira-Rey and Skare, 2001). RT-PCR analysis could not detect *bbk07* gene expression in the mutant isolate (Fig 10C). Anti-BBK07 sera generated previously (Coleman and Pal, 2009) was unable to detect BBK07 protein in the mutant isolate (Fig 10D). The insertion of the antibiotic cassette successfully inactivated transcription of the *bbk07* gene, and expression of BBK07 protein.

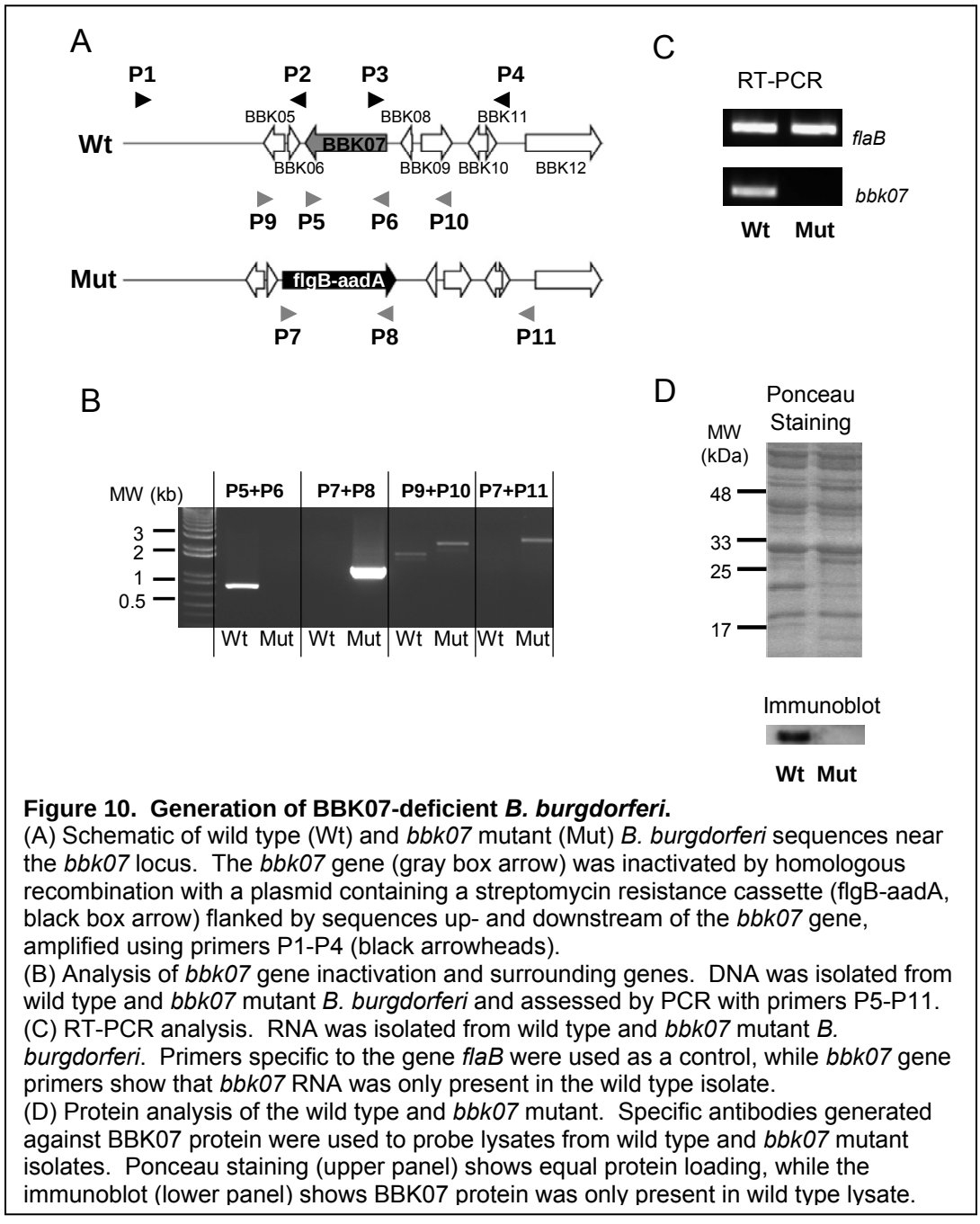

Figure 10. Generation of BBK07-deficient *B. burgdorferi*.
(A) Schematic of wild type (Wt) and *bbk07* mutant (Mut) *B. burgdorferi* sequences near the *bbk07* locus. The *bbk07* gene (gray box arrow) was inactivated by homologous recombination with a plasmid containing a streptomycin resistance cassette (flgB-aadA, black box arrow) flanked by sequences up- and downstream of the *bbk07* gene, amplified using primers P1-P4 (black arrowheads).
(B) Analysis of *bbk07* gene inactivation and surrounding genes. DNA was isolated from wild type and *bbk07* mutant *B. burgdorferi* and assessed by PCR with primers P5-P11.
(C) RT-PCR analysis. RNA was isolated from wild type and *bbk07* mutant *B. burgdorferi*. Primers specific to the gene *flaB* were used as a control, while *bbk07* gene primers show that *bbk07* RNA was only present in the wild type isolate.
(D) Protein analysis of the wild type and *bbk07* mutant. Specific antibodies generated against BBK07 protein were used to probe lysates from wild type and *bbk07* mutant isolates. Ponceau staining (upper panel) shows equal protein loading, while the immunoblot (lower panel) shows BBK07 protein was only present in wild type lysate.

Inactivation of *bbk07* does not affect persistence or virulence of *B. burgdorferi* in mice. *bbk07* gene inactivation was performed in a fully infectious *B. burgdorferi* isolate in order to study the role of BBK07 during infection. It has been previously reported that the plasmid lp36, which contains the *bbk07* gene, is nonessential for *B. burgdorferi* persistence in the tick vector but plays an important role during mammalian infection (Jewett et al., 2007). Groups of mice were infected with either wild type or BBK07-deficient *B. burgdorferi* by intradermal needle inoculation of 10^5 spirochetes per mouse. Bacterial load was measured in a blood sample taken 5 days after infection to measure hematogenous dissemination. One and three weeks after infection the pathogen load was also measured in the heart, tibiotarsal joint, and skin. Either wild type or mutant spirochetes were cultured from bladder, spleen, and skin samples from all mice sacrificed 7 days after infection. In order to measure the relative amounts of live *B. burgdorferi* in the samples, total RNA was isolated from tissue samples and the pathogen burden was measured by qRT-PCR (Fig. 11A). To assess the severity of arthritis caused by the wild type and BBK07-deficient *B. burgdorferi*, the diameter of the tibiotarsal joints were measured weekly using a precision digital caliper (Fig. 11B). Inactivation of the *bbk07* gene did not significantly affect bacterial load or severity of disease in two independent experiments. Therefore, the inactivation of *bbk07* did not significantly alter the infectivity or virulence of *B. burgdorferi*.

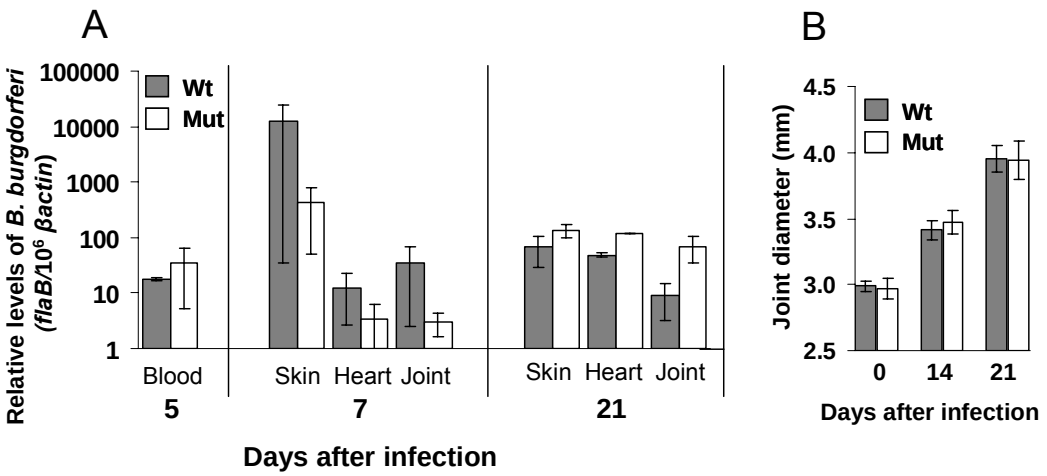

Figure 11. Inactivation of *bbk07* does not affect spirochete persistence or disease in the murine host.

(A) Mice (3 animals/time point) were infected with either wild type or *bbk07* mutant *B. burgdorferi* and total RNA was isolated from blood, skin, heart, and joint samples. Pathogen load in each sample was assessed by qRT-PCR analysis of *B. burgdorferi flaB* normalized to murine *β-actin* using the $2^{-\Delta\Delta Ct}$ method. The bars represent the mean and SEM of 4 qRT-PCR reactions of two independent mouse experiments.

(B) The severity of arthritis in the infected mice was assessed by measuring the largest diameter of the tibiotarsal joints with precision calipers. The bars represent the mean and SEM of two independent mouse experiments and at least 3 mice from each experiment.

Table 3. Oligonucleotide primers used in Chapter 4.

Sequence (5' to 3')	Purpose
TTCAATCAGGTAACGGCACA	Forward primer for *B. burgdorferi flaB* Quantitative RT-PCR (qRT-PCR).
GACGCTTGAGACCCTGAAAG	Reverse primer for *B. burgdorferi flaB* qRT-PCR.
AGAGGGAAATCGTGCGTGAC	Forward primer for mouse *β-actin* qRT-PCR.
CAATAGTGATGACCTGGCCGT	Reverse primer for mouse *β-actin* qRT-PCR.
AGAGAGAG*GCGGCCGC*TTTTTAAGCTC TTCTTCAGAATA	Primer P1, used to amplify and clone the 5' flanking region to generate the BBK07-deficient mutant. The italicized NotI site is included for cloning purposes.
GGA*CTGCAG*TTTAATAATTAAAACTGGG CATTT	Primer P2, used to amplify and clone the 5' flanking region to generate the BBK07-deficient mutant. The italicized PstI site is included for cloning purposes.
TTT*GCTAGC*TCTTCCGGAGTTAATTTTG TGAT	Primer P3, used to amplify and clone the 3' flanking region to generate the BBK07-deficient mutant. The italicized NheI site is included for cloning purposes.
ATT*GGTACC*AAATTTTAATGCTACTAAA AGATAATGT	Primer P4, used to amplify and clone the 3' flanking region to generate the BBK07-deficient mutant. The italicized KpnI site is included for cloning purposes.
TAATTATTAAAGCACAAATGTATGGCC	Primer P5, forward primer used to confirm the inactivation of the *bbk07* gene.
AGGAGGTGTTTACAAATGAGTAAACT	Primer P6, reverse primer used to confirm the inactivation of the *bbk07* gene.
AGGCTGCAGCGAGCTTCAAGGAAGA	Primer P7, forward primer used to confirm the inactivation of the *bbk07* gene.
AGGGCTAGCATTATTTGCCGACTACC	Primer P8, reverse primer used to confirm the inactivation of the *bbk07* gene.
GCTTTCACTGTTTAGATGCATAAGGA	Primer P9, forward primer used to confirm the inactivation of the *bbk07* gene.
TTGAGGTAAAATGGGGCAAA	Primer P10, reverse primer used to confirm the inactivation of the *bbk07* gene.

4.4 Discussion

The *B. burgdorferi* gene *bbk07* is located on lp36, a plasmid known to contain genes that are essential and or thought to facilitate mammalian infection (Jewett et al., 2007). BBK07 protein has been shown to be expressed on the surface of the bacterium, and expressed during infection (Coleman and Pal, 2009). In order to study its contribution to microbial persistence and disease, I have interfered with BBK07 function using two strategies. First, the effects of specific antibody interference of BBK07, as well as the vaccine potential of BBK07, were tested by immunizing mice with a recombinant fragment of BBK07. The mice were infected with *B. burgdorferi* and the protective immunity assessed. Second, an infectious *B. burgdorferi* isolate deficient in BBK07 expression was generated to assess the role of BBK07 in spirochete infectivity and the pathogenesis of LD. The effects of *bbk07* gene inactivation and antibody interference were assessed using the murine laboratory model of LD. Neither strategy had discernable effects on virulence or persistence, leading me to conclude that BBK07 is not essential for *B. burgdorferi* infection. This could indicate, among other possibilities, that BBK07 function is redundant and is compensated by other proteins, or that the laboratory model of LD does not provide an opportunity to observe the true function of BBK07 in the *B. burgdorferi* life cycle.

*B. burgdorfer*i is an extracellular spirochete which is able to persist and chronically infect mammals despite provoking a robust humoral immune response. This

ability to avoid immune clearance is not well understood, but may be due, in part, to antigen variation in the population. Infection-induced genetic variation in the gene *vlsE* can potentially cause each spirochete to display a different VlsE on their surface. Heterogeneity in the sequences of outer surface proteins such as OspC and BmpA may also allow for a more diversified antigenic profile of the *B. burgdorferi* inoculum. Though possessing a relatively small genome, approximately 8% of genes are predicted to encode lipoproteins, a relatively large percentage when compared to other extracellular pathogens. These lipid-modified proteins can anchor to the outer membrane and be surface exposed. It has been previously shown that some genes are differentially regulated on a single cell level, with individual cells in the same population displaying a different surface protein profile.

The abundance of lipoproteins encoded by the *B. burgdorferi* genome suggests a possible mechanism for enhancing humoral immune evasion. *B. burgdorferi* could have a large repertoire of surface proteins, including the lipoprotein BBK07, that are differentially expressed by the spirochete population in order to increase the chances of surviving the immune response. A large enough family of surface proteins could allow the inactivation of one or several genes without discernable effect in the murine laboratory model. Functionally redundant protein families in *B. burgdorferi* could explain the myriad of experiments that have reported little to no effects after gene deletion experiments, including BbCRASP-2 (Coleman et al., 2008), BBK32 (Li et al., 2006), OspD (Li et al., 2007a; Stewart et al., 2008), LuxS (Hubner et al., 2003), ChbC (Tilly et al., 2004), BBA36 (Revel et al., 2005). Another possible explanation is that

BBK07 is indeed important for *B. burgdorferi* to persist in the murine host, but that the laboratory model of LD does not provide an opportunity to study the true function of BBK07. While inbred mice, such as the C3H mice used in these studies, display many of the same symptoms that are common in human LD, wild mice such as *Peromyscus leucopus* are not susceptible to disease, and act as the natural reservoir for *B. burgdorferi*. Future studies are required to assess the possibility that BBK07 provides a selective advantage towards persistence in wild mice.

Though the lipoprotein BBK07 is expressed during infection, is immunogenic, and is surface exposed, these data show that immunization with surface exposed amino-terminal half of BBK07 does not interfere with pathogen persistence in the laboratory model of LD. As a result, vaccination with the BBK07 fragment also does not modulate the severity of disease. Furthermore, an isogenic mutant lacking BBK07 was indistinguishable from wild type *B. burgdorferi* during murine infection. In conclusion, these current data suggest that the lipoprotein BBK07 is a nonessential for mouse infectivity, at least in C3H mice, and is not likely to be effective as a vaccine target.

Chapter 5: BBK07 as a novel serodiagnostic marker of Lyme disease

Abstract

Lyme disease (LD) is a common tick-borne infection caused by the bacterial pathogen *Borrelia burgdorferi*. Current laboratory diagnostic tests mostly use borrelial lysates, as well as recombinant and synthetic antigens to detect serum antibodies against *B. burgdorferi* as an indicator of infection. These immunoassays are not entirely effective to detect the infection, especially in the early stages of disease. BBK07, a novel lipoprotein of *B. burgdorferi*, is evaluated as a potential serodiagnostic marker of LD. I show that the *bbk07* gene is expressed at extremely low levels *in vitro* and in ticks, but is dramatically induced by spirochetes once introduced into the host, and is highly expressed throughout mammalian infection. In contrast, the expression of *bbk12,* a paralog of *bbk07* with 87% amino acid identity, although expressed *in vitro*, remained undetectable *in vivo* throughout murine infection and in ticks. BBK07 is localized in the outer membrane and the amino-terminal domain of the antigen is exposed on the microbial surface. A truncated BBK07 protein representing the amino-terminal domain is able to effectively detect antibodies to *B. burgdorferi*, both in experimentally infected mice and in humans. I show that BBK07-based detection is not only sensitive but also highly specific against *B. burgdorferi* infection in human patients from North America. Using an overlapping peptide library of the full-length BBK07 antigen, I identified the immunogenic epitopes of BBK07 during human infection using a human serum panel. I

show that a select combination of immunogenic peptides housed in the amino-terminal region of the protein significantly enhanced BBK07-based diagnostic accuracy when compared to the full-length recombinant protein. An independent serum panel collected from LD patients in the early stages of disease was tested using the BBK07 peptide combination, as well as previously established *B. burgdorferi* serodiagnostic peptides: VlsE-derived C6 and OspC-derived pepC10. While BBK07 peptides had a lower overall sensitivity than the C6 or pepC10 peptides, as determined by IgG or IgM ELISA, respectively, a subset of samples highly reactive to BBK07 peptides failed to recognize either VlsE or OspC peptides. This suggests that BBK07 peptides could be useful to complement the efficacy of current C6 and pepC10-based serodiagnostic assays. Finally, using a panel of canine sera, I show that BBK07 peptide-based serodiagnosis is highly effective for LD diagnosis in infected dogs. Together, these data show that peptides from the *B. burgdorferi* surface protein BBK07 are highly specific and sensitive serodiagnostic markers, and suggest their future use in LD diagnostic assays.

5.1 Introduction

Since the identification of *Borrelia burgdorferi* as the causative agent of Lyme disease (LD) over 25 years ago the number of reported cases of LD has increased steadily (Steere et al., 1977b; Bacon et al., 2008). Every year more than 20,000 patients are diagnosed in the United States, and an estimated 2 million LD serological tests are performed (Aguero-Rosenfeld et al., 1993; Bacon et al., 2008). Difficulties in diagnosis have long complicated the treatment of LD, as the bite of an infected tick may go unnoticed by the patient, and the clinical manifestations of LD can significantly vary amongst diagnosed patients (Steere et al., 2004). Common symptoms such as fever, malaise, and arthritis can resemble those caused by other conditions, further complicating diagnosis. Antibiotic therapy is highly effective, especially if administered in the early stages of LD, however, serious complications can result from false diagnoses and inappropriate treatment (Steere et al., 1993; Ettestad et al., 1995; Tugwell et al., 1997; Brown et al., 1999; Patel et al., 2000). There is no commercially available vaccine for human LD, so the development of accurate, sensitive laboratory diagnostics is an important goal of LD research.

While many laboratory methods have been used to assess *B. burgdorferi* infection, direct detection of the bacterium is difficult due to the low pathogen load in clinical samples (Johnston et al., 1985; Aguero-Rosenfeld et al., 2005). Likewise, the extremely slow growth of *B. burgdorferi*, the high cost and the labor-intensive procedure needed to culture this bacterium have limited the effectiveness of culture as a diagnostic

tool (Nadelman and Wormser, 1998; Stanek and Strle, 2003). PCR detection is possible (Schwartz et al., 1992), but not widely used for diagnosis, due primarily to low sensitivity in tissues, such as cerebrospinal fluid and blood (Aguero-Rosenfeld et al., 2005). Instead, seroreactivity is currently the primary method of laboratory diagnosis of LD (Aguero-Rosenfeld et al., 2005). If the patient does not present erythema migrans, a pathognomonic skin rash, a two-tiered serological approach for LD diagnosis has been recommended by the Centers for Disease Control (CDC, 1995). A tested serum that is considered positive or equivocal by a sensitive method, most commonly enzyme-linked immunosorbent assay (ELISA), is then subsequently tested by an immunoblot analysis against *B. burgdorferi* lysate. The latter method is a specific test, recommended in order to reduce the number of false-positives that are possible using only commercial enzyme assays (Ledue et al., 1996).

Although ELISA using whole-cell lysates is the most frequently used format for the sensitive first-tier testing (Aguero-Rosenfeld et al., 2005), the outcome is inherently limited by difficulties in standardization. The antigenic profile of *in vitro*-grown *B. burgdorferi* can significantly vary by growth phase, number of passages, composition of the complex culture media amongst other factors resulting in substantial batch-to-batch variation in ELISA kits (Schwan et al., 1988; Ramamoorthy and Philipp, 1998). With millions of serum samples tested for LD each year, standardization and automation of serological testing are one of the major goals of LD research (Aguero-Rosenfeld et al., 2005). It has been suggested that dogs may serve as sentinels for LD, as they can be at a higher risk of tick exposure (Lindenmayer et al., 1991). Since dogs do not display the

erythema migrans rash, laboratory diagnosis is important in cases of canine LD, further increasing the demand for serological testing (Appel et al., 1993). Recombinant and synthetic antigen ELISA kits ease standardization, are amenable to automation, and may improve specificity by concentrating *B. burgdorferi*-specific epitopes.

Many recombinant *B. burgodorferi* antigens and various fragments thereof have been evaluated as serodiagnostic markers for LD, including OspC (Padula et al., 1994), BmpA (Simpson et al., 1990), VlsE (Lawrenz et al., 1999), BBK32 (Heikkila et al., 2002), L25 (Mueller et al., 2006), P37 (Magnarelli et al., 2000) and DbpA (Goettner et al., 2005). OspC is exposed on the *B. burgdorferi* surface, is produced during early infection, and is highly immunogenic (Aguero-Rosenfeld et al., 1993; Dressler et al., 1993; Padula et al., 1994; Engstrom et al., 1995). A peptide fragment termed pepC10, containing a conserved immunogenic epitope, has been developed for serodiagnosis (Mathiesen et al., 1998). Though immunogenic, significant protein sequence heterogeneity exists among *B. burgdorferi* isolates, constituting several serotypes, which limit the effectiveness of both OspC (Earnhart et al., 2005) and BmpA as serodiagnostic markers (Roessler et al., 1997). VlsE is a dominant surface-exposed antigen of *B. burgdorferi*, a lipoprotein that undergoes antigenic variation by genetic recombination with silent *vls* cassettes (Zhang et al., 1997). Expressed throughout late infection, VlsE and C6, a conserved peptide fragment of VlsE, have been evaluated as serodiagnostic markers for LD (Lawrenz et al., 1999; Liang et al., 2000; Embers et al., 2007).

These studies suggest that while the use of recombinant proteins can reduce cross-reactivity, thereby enhancing specificity, the use of only select antigens can reduce the sensitivity of the diagnostic test (Magnarelli et al., 1996). Some of the highest sensitivities reported thus far have used several antigens in combination to enhance diagnostic accuracy (Burbelo et al., ; Rasiah et al., 1994; Rauer et al., 1998; Kaiser and Rauer, 1999; Bacon et al., 2003). However, there remains a need for improvement in sensitivity, especially for detection during the earliest stages of disease (Aguero-Rosenfeld et al., 1993). The addition of new immunogenic epitopes could allow these tests to eventually supplant the two-tiered approach, improving both the efficacy and cost of LD testing.

In an effort to more completely catalogue antigens produced during infection, a recent study by Barbour *et al.* used synthetic protein arrays to test the immunogenicity of the majority of *B. burgdorferi* open reading frames (Barbour et al., 2008). Though most open reading frames were not measurably immunogenic, they identified several novel antigens, including BBK07 and BBK12, putative lipoproteins from the linear plasmid lp36. These proteins are extremely similar in sequence, though BBK07 is slightly larger than BBK12 (250 and 232 amino acids, respectively) (Fraser et al., 1997b). The genes are members of paralogous family 59, and are 87% identical in their overlapping amino acid sequences. While both BBK07 and BBK12 were identified as immunogens and potential antigenic markers, a detailed characterization of their expression and the resulting immune response was not explored. I sought to characterize the expression,

surface localization and immune response against BBK07 to further evaluate its inclusion as diagnostic marker to improve the accuracy and sensitivity of LD serodiagnosis.

I then tested the sensitivity and specificities of BBK07-based diagnosis using serum samples from North American and European patients with diagnosed LD and several other conditions including syphilis and autoimmune diseases. I also assessed the serodiagnostic abilities of BBK07 using a full-length protein and an overlapping peptide library, identifying the most immunogenic epitopes of BBK07. I demonstrate that serum testing using a combination of peptides was superior to full-length BBK07 protein. Finally, I show that the peptides are able to detect both canine and human LD effectively, even during the early stages of the disease. The IgG or IgM ELISA further show that a cohort of human LD sera failed to recognized VlsE-derived C6 or OspC-derived pepC10 peptides but exclusively reacted with BBK07 peptides suggesting their potential for enhancing diagnostic sensitivity of early LD.

5.2 Materials and methods

Bacteria, ticks and mice. Isolate A3, a clonal derivative of *Borrelia burgdorferi* B31 M1 and a generous gift from Dr. Patricia Rosa was used throughout the study. Bacteria were grown in BSK-II media at 34°C. The *Ixodes scapularis* ticks used in this study were maintained in the laboratory as described (Pal et al., 2004a). C3H/HeN mice were purchased from the National Cancer Institute. All animal procedures were performed in compliance with the guidelines and with the approval of the Institutional Animal Care and Use Committee. For generation of immunized serum, each mouse was injected with 100 μg of *B. burgdorferi* sonicate (5 mice/group) intradermally. As injections with lysed spirochetes were performed without an adjuvant, all booster injections were performed at weekly intervals for 9 weeks. Polyclonal antibodies against truncated BBK07 protein representing amino terminal region of the mature protein (BBK07N) were obtained by injecting mice intradermally with recombinant protein (10 μg/animal) emulsified in complete Freund's adjuvant once, and twice in incomplete Freund's adjuvant (Sigma) at 10 day intervals. Serum was collected and pooled 10 days after final injection (Coleman et al., 2008). Infected mouse serum was obtained by needle inoculation with Isolate A3, 297 (Pal et al., 2004b), or clinical isolates B408, B491, B500, B515, BL203, or BL206 isolated from human patients as described (Wang et al., 2002).

Purification of recombinant proteins. The recombinant protein fragment BBK07N, containing amino terminal part of the protein and excluding the signal peptide, amino acids 18-142, was fused to an N-terminal 6-histidine tag for purification on the

pET302/NT-His Champion vector (Invitrogen). The following oligonucleotide primers were used to construct the expression vector: forward primer (5' AA*T CTA GA*A TGT GGC ATG TAG ACA ATC CCA TTG 3', *XbaI* site italicized), reverse primer (5' CCG *GGA TCC* ATT ACA TCT TTA GTC CAT TCT T 3', *BamHI* site italicized). Purification was performed using commercial cell lysis buffer (FastBreak, Promega) and MagneHis nickel particles (Promega) according to the manufacturer's instructions. Recombinant VlsE was a generous gift from Fang Ting Liang from Louisiana State University. Recombinant BmpA (Pal et al., 2008b), Lp6.6 (Lahdenne et al., 1997), OspC (Pal et al., 2004b) and BbCRASP-2 (Coleman et al., 2008) were purified as detailed. Full-length BBK07 protein was produced in insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen). The complete BBK07 open reading frame without the native signal peptide was amplified using the following primers, with restriction sites italicized: (forward primer with *BamHI* site, TTT C*GG ATC C*AT GCA CCA CCA CCA CCA CCA CTG GCA TGT AGA CAA TCC CAT TG; reverse primer with *XhoI* site, CCC C*CT CGA G*AA TTA ATT ATT AAA GCA CAA ATG T). The resulting amplicon was inserted into the pFASTBAC-1 vector. A recombinant bacmid was generated with the amino terminal histidine-tagged BBK07 driven by the polyhedrin promoter. Transfection and protein expression were performed in Sf9 cells, according to the manufacturer's instructions. The protein was purified using nickel resins (Probond system, Invitrogen) to 95% purity, as assessed by SDS-PAGE analysis.

Gene expression analysis. Infected ticks (3 ticks per time point), as well as infected mouse skin, hearts, and tibiotarsal joints (3 mice per time point) were homogenized using

mortar and pestle under liquid nitrogen, and total RNA extracted using TRIzol reagent (Invitrogen). RNA from log-phase *in vitro*-grown *B. burgdorferi* (10^7 spirochetes/ml) was also isolated using TRIzol reagent (Invitrogen). The purified RNA was treated with DNase I (NEB) to reduce DNA contamination. One microgram of total RNA from each sample was used to synthesize cDNA using AffinityScript first-strand cDNA synthesis (Stratagene). Quantitative RT-PCR (qRT-PCR) analysis was performed on 50 µg of each cDNA using iQ SybrGreen Supermix (BioRad). To help protect against DNA contamination, cDNA was compared to an equal concentration of template RNA to measure the contribution of DNA to the final results. Standard curves for *flaB* and *bbk07* were generated using *B. burgdorferi* genomic DNA purified by DNeasy Blood and Tissue Kit (Qiagen). The following primers were used for qPCR: *flaB* (5' TTC AAT CAG GTA ACG GCA CA, GAC GCT TGA GAC CCT GAA AG 3'), *bbk07* (5' CCT ATT TCA AGG GCG TGA GC, TAT GGC CAT TGC TGC ATT CT 3'), *bbk12* (5' GCT GAA AAT TCG GTA AGC GTT T, TAA GTT CGC TGC ATA CAC CTT CA 3').

Proteinase K accessibility assay. Proteinase K accessibility assays were performed as described (Brooks et al., 2006), with the following modifications. *B. burgdorferi* (1×10^9) were gently washed three times in 1 ml of PBS (pH 7.4) and collected by centrifugation at 4,000×g for 5 min. Washed spirochetes were then gently resuspended in 100 µl of PBS and split into two equal 50 µl volumes. One aliquot received 10 µg of proteinase K (PK) (Sigma) in PBS while the other aliquot received an equal volume of PBS without PK. Both aliquots were incubated for 20 minutes at room temperature, and then washed

3 times with 1 ml PBS with 1 mM phenylmethylsulfonylfluoride (Sigma) to stop PK activity. After washing, the spirochetes were resuspended in PBS and used for immunoblot analysis. Polyclonal BBK07N antisera was used for immunoblot at a 1:2000 dilution in 5% skim milk. FlaB and OspA antisera was used as described (Yang et al., 2009).

Serum. Serum samples used in the line blot assay were provided by Genzyme Virotech (Germany). For ELISA, the healthy control sera included 25 samples from normal individuals that tested negative for LD, which were provided by CDC and Marylou Breitentein at Yale University. These control sera were used to define the cutoff value in each assay (mean plus 3 SD). Additional infected serum panels were provided by the CDC. Serum panel 1 consisted of 33 serum samples collected from human patients diagnosed with LD, between 2 weeks and 13 years after the onset of symptoms. Serum panel 2 consisted of 60 samples collected from 20 culture-positive patients that presented to medical doctors with erythema migrans. Serum was collected at the first doctor visit after the onset of disease (day 0), and at subsequent visits, 10 and 20 days thereafter. Canine serum samples were provided by the Synbiotics Corporation, and consisted of control and infected samples, as previously defined by serology using commercial ELISA.

ELISA and immunoblotting. For ELISA, antigens were diluted in 50 mM carbonate-bicarbonate buffer, pH 9.6, and coated on Maxisorp microtiter plates (Nunc).

Recombinant proteins and *B. burgdorferi* lysate were coated using 100 ng per well, and all synthetic peptides using 500 ng per well. BBK07 peptides and the C6 peptide were coated directly on ELISA plates. Although the BBK07 peptides were biotinylated at the N-terminus, experiments using streptavidin-coated plates did not influence the sensitivity of the assay (data not shown). However, in experiments using the pepC10 peptide, the plates were first coated with 400 ng per well of streptavidin (MP Biomedicals) before the addition of the peptide, in order to enhance sensitivity. The mean OD values of the control sera plus 3 SD was considered the cutoff value to determine positivity in each sample. The plates were blocked using 1% bovine serum albumin in PBS-T (phosphate buffered saline with 0.05% Tween-20), and plates were washed extensively between all steps with PBS-T. Human and canine sera were diluted in 1:200 or 1:10, respectively, in 1% BSA in PBS-T. Immunoblots were performed using nitrocellulose membranes blocked with 5% skim milk in PBS-T, using murine serum dilutions from 1:200 to 1:2000. Secondary antibodies against IgG conjugated to horseradish peroxidase were used with the following dilutions: goat anti-mouse IgG 1:10000, goat anti-human IgG 1:5000, goat anti-human IgM 1:20000, and goat anti-dog 1:2000 (KPL). All steps were carried out either for one hour at 25°C or overnight at 4°C. ELISA results were quantified using SureBlue TMB Substrate and TMB Stop Solution (KPL).

For line blot assay, recombinant protein was transferred to nitrocellulose membranes by a microdispensing method as described (Goettner et al., 2005). Strips were incubated with human sera (dilution 1:100) and the binding of specific antibodies was detected by using alkaline phosphatase-conjugated goat anti-human IgG serum

(1:100; Genzyme Virotech, Germany). Immunoreactive bands were visualized with the addition of tetramethylbenzidine substrate. Cutoffs were based on a standardized band intensity scale (Genzyme Virotech GmbH, version VI 0803093) of 0 to 5, with 5 as the most intense and 0 as no band visible. Line blots were considered positive if the band intensity was >2.

Peptide synthesis. A total of 23 overlapping peptides (P1-P23) encompassing the mature BBK07 protein were commercially synthesized (PEPscreen system, Sigma). The peptides were 14 amino acids in length, with an overlap of 4 amino acids on the amino and carboxyl terminal sides. The C6 peptide (CMKKDDQIAAAMVLRGMAKDGQFALK) (Liang et al., 1999) and pepC10 peptide (PVVAESPKKP) (Mathiesen et al., 1998) were also commercially synthesized (FlexPeptide system,GenScript USA). All peptides were labeled with biotin at the amino-terminus, and were dissolved in dimethyl sulfoxide, aliquoted and stored at -20°C until use.

Statistics. Results are expressed as the mean ± standard error of the mean (SEM). The significance of the difference between the mean values of the groups was evaluated by two-tailed Student *t*-test. MedCalc (MedCalc Software, Mariakerke, Belgium) was used in the comparison of diagnostic tests. Diagnostic performance of each antigen was compared pairwise using area under curve (AUC) from receiver operator characteristic (ROC) analysis, by the method of DeLong et al. (DeLong et al., 1988).

5.3 Results

***bbk07,* but not the paralogous gene *bbk12,* is selectively expressed in the mammal during the infection cycle of *B. burgdorferi*.** The paralogous gene products *bbk07* and *bbk12* have recently been identified as potential immunogens of *B. burgdorferi* (Barbour et al., 2008). The genes are highly homologous, with 87% amino acid identity in their overlapping sequences (Fraser et al., 1997b). Due to the nearly identical sequences of BBK07 and BBK12, it is unclear if the host immune response is directed against either or both genes. To ascertain their individual expression patterns, I developed two sets of oligonucleotide primer pairs targeting variable regions of each gene, which specifically amplified either *bbk07* or *bbk12* with low cross-reactivity, as confirmed by the DNA sequencing of the corresponding amplicons. These primers were then used to determine the relative expression levels of each gene in cultured spirochetes or infected host tissue by qRT-PCR analysis. While both genes were transcribed at relatively low levels *in vitro*, only *bbk07* was detectable *in vivo*, as shown in infected mouse dermis 1 week after inoculation (Fig. 12). Strikingly, the transcriptional level of *bbk07* is more than 100 fold higher in the infected host tissue than *in vitro*.

Because of the relatively high transcription level of *bbk07* in the infected host tissue, I then studied the expression of *bbk07* in the *B. burgdorferi* life cycle, covering the first 4 weeks of murine infection. Total RNA was isolated from experimentally infected

tick and mouse tissues to generate cDNA representative of important stages in the *B. burgdorferi* life cycle: transmission from infected ticks, murine infection, acquisition by naïve ticks, and persistence through the tick molt. While consistently expressed in multiple murine tissues during the first 4 weeks of murine infection, the transcription of *bbk07* was dramatically reduced below the limit of detection in all tested stages of ticks (Fig. 13). The same RNA samples did not contain detectable quantities of *bbk12* transcripts at any tissues or time point examined.

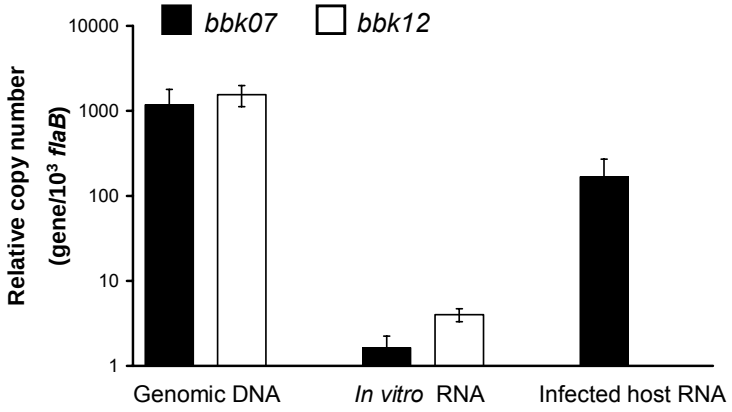

Figure 12. *bbk07* is selectively expressed during infection.
bbk07 and *bbk12* expression by *B. burgdorferi*. qRT-PCR was performed using gene-specific primers to determine if *bbk07*, *bbk12*, or both are transcribed by *B. burgdorferi in vivo* and *in vitro*, normalized to *flaB*. Both gene transcripts were detectable at relatively low levels *in vitro*, but only *bbk07* transcripts were present in infected murine skin samples 1 week after inoculation.

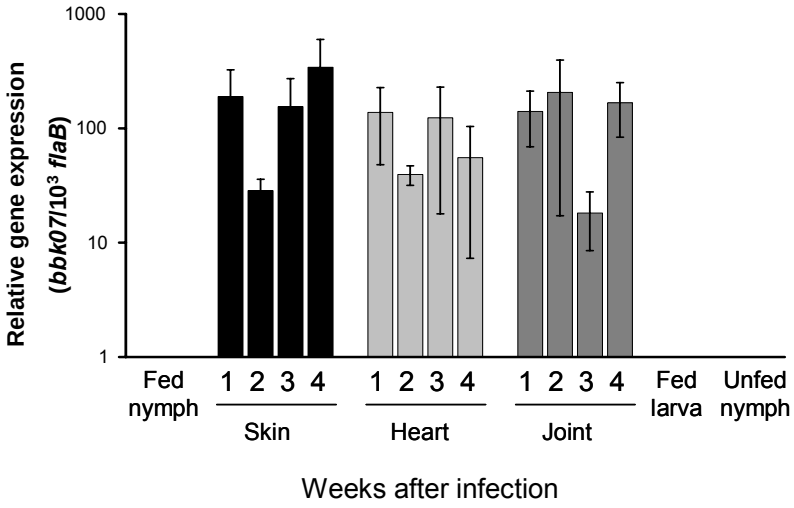

Figure 13. *bbk07* expression pattern *in vivo*.
The transcriptional profile of *bbk07* throughout representative stages of the life cycle of *B. burgdorferi* was measured by qRT-PCR. Mice (3 mice per time point) and ticks (3 ticks per time point) were infected with *B. burgdorferi*. Total RNA was isolated from infected tissues representing the complete life cycle of *B. burgdorferi*: fed nymphs, infected murine tissues collected at weekly intervals through four weeks after infection, feeding larva, and unfed nymphs. *bbk07* was expressed in all murine tissues tested but was undetectable in tick samples. Bars represent the mean and SEM of four qPCR measurements from two independent infection experiments.

Amino-terminal region of BBK07 is surface exposed and immunogenic. Since *bbk07* is annotated as a lipoprotein, which might be exposed on the spirochete surface, I next assessed the surface localization of BBK07. Expressing a full-length or truncated protein representing carboxy-terminal half of BBK07 proved difficult in *Escherichia coli*, however, an amino-terminal fragment could be purified in sufficient quantities and used for further experimentation. This fragment contained the amino terminus through the first half of the mature protein, referred to as BBK07N (Fig. 14A). Specific antiserum was generated by immunizing mice with BBK07N and adjuvant. In agreement with a previous study showing the immunogenicity of *in vitro* translated BBK07 (Barbour et al., 2008), BBK07N also evoked a robust immune response and BBK07N anti-serum

recognized both purified BBK07N and native BBK07 from *B. burgdorferi* lysate (Fig. 14B).

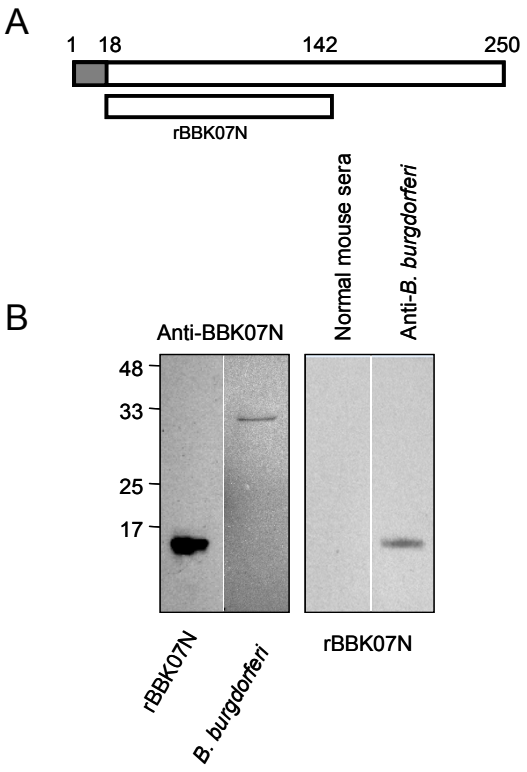

Figure 14. Amino terminal region of BBK07 is immunogenic.
(A) Schematic of BBK07, showing location of recombinant amino-terminal BBK07 protein fragment used in the current study. A protein fragment lacking the putative signal peptide (grey box) starting from amino-terminal to half of the protein length was purified, and termed BBK07N.
(B) Generated mouse anti-BBK07N sera recognizes recombinant truncated protein and native BBK07 (left panels), while recombinant BBK07N detects BBK07-specific antibody in infected mouse serum two weeks after infection (right panels). Mice were immunized with BBK07N in order to generate BBK07N antiserum. The resulting serum was used to probe 100 ng of purified BBK07N and 1 µg of *B. burgdorferi* cell lysate.

To test the surface exposure of BBK07, a proteinase K accessibility assay was performed (Coleman et al., 2008). Intact *B. burgdorferi* were incubated with and without proteinase K, and probed with FlaB, OspA or BBK07N antiserum. FlaB, a known

subsurface protein, was not degraded, but both the surface protein OspA and BBK07 were significantly degraded, suggesting that the amino terminal region of BBK07 is surface exposed (Fig. 15).

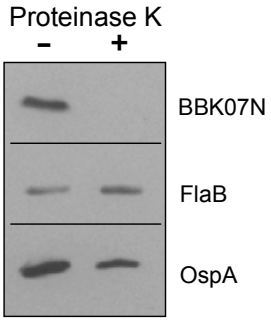

Figure 15. Surface exposure of BBK07. Viable *B. burgdorferi* cells were incubated with (+) or without (-) Proteinase K and subjected to immunoblotting using antiserum against BBK07N, FlaB and OspA. FlaB and OspA were used as subsurface and surface controls, respectively. While the levels of FlaB did not decrease, both OspA and BBK07 showed degradation in the presence of Proteinase K.

BBK07-specific immune response is pronounced during active borrelial infection but absent in hosts immunized with lysed pathogens. Because qRT-PCR analysis indicated a dramatic induction of *bbk07 in vivo* during early infection, I next assessed kinetics of BBK07 antibody development in the host over the first nine weeks of *B. burgdorferi* infection. As qRT-PCR analysis indicated minor expression of *bbk07 in vitro*, I also assessed, in parallel, BBK07 antibody development in mice immunized with sonicated spirochetes, in order to test whether BBK07 could differentiate infected hosts from ones vaccinated with killed pathogens. To accomplish this, groups of mice (5 animals/group) were needle-inoculated with a single *B. burgdorferi* inoculum (10^5 cells/mice). In parallel, another group of mice (5 animals/group) were injected with

sonicated *B. burgdorferi* (100 µg/mice) at 7 days intervals for a total of 9 weeks. Serum samples were collected and pooled weekly. Equal amounts of *B. burgdorferi* lysate or BBK07N were used to detect specific antibodies present in each serum sample by ELISA (Fig. 16A). As a negative control, antibody development against the *B. burgdorferi* antigen Lp6.6, which is abundant *in vitro* but known to be downregulated during murine infection, was also measured (Lahdenne et al., 1997). As expected, antibodies against *B. burgdorferi* lysate, but not against Lp6.6, were detected in infected mice (Lahdenne et al., 1997; Brooks et al., 2003). BBK07 provoked a robust antibody development that was detectable after one week, and remained elevated throughout the infection. In contrast, the mice immunized weekly with lysed spirochetes produced a low BBK07 antibody response that did not increase over the course of the experiment, while Lp6.6 provoked a robust antibody response (Fig. 16B). In order to confirm that the immunogenicity of BBK07 is not confined to needle-borne artificial murine infection, groups of naïve mice were infected by tick bite. Serum samples were collected after 4 weeks of infection, and tested by ELISA (Fig. 16C). The serum contained a similar response against both lysate and BBK07, indicating that the antibody response against BBK07 does not depend on route of infection.

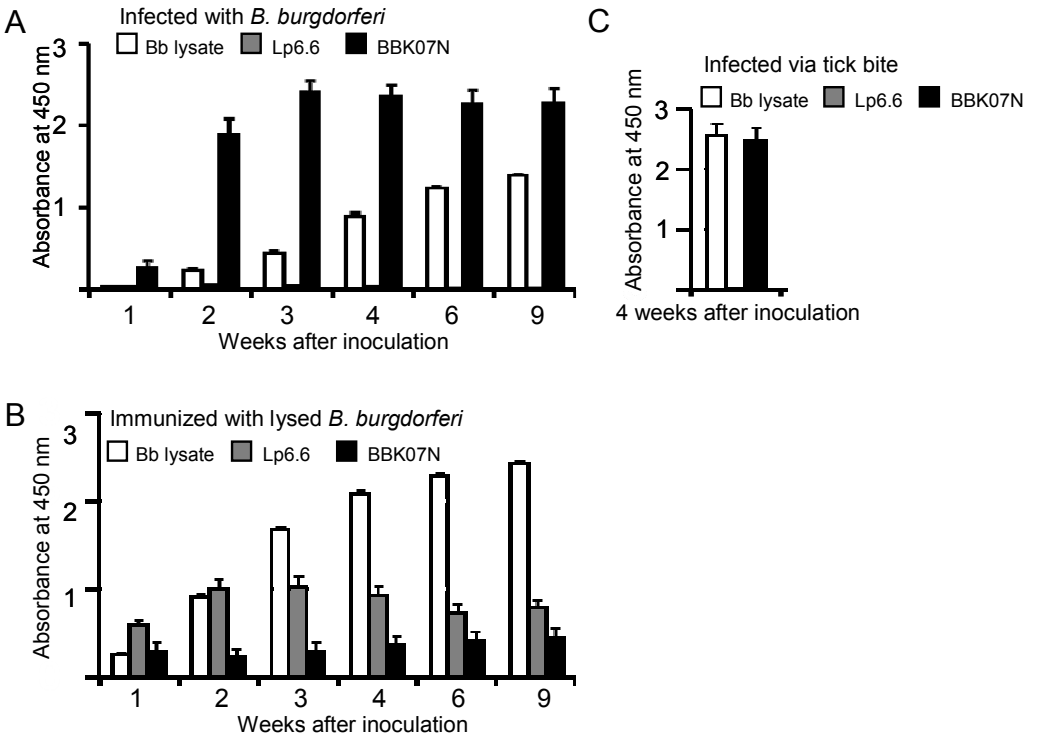

Figure 16. BBK07-specific antibody develops early during infection but absent in hosts immunized with lysed pathogens.
(A) BBK07 antibodies are detectable during early infection. Serum samples from mice needle-inoculated with *B. burgdorferi* were collected at several time points after infection. The immune response to *B. burgdorferi* lysate, purified Lp6.6 and BBK07N proteins was measured by ELISA.
(B) Mice immunized with sonicated *B. burgdorferi* generate an insignificant immune response against BBK07. Mice were immunized weekly with *B. burgdorferi* lysate and serum samples were tested by ELISA.
(C) BBK07 antibodies present during natural tick-borne infection of mice. Mice were infected by tick bite, and serum samples tested by ELISA. The immune response was similar to that of the needle-infected mice. The bars represent the mean and SEM of duplicate measurements.

Evaluation of BBK07N as a diagnostic marker for *B. burgdorferi* infection in murine hosts. The robust and specific immune response provoked by BBK07 led me to investigate a possible diagnostic use of BBK07N. Using mouse serum collected 2 weeks after infection, I compared the immunogenicity of BBK07N to several other immunogenic *B. burgdorferi* antigens, such as VlsE, OspC, BmpA and BbCRASP-2. As

controls, *B. burgdorferi* lysate and Lp6.6 were also included in the assay. To measure the relative immunogenicity of each antigen, equal amounts of proteins and lysate were used in an ELISA, probed with the infected mouse serum (Fig. 17). Due to the high antibody titers detected by BBK07N and VlsE, which quickly reached upper detection limit of the assay, the reaction was stopped shortly (1 minute) after the addition of chromogenic substrate. As expected, naïve sera had low reactivity to all antigens. Amongst all antigens tested, BBK07N reflected the most robust immune response, proving more sensitive than several antigens currently used in LD diagnosis.

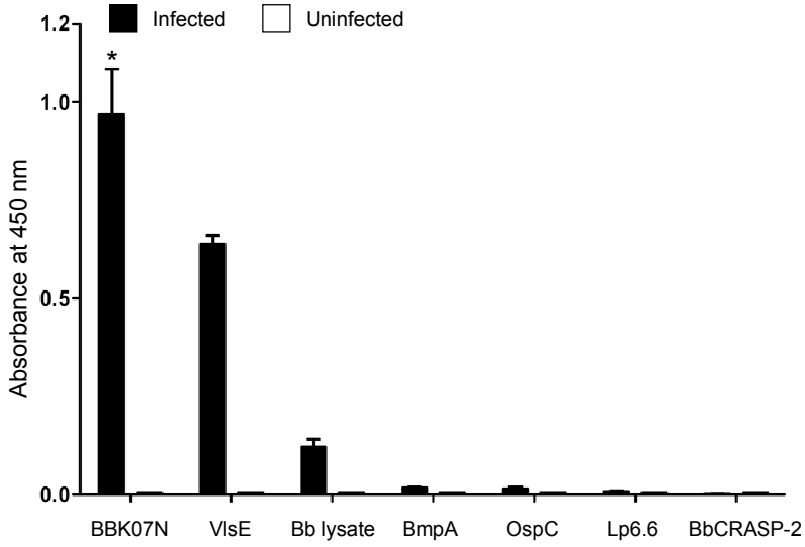

Figure 17. Comparative immunogenicity of BBK07 against other antigens in mice. Pooled sera from groups of two-week post-infection (black bars) and uninfected (white bars) mice were used to probe 100 ng of purified proteins or sonicated *B. burgdorferi* lysate and the levels of specific antibody responses were measured by ELISA. Naïve mouse sera had low reactivity to all antigens. As wells containing BBK07N and VlsE quickly reached the upper detection limit of the assay, the reaction was stopped 1 minute after the addition of the chromogenic substrate. The bars represent the mean and SEM from duplicate measurements of two independent mouse experiments. Except for VlsE, differences in antibody responses against BBK07N are significantly higher than other antigens (*$P < 0.05$).

BBK07 is highly sensitive and specific against *B. burgdorferi* infection in North American and European patients. As BBK07N protein proved highly reactive in infected murine sera, I next evaluated if it could be used for detection of human LD in North America. While *bbk07* is highly conserved among *B. burgdorferi* sensu stricto isolates present both in North America and Europe, there are additional borrelial genospecies that cause LD, also known as Lyme borreliosis (LB) in Europe, such as *B. garinii* and *B. afzelii*. The latter species do not contain linear plasmid 36 (plasmid k) that harbors the *bbk07* gene, and lack an orthologue of BBK07. Therefore, while BBK07 serodiagnosis could be ineffective in Europe for universal diagnosis of LB, but it could be useful in discriminating *B. burgdorferi* infection from other genospecies. To test this hypothesis, the recombinant BBK07N was tested using a line blot assay which is widely used for serodiagnosis of LB in Europe (Hunfeld and Kraiczy, 2009). To assess the specificity of BBK07-based diagnosis, a set of 20 German and United States patients with diagnosed infection, as well as serum from normal healthy individuals, pregnant women, and patients with other conditions, including autoimmune diseases, and syphilis were compared in a line blot assay and the results were scored using a standardized band intensity scale (Table 4). While BBK07 was highly sensitive (90%) in detecting *B. burgdorferi* infection in serum from the United States, the German patients with LB, other diseases, or healthy controls showed minor reactivity. These data underscore the high sensitivity and specificity of BBK07-based diagnosis of *B. burgdorferi* infection in US patients. The observed lower sensitivity of BBK07 reactivity in European LB patient sera likely results from the infection with other *B. burgdorferi* sensu lato species.

Table 4. Assessment of serodiagnosis potential of BBK07N in a Line blot assay.

Serum panel		BBK07N line blot results	
US sera	Lyme IgG positive	18/20	90%
German sera	Early Lyme Disease	1/20	5%
	Neuroborreliosis	1/20	5%
	Lyme arthritis	2/20	10%
	ACA	4/20	20%
Control sera	Healthy blood donors	0/20	0%
	EBV infections	0/10	0%
	Syphilis infections	0/10	0%
	Pregnant women	0/10	0%
	Autoimmune diseases	1/10	10%
	Rheumatoid factor	0/10	0%

Human serum samples were analyzed by a line blot assay containing various amounts of the recombinant fragment BBK07N. ACA, Acrodermatitis chronica atrophicans; EBV, primary Epstein Barr virus infection.

BBK07-specific immune response is elicited during murine infection with several clinical isolates of *B. burgdorferi* sensu stricto. In order to further characterize the diagnostic potential of BBK07, I measured its conservation and immunogenicity in multiple clinical strains, which were isolated from human patients from North America (Wang et al., 2002). The *B. burgdorferi* clinical isolates were grown in BSK medium, were injected into mice, and serum was collected at 2 or 3 weeks following infection. Infected sera were used to probe recombinant BBK07N protein by immunoblot analysis (Fig. 18). A BBK07-specific antibody response was highly detectable in mice infected with the *B. Burgdorferi* B31 laboratory isolate A3, as well as all clinical isolates. These data highlight the ability of BBK07-based serodiagnostic assay to detect LD infection with multiple clinical isolates of *B. burgdorferi* sensu stricto.

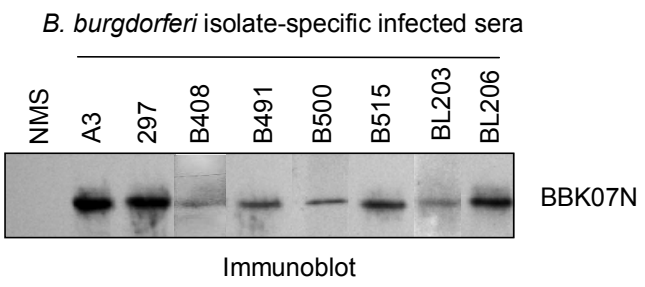

Figure 18. Conservation of BBK07 immunoreactivity in clinical *B. burgdorferi* isolates.
Mice were infected with several *B. burgdorferi* isolates isolated from infected human patients in North America. Serum from the infected mice was collected and used to probe 200 ng of recombinant BBK07N protein to test for an immune response against BBK07. A BBK07-specific immune response was detected in the widely-used laboratory strain (A3), as well as each mouse infected with a human isolate (297 through BL206). No antibodies were detected using normal mouse serum (NMS).

Identification of immunogenic epitopes of BBK07. I next mapped the immunodominant epitopes of BBK07 using human sera. To accomplish this, a library consisting of 23 overlapping peptides was synthesized, covering the full-length mature BBK07 (Table 5). Each peptide was tested for ELISA reactivity in serum samples from 25 healthy donors, and 33 patients diagnosed with LD from serum panel 1. Given the number of peptides and the limited supply of serum, all peptides were screened once with each serum sample, and the most immunogenic peptides were repeated in three independent experiments to validate the results. Results indicated that peptides P1 (CKWHVDNPIDEATA), P5 (ITKLTPEELENLAK), and P7 (EKSKKEIEDQKNTK) harbored the most immunodominant epitopes recognized by the infected sera (Fig. 19A). Samples were considered positive if they exceeded the cutoff value in at least 2 out of 3 independent ELISA (Fig. 19B). I speculated that additional testing of other peptides was unlikely to significantly increase sensitivity, as the serum samples reactive to other peptides were also reactive to peptides P1, P5 or P7. I therefore focused my attention to

these 3 peptides and repeated measurements confirmed that amongst the P1, P5 and P7 peptides, P5 housed the most immunogenic epitopes and was able to distinguish 24% (8 out of 33) of the LD diagnosed patient sera from control. In order to assess if the combination of the three most immunogenic peptides increased the sensitivity of the assay, the peptides were mixed and tested against the serum panel (Fig. 19B). The results indicated that the combination of 3 peptides had a synergistic effect and increased the diagnostic accuracy to 52% (P1P5P7, 17 out of 33), significantly increasing the test performance by receiver operator characteristic (ROC) analysis ($P<0.05$).

Table 5. Amino acid sequences of BBK07 peptide library

Peptide name	Amino acid sequence
P1	CKWHVDNPIDEATA
P2	EATAESKSALTSVD
P3	TSVDQVLDEISEAT
P4	SEATGLSSEKITKL
P5	ITKLTPEELENLAK
P6	NLAKEAQDDSEKSK
P7	EKSKKEIEDQKNTK
P8	KNTKESKNIEVKDT
P9	VKDTPRLIKLIKNS
P10	IKNSSEKIDSVFQT
P11	VFQTLINIGYNATY
P12	NATYAAKSNLKNGL
P13	KNGLKMVKLLDELL
P14	DELLKISVSSNGDK
P15	NGDKSTQKYNELKT
P16	ELKTVVNRFNAENS
P17	AENSAIKVPLENGS
P18	ENGSKIEAKKCIKT
P19	CIKTLMTNVETYFK
P20	TYFKGVSTELKDKK
P21	KDKKDDKYTKILAA
P22	ILAALSEAANKIEN
P23	KIENAAMAIHLCFNN

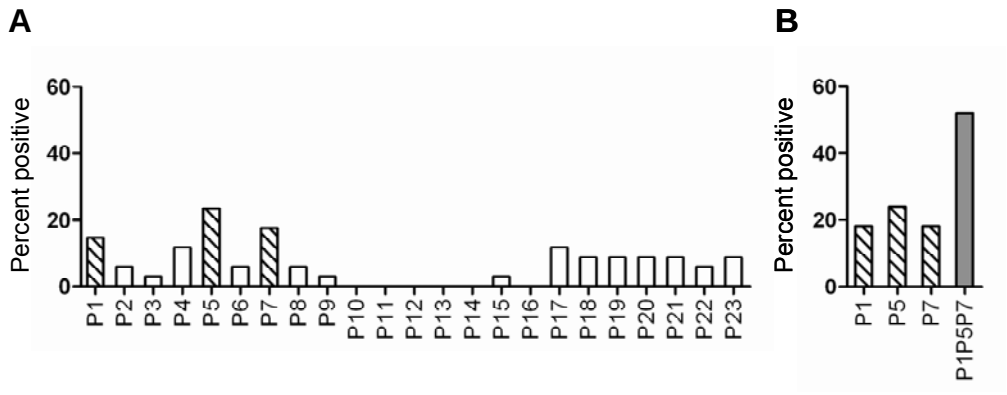

Figure 19. Identification of immunodominant epitopes of BBK07.
(A) Epitope mapping of BBK07 using infected human sera. A library of 23 overlapping BBK07 peptides was screened for reactivity in sera from 33 patients diagnosed with Lyme disease (LD) or from 25 healthy donors. Microtiter wells were coated with individual BBK07 peptides (500 ng/well) and incubated with individual patient or healthy donor serum (1:200 dilutions). The signal was generated using goat secondary antibodies against human IgG conjugated to horseradish peroxidase at the dilution of 1:5000. The cutoff value for each peptide was the mean plus 3 SD of the healthy control sera. Bars represent the percentage of diagnosed serum samples above the cutoff value.
(B) A peptide combination significantly enhances BBK07-based serodiagnostic ability compared to individual peptides. Peptides P1, P5, P7, and all 3 peptides combined (P1P5P7) were tested in three independent ELISA measurements for reactivity with human serum. Serum samples were considered positive if above the cutoff value in at least 2 out of 3 experiments.

A peptide combination of BBK07 provides greater diagnostic accuracy than recombinant BBK07. I have demonstrated that a combination of BBK07 peptides and a recombinant amino-terminal fragment of BBK07 (Coleman and Pal, 2009) are effective in LD diagnosis. To see if the presence of conformational epitopes could improve the sensitivity of BBK07-based serodiagnosis, I produced recombinant full-length BBK07 in insect cells using a baculovirus expression system. The full-length protein was tested against serum panel 1 as detained before, and its reactivity was compared to the recombinant amino-terminal fragment BBK07N, and the BBK07 peptide combination. *B. burgdorferi* lysate was also included as a positive control. Three independent measurements were performed, which produced similar results (data not shown) and a

representative experiment is shown (Fig. 20). While I expected that the full-length BBK07 (13 out of 33, 39%) should provide more epitopes for antibody recognition, the amino-terminal fragment BBK07N (14 out of 33, 42%) shows no significant reduction in diagnostic accuracy, as assessed by statistical analysis (P=0.181). However, the peptide combination (17 out of 33, 52%) showed a superior diagnostic accuracy than either BBK07N or full-length BBK07. Statistical analyses further indicated that there was a significant improvement in test performance from the full-length protein to the peptide combination (P=0.048), but not between the peptide combination and the recombinant fragment BBK07N (P=0.252).

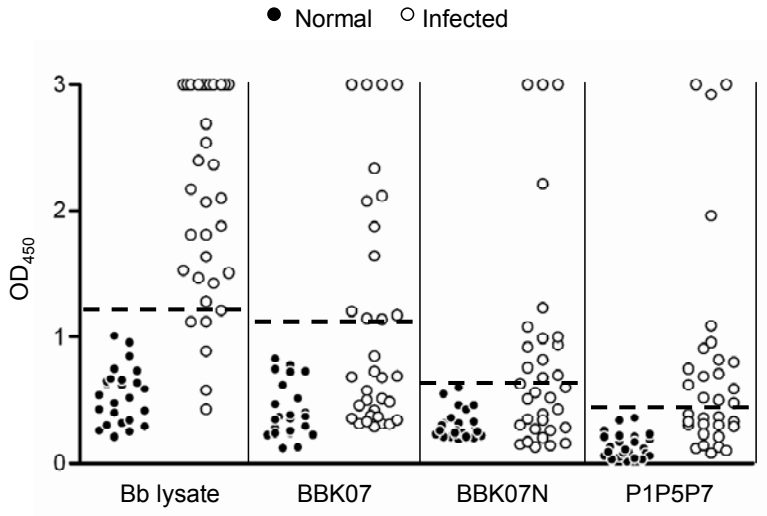

Figure 20. Immunoreactivities of full-length or truncated BBK07 proteins and peptides to individual Lyme disease patient sera.
Microtiter wells were coated with 100 ng of *B. burgdorferi* lysate (Bb lysate), recombinant full-length BBK07 protein (BBK07), the N-terminal BBK07 fragment (BBK07N), or a combination of immunodominant peptides P1, P5, and P7 (P1P5P7, 500 ng each). Immunoreactivity of individual serum samples from healthy donors (black circle) and from patients diagnosed with LD (white circle) was measured by ELISA. The horizontal dashed line indicates the cutoff value calculated for each antigen from the healthy sera. Values higher than 3 are represented on the graph as 3 for clarity of presentation.

BBK07 peptide-based diagnosis is effective during early infection. I next assessed if BBK07-based diagnosis is effective in early LD infection. Since the peptide combination

displayed the highest BBK07-based serodiagnostic efficiency, this was used in the study. Serum samples (serum panel 2) were collected from patients displaying erythema migrans on their first visit to a physician. Biopsies from all patients were later culture positive for *B. burgdorferi*, and serum from each patient was collected again at 10 and 20 days after the first visit (day 0) to the doctor. Note that the serum is first collected from the patients upon their first visit to a physician, and not the time of initial exposure. Therefore, while it is not known how long after infection the serum samples were collected, the appearance of erythema migrans on their first visit to a physician makes it likely to be within the first few weeks of *B. burgdorferi* infection (Steere, 2006). The control sera from serum panel 1 were used to define the cutoff for each antigen as detailed before, and each antigen was used to test serum panel 2 in three independent measurements. The BBK07 peptide combination P1P5P7 had a similar diagnostic accuracy detecting IgG at day 10 as observed using serum panel 1 (10 out of 20, 50%) (Fig. 21). To further assess the effectiveness of the P1P5P7 peptide combination in the diagnosis of early disease, it was tested for both IgM and IgG reactivity using appropriate controls, such as the OspC peptide pepC10 for IgM (Mathiesen et al., 1998) and the VlsE peptide C6 for IgG detection (Liang et al., 1999). The results show that, compared to BBK07, the peptides pepC10 and C6 showed higher overall sensitivities in detecting IgM and IgG, respectively (Table 6). However, a subset of patients that displayed negative reactivity to the pepC10 and C6 peptides positively reacted against P1P5P7 combination (Table 6). These data indicate a heterogeneity in the immune response of the tested patients, and suggests the potential use of BBK07 peptides for enhancing the diagnostic accuracy of LD detection.

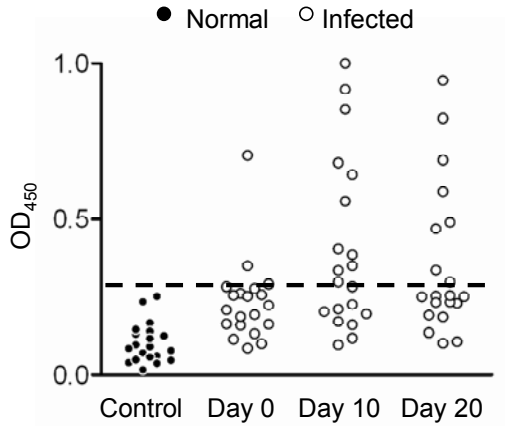

Figure 21. BBK07 antibody development in LD patients during early infection. Early immunoreactivity to BBK07 peptides. The BBK07 peptide combination (P1P5P7) was assessed for immunoreactivity against the same cohort of human patients during early LD. Sera from a group of twenty patients experiencing the early symptoms of LD were collected during the initial doctor visit (Day 0) and two subsequent visits (Day 10) and (Day 20) and were used in the assay. Sera from twenty normal healthy controls were used to establish a cutoff value, and a representative experiment is shown.

Table 6. Individual patient reactivity against BBK07 (P1P5P7), OspC (pepC10) and VlsE (C6) peptide-based serodiagnosis of early LD

IgM	**P1P5P7**			**pepC10**		
Patient	Initial visit	Day 10	Day 20	Initial visit	Day 10	Day 20
1	**-**	**+**	**+**	**-**	**-**	**-**
2	-	-	-	-	+	+
3	-	-	-	-	+	+
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	+	+
9	-	-	-	-	-	-
10	-	-	-	+	+	+
11	-	-	-	-	-	-
12	-	-	-	+	+	+
13	-	+	-	-	+	+
14	-	+	-	-	+	+
15	-	-	-	+	+	+
16	-	+	-	-	+	-
17	-	-	-	-	-	-
18	**-**	**-**	**+**	**-**	**-**	**-**
19	-	-	-	-	-	-
20	-	-	-	-	+	+

IgG	**P1P5P7**			**C6**		
Patient	Initial visit	Day 10	Day 20	Initial visit	Day 10	Day 20
1	-	+	+	+	+	+
2	-	+	-	+	+	+
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	+	+
6	-	-	-	+	+	+
7	-	-	-	+	-	-
8	-	-	-	-	+	+
9	**+**	**+**	**+**	**-**	**-**	**+**
10	-	-	-	+	+	+
11	-	+	-	+	+	+
12	-	-	-	+	+	+
13	-	+	+	-	+	+
14	**-**	**+**	**+**	**-**	**-**	**-**
15	-	+	+	+	+	+
16	-	+	+	-	+	+
17	+	+	+	+	+	+
18	**-**	**+**	**+**	**-**	**-**	**-**
19	-	-	-	+	+	+
20	+	-	-	+	+	+

BBK07 peptide-based diagnosis is highly effective in canine LD. As BBK07 immunoreactivity is an effective serodiagnostic marker for the detection of *B. burgdorferi* infection in mice and humans (Coleman and Pal, 2009), I next assessed if the antigen could be used to detect LD in dogs that are routinely screened for LD infection. A panel of 55 uninfected and 46 *B. burgdorferi*-infected canine serum samples was assessed using the BBK07 peptides P1P5P7 in an ELISA as before. The assay was repeated with similar results, and a representative experiment is shown (Fig. 22). The results show that, like humans, a peptide from BBK07 is also effective (35 out of 46, 76%) for the detection of LD in dogs.

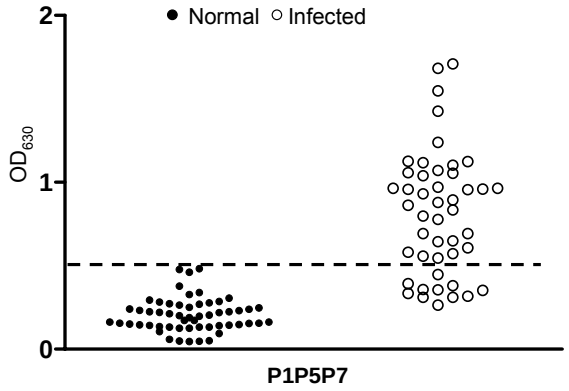

Figure 22. Immunodominant BBK07 peptides as a potential antigenic target for serodiagnosis of canine Lyme disease. Canine sera from healthy individuals (black circles, n=55) and ones diagnosed with Lyme disease (open circles, n=46) were used to assess immunoreactivity of the BBK07 peptide combination P1P5P7 by ELISA. Optical density values of control dog serum were used as controls to set the cutoff value for the antigen (horizontal dashed line).

5.4 Discussion

The identification and characterization of *in vivo* antigens of *B. burgdorferi* is central to the improvement of current laboratory diagnostics for LD. A previous study identified BBK07 and BBK12 as novel immunogenic antigens of *B. burgdorferi* (Barbour et al., 2008). I further extend the observation and establish that *bbk07*, but not highly similar paralogous member *bbk12*, was expressed at relatively high levels *in vivo*. I show that a recombinant protein representing the amino-terminal region of BBK07 was able to provoke a specific antibody response against the native protein, providing antibodies that were then used to demonstrate the surface exposure of the amino-terminal region of BBK07. The recombinant protein could, accordingly, detect a specific antibody response to active infection with *B. burgdorferi*. As *bbk07* had negligible expression *in vitro*, I show that this antigen could be useful in discriminating antibody development during active infection versus hosts vaccinated with killed pathogen preparations. The detected antibody response during murine infection was more robust than that detected by several previously characterized serodiagnostic antigens (Bacon et al., 2003; Aguero-Rosenfeld et al., 2005).

To further evaluate the potential of BBK07 as a serodiagnostic marker, I assessed its sensitivity and specificity using diverse sets of serum collected from the United States and Germany where patients were chosen from diagnosed LD, several relevant and cross-reactive diseases, and normal healthy controls. When mice were infected with seven

different clinical isolates of *B. burgdorferi* originally cultured from human patients, each isolate prompted a robust antibody response to BBK07, suggesting wide conservation of BBK07 immunogenicity amongst clinical isolates. Using a library of 23 overlapping peptides and a human LD serum panel, I have identified most immunodominant epitopes of BBK07. I also compared the reactivity of BBK07 peptides and proteins and show that the combination of three amino terminal peptides is a significantly more effective serodiagnostic marker than the full-length antigen. I then show that this peptide combination can be used as a marker in the earliest stages of disease, effectively diagnosing some patients that lack reactivity to the OspC peptide (pepC10) or VlsE-derived C6 peptide. Finally, I show that these results may be applicable to other non-human species at high risk of *B. burgdorferi* infection, as a peptide from BBK07 was able to effectively detect LD in canine serum samples. Overall, these data identified most immunodominant epitopes of BBK07 and highlighted the usefulness of a peptide combination of BBK07 as potential components of a highly sensitive and specific test for human and canine LD.

These data completely support a previous study showing that BBK07 is highly immunogenic during LD (Barbour et al., 2008). However, plasmid lp36, which contained *bbk07* locus could be lost during *in vitro* growth (Jewett et al., 2007), and these data showing extremely low *in vitro* expression of *bbk07* suggest that BBK07 is under-represented in tests using *in vitro* grown *B. burgdorferi* (Barbour et al., 2008). The inclusion of BBK07 as a diagnostic marker could increase serodiagnostic sensitivity in human patients while maintaining the high specificity afforded by recombinant antigen

tests. The low *in vitro* expression of *bbk07* and undetectable immune response against sonicated borrelial cells suggests additional use of BBK07 in animal LD diagnosis. An animal LD vaccine is commercially available that utilizes killed *in vitro* grown *B. burgdorferi* (Chu et al., 1992; LaFleur et al., 2009). The presence of BBK07 antibodies could serve as an indicator of active infection, as BBK07 reactivity is unlikely in vaccinated animals immunized with cultured organisms (Gauthier and Mansfield, 1999). Thus, BBK07 could differentiate between infected and vaccinated animals. Sequence analysis also indicates that *Borrelia garinii* and *Borrelia afzelii*, the most prevalent causative agents of LD in Europe and Asia, lack an ortholog to BBK07 therefore, BBK07 reactivity could be used to discriminate human LD caused by *B. burgdorferi* from that caused by other strains. As expected, these data show that patients from North America had a higher reactivity than European patients.

The effectiveness of some *B. burgdorferi* antigens has been reduced by sequence variation in the bacterial population, including VlsE (Iyer et al., 2000), OspC (Earnhart et al., 2005), and BmpA (Roessler et al., 1997). These data indicate that BBK07 immunoreactivity is detectable across the *B. burgdorferi* isolates present in North America, but not in European LD patients. Although the *bbk07* gene is highly conserved in *B. burgdorferi* sensu stricto isolates in United States, the linear plasmid carrying the *bbk07* gene or an ortholog thereof is absent in major *B. burgdorferi* sensu lato strains prevalent in Europe. Therefore, while BBK07 diagnosis is unlikely to be effective in Europe, reactivity to BBK07 or similar antigens absent in other *B. burgdorferi* sensu lato species could aid physicians or researchers in differentiating between individuals infected

with *B. burgdorferi* or the European strains (Barbour et al., 2008). As immunoreactivity could potentially vary across mammalian species, the effectiveness of a BBK07 peptide to diagnose canine LD also testifies to the future use of BBK07-based diagnosis in companion animals.

These data show that BBK07 peptides could be used to correctly diagnose nearly 50% of the tested human sera. While this is well below the sensitivity required of a stand-alone test, it suggests that BBK07 peptides could be a valuable addition to a future multi-antigen test. Though many other *B. burgdorferi* antigens have been studied for their diagnostic potential (Aguero-Rosenfeld et al., 2005), no single antigen has proved sensitive enough to replace the current two-tiered testing recommended by the CDC, especially in the early stages of LD. Therefore, several studies have evaluated antigen combinations intended to increase the test sensitivity (Aguero-Rosenfeld et al., 2005). Peptide antigens are highly desirable in this application, as a reduced antigen size can minimize the chances of cross-reactivity and maximize the concentration of important epitopes. Additionally, compared to bacterial antigens used in serodiagnosis, the use of synthetic peptides has the advantage of less cross-reactivity to uninfected humans exposed to microbial infection. These data show that a combination of several BBK07 peptides is more effective than the full-length recombinant BBK07 in LD diagnosis, and thus is an attractive candidate for future peptide diagnostics.

My primary screen of the peptide library showed that the most immunogenic peptides of BBK07 were located on the amino-terminus of the protein. This finding is in

agreement with my data showing that an amino-terminal recombinant fragment of BBK07, BBK07N, had a similar diagnostic accuracy to that of the full-length protein. My previous work also indicated that the amino-terminal half of BBK07 was accessible to protease degradation on the surface of *B. burgdorferi* cells. Taken together, these data confirm that the amino-terminal region is highly antigenic, and thus is a prime target for the host's immunological response, thus housing the most immunodominant epitopes of BBK07. Two of the most effective BBK07 peptide antigens, P5 and P7, are situated only six amino acids apart on the amino-terminal region of BBK07. Their proximity immediately suggests that a longer peptide, encompassing P5, P6, and P7, could effectively harness the linear epitopes of each on a single molecule. In addition, the longer peptide could form limited conformational epitopes present in this region of the protein, further increasing sensitivity.

BBK07 peptide diagnosis could be enhanced by improvements in antibody detection techniques. In this study, I utilized a simple ELISA test, absorbing the peptides directly on the plastic surface in a high pH buffer. The sensitivity of the test is then dependent on the ability of the plate to bind and present the peptide. In this assay, each peptide was biotinylated on their amino-terminus, but streptavidin-coated plates did not increase the sensitivity of the test. Chemical conjugation to solid surfaces or fusions with carrier proteins have been used in the past to improve peptide binding and display, and could be applied to further enhance sensitivity of BBK07 peptide-based diagnosis (Gomara and Haro, 2007). A recent study demonstrated enhanced sensitivity of LD detection using a Luciferase Immunoprecipitation System (Burbelo et al., 2010). This

technique is performed entirely in the liquid phase, and has a broad dynamic detection range. Their highest sensitivity was achieved using a synthetic peptide hybrid containing epitopes from the *B. burgdorferi* proteins OspC and VlsE. These data show that BBK07 peptides were able to detect an antibody response in several patients that did not react with the VlsE peptide C6 or the OspC peptide pepC10. Therefore, it is likely that the inclusion of BBK07 peptides could enhance the sensitivity of other tests containing these antigens. In conclusion, these data show that despite their small size, peptides from BBK07 are more effective than the full-length BBK07 protein, have the ability to detect both late and early human infections, and can detect a variety of *B. burgdorferi* infectious isolates. These factors, combined with their unique ability to detect *B. burgdorferi* in patients not reactive to C6 or pepC10 peptides, suggest that epitopes from BBK07 could be developed into a sensitive and specific serodiagnostic marker for LD.

Portions of this chapter have been published previously:

Coleman AS, Pal U. (2009) BBK07, a dominant i*n vivo* antigen of *Borrelia burgdorferi*, is a potential marker for serodiagnosis of Lyme disease. Clin. Vaccine Immunol. 16(11): 1569-75

Coleman AS, Rossmann E, Song H, Lamichhane CM, Iyer R, Schwartz I, Pal U. (2010) BBK07 immunodominant peptides as serodiagnostic markers of human and canine Lyme disease. Submitted to Clin. Vaccine Immunol. (CVI00461-10 Version 1)

Chapter 6: Conclusions

B. burgdorferi is a unique pathogen, able to adapt and persist in both mammal and arthropod hosts. Many abilities of this spirochete are poorly understood, and since the vast majority of *B. burgdorferi* proteins have little similarity among well-studied proteins, the function of most gene products and the exact mechanisms of bacterial persistence have been difficult to elucidate. *B. burgdorferi* is a clinically important pathogen, causing an emerging infectious disease known as Lyme disease, or Lyme borreliosis. If not promptly treated, *B. burgdorferi* infection can lead to chronic arthritis, carditis, and neurological pathologies that are sometimes resistant to antibiotic treatment. The identification and characterization of important gene products of *B. burgdorferi* could result in improved diagnostic or therapeutic approaches to Lyme disease, as well as a greater understanding of this mysterious and highly evolved pathogen.

I hypothesized that the *in vivo*-expressed surface proteins of *B. burgdorferi* contributed to persistence and virulence through interactions with the host environment. By analyzing the *B. burgdorferi* transcriptome *in vivo*, I mapped the transcriptional profiles of a selected set of genes encoding predicted surface proteins. Of those expressed highly in a mammalian host, I selected two lipoproteins, BbCRASP-2 and BBK07, for further study. Though previous research had suggested a role for BbCRASP-2 in complement resistance, I showed that BbCRASP-2 was nonessential for complement

resistance *in vitro* or infectivity *in vivo*. I also showed that the surface lipoprotein BBK07 was not required for infectivity, and that BBK07 immunization did not protect the host from infection or disease. Together, these results suggest that the surface proteins of *B. burgdorferi* may work together in functionally redundant families. While these lipoproteins may confer an evolutionary advantage over a longer time scale, they can be partially compensated for, resulting in a phenotype too subtle to detect in the laboratory mouse model. Finally, I showed that BBK07 is an immunodominant antigen of *B. burgdorferi*, and that BBK07-based diagnostics have the potential to improve currently available LD serological testing.

Future directions

While I was unable to find a phenotype in the gene-deletion mutants I generated, the functions of BbCRASP-2 and BBK07 remain an enigma. My transcriptional analysis can be used and extended to select other possible surface proteins for similar mutagenesis experiments. Whole-genome transposon mutagenesis could significantly speed the rate at which genes can be characterized in this way. *B. burgdorferi* is highly evolved to persist in mammals for years, one of the few extracellular bacteria capable of this feat. This unique ability combined with the plethora of *B. burgdorferi* outer surface proteins suggests that functional proteins are present on the surface of the bacterium. Once identified, the mechanisms and host ligands can be identified, providing information about *B. burgdorferi*, as well as our own immune system, that could be applicable to other diseases. More research on the surface proteins of *B. burgdorferi* is warranted to

further understand the biology of host-pathogen interactions. These studies could lead to the development of novel diagnostic and preventative strategies to combat spirochete infection.

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