ABSTRACT

Title of Document: CHARACTERIZATION OF *BORRELIA BURGDORFERI* GENE PRODUCT BBD18 FOR ITS ROLE(S) IN PATHOGEN BIOLOGY AND INFECTIVITY

Faith Kung, Doctor of Philosophy, 2016

Directed By: Associate Professor Utpal Pal,
Department of Veterinary Medicine

bbd18 is a differentially expressed *Borrelia burgdorferi* gene that is transcribed at almost undetectable levels in spirochetes grown *in vitro* but dramatically upregulated during tick infection. The gene also displays low yet detectable expression at various times in tissues of murine hosts. As the gene product bears no homology to known proteins, its biological significance remains enigmatic. To understand the gene function, we created isogenic *bbd18*-deletion mutants as well as genetically-complemented isolates from an infectious wild-type *B. burgdorferi* strain. Compared to parental isolates, *bbd18* mutants - but not complemented spirochetes - displayed slower *in vitro* growth. The *bbd18* mutants also reflect significantly reduced ability to persist or remain undetectable both in immunocompetent and SCID mice, yet were able to survive in ticks. This suggests

BBD18 function is essential in mammalian hosts but redundant in the arthropod vector. Notably, although *bbd18* expression and *in vitro* growth defects are restored in the complemented isolates, their phenotype is similar to the mutants - being unable to persist in mice but able to survive in ticks. Despite low expression in cultured wild-type *B. burgdorferi*, *bbd18* deletion downregulated several genes. Interestingly, expression of some, including *ospD* and *bbi39*, could be complemented, while that of others could not be restored via *bbd18* re-expression. Correspondingly, *bbd18* mutants displayed altered production of several proteins, and similar to RNA levels, some were restored in the *bbd18* complement and others not. To understand how *bbd18* deletion results in apparently permanent and noncomplementable phenotypic defects, we sought to genetically disturb the DNA topology surrounding the *bbd18* locus without deleting the gene. Spirochetes with an antibiotic cassette inserted downstream of the gene, between *bbd17* and *bbd18*, were significantly attenuated in mice, while a similar upstream insertion, between *bbd18* and *bbd19*, did not affect infectivity, suggesting that an unidentified *cis* element downstream of *bbd18* may encode a virulence-associated factor critical for infection.

CHARACTERIZATION OF *BORRELIA BURGDORFERI* GENE PRODUCT BBD18 FOR ITS ROLE(S) IN PATHOGEN BIOLOGY AND INFECTIVITY

By

Faith Kung

Dissertation submitted to the Faculty of the Graduate School of the University of Maryland, College Park, in partial fulfillment of the requirements for the degree of
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Advisory Committee:
Professor Utpal Pal, Chair
Professor George Belov
Professor Kevin McIver
Professor Daniel Nelson
Professor Meiqing Shi

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Dedication

I dedicate this dissertation to my family and friends who have supported and encouraged me in every step of my educational journey.

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List of Abbreviations

µg	Microgram
µl	Microliter
°C	Degrees Celsius
Amp	Ampicillin
APS	Ammonium persulfate
bp	base pair
BSA	Bovine serum albumin
BSK	Barbour-Stoenner-Kelly medium
cDNA	Complementary DNA
CDC	Centers for Disease Control and Prevention
Com	*bbd18* complement
cp	Circular plasmid
Ct	Threshold cycle
DMC	Dialysis membrane chamber
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
Erp	Exported repetitive protein
FlaB	Flagellin B
gDNA	Genomic DNA

GST	Glutathione-S-transferase
h	Hour(s)
HRP	Horseradish peroxidase
Kan	Kanamycin
kan D	*kan* downstream of *bbd18*
kan U	*kan* upstream of *bbd18*
kD	Kilodalton
LB	Luria broth
lp	Linear plasmid
LPS	Lipopolysaccharide
MCS	Multiple cloning site
min	Minute(s)
ml	Milliliter
mm	Millimeter
Mn	Manganese
mRNA	Messenger RNA
Mut 1	*bbd18* mutant 1
Mut 2	*bbd18* mutant 2
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
ori	Origin of replication
Osp	Outer surface protein
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline + 0.05% Tween 20
PCR	Polymerase chain reaction
pI	Isoelectric point
PSIPRED	Psi-blast based secondary structure prediction
qPCR	Quantitative (real-time) PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription PCR
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TBE	Tris base/boric acid/EDTA
TCA	Trichloroacetic acid
TCS	Two-component system
TEMED	Tetramethylethylenediamine
WT	Wild type
Zn	Zinc

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Chapter 1: Introduction

A portion of the following chapter was previously published:

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1.1 Lyme disease

Lyme disease, caused by the bacterial pathogen *Borrelia burgdorferi*, is currently regarded as the most prevalent vector-borne infection in the United States and parts of Europe and Asia (Piesman and Gern 2004; Piesman and Eisen 2008; Rizzoli, Hauffe et al. 2011; Levi, Kilpatrick et al. 2012). The U.S. CDC reports that from 2005 to 2014, there were 20,000 to 30,000 confirmed cases of Lyme disease a year in the U.S (Fig. 1). This is a severe underestimate, as the number of Americans diagnosed with Lyme disease is about 300,000 a year (www.cdc.gov/).

Figure 1. Reported cases of Lyme disease in the United States in 2014 (CDC).

Recently, a new pathogenic *B. burgdorferi* sensu lato genospecies that causes Lyme borreliosis with unusually high spirochetemia more characteristic of relapsing-fever *Borrelia* was discovered in the Upper Midwest (Dolan, Hojgaard et al. 2016; Pritt, Mead et al. 2016). The microbe survives in a complex enzootic cycle involving tick vectors belonging to the *Ixodes ricinus* species complex and a variety of mammalian hosts, usually small rodents (Burgdorfer, Barbour et al. 1982; Anderson 1991; Anguita, Hedrick et al. 2003; Pal and Fikrig 2003). In North America, the primary tick vector is *I. scapularis*. During the blood meal engorgement, *B. burgdorferi* migrate from the vertebrate host dermis into the tick gut, where they continue to persist through the intermolt period. As infected ticks feed on a subsequent host, spirochetes exit the gut, invade the salivary glands, and along with tick saliva, transmit to the skin. From the dermis, *B. burgdorferi* disseminate to several internal organs, including the joints, heart, and nervous system (Steere, Coburn et al. 2004; Tilly, Rosa et al. 2008; Radolf, Caimano et al. 2012; Stanek, Wormser et al. 2012). According to the CDC, arthritis is a frequent clinical complication of Lyme disease that is reported in at least 30% of untreated and confirmed human cases. However, borrelial infection is also responsible for other mild to severe conditions, such as a pathognomonic skin rash, carditis, and various neurological symptoms (Barthold, Beck et al. 1990; Barthold, deSouza et al. 1993; Nadelman and Wormser 1998; Steere and Glickstein 2004; Barthold, Diego et al. 2010; Radolf, Salazar et al. 2010).

Borrelia species commonly associated with Lyme disease are very diverse - both genetically and antigenically - and include a number of genospecies, with the

most well-known being *B. burgdorferi* sensu stricto (prevalent throughout the United States and Europe) and *B. afzelii* and *B. garinii* (primarily distributed in Europe and parts of Asia) (Piesman and Gern 2004). Despite significant strain diversity, Lyme disease pathogens are only transmitted by a single group of ticks - the *I. ricinus* complex - thereby suggesting the evolution of a highly specific relationship between the vector and the pathogen (de Silva, Tyson et al. 2009; Pal and Fikrig 2010).

Due to their unique evolutionary divergence from related arthropod and bacterial species respectively (Nava, Guglielmone et al. 2009), the biology and possibly complex relationship between *B. burgdorferi* and *I. scapularis* may not be understood simply by applying many traditional concepts of microbiology or host-pathogen interaction and therefore warrant in-depth experimental studies. Availability of excellent animal models, development of targeted gene inactivation and gene silencing tools for the pathogen (Rosa, Tilly et al. 2005) and vector (de la Fuente, Kocan et al. 2007; Pal and Fikrig 2010), as well as the completion of the *I. scapularis* genome sequencing project (Hill and Wikel 2005; Pagel Van Zee, Geraci et al. 2007; Nene 2009) have provided researchers increasing opportunities to study the intricate biology of *Borrelia*-tick interactions, both in the vector and at the interface with the mammalian host.

Revealing novel features of the vector-pathogen interaction will have a direct impact on translational studies for the effective management of *B. burgdorferi* infection. Despite the fact that incidence of Lyme disease has been ever increasing (Levi, Kilpatrick et al. 2012) and that inherent difficulties exist in achieving timely diagnosis and administering appropriate treatment, a vaccine to prevent the infection

in humans is still unavailable (Marconi and Earnhart 2010). Thus, some of the current efforts are directed towards a better understanding of the distinct phases of the borrelial life cycle, especially within the vector, as well as the identification of pathogen or vector proteins as potential vaccine candidates (Pal and Fikrig 2010). The subject of *B. burgdorferi* interactions with its host or vector has been discussed in several publications (Hu and Klempner 1997; Schwan and Piesman 2002; Anguita, Hedrick et al. 2003; Pal and Fikrig 2003; Coburn, Fischer et al. 2005; Fikrig and Narasimhan 2006; Pal, Anderson et al. 2006; Hovius, van Dam et al. 2007; de Silva, Tyson et al. 2009; Tyson and Piesman 2009; Pal and Fikrig 2010; Radolf, Caimano et al. 2012).

1.2 Infection cycle

B. burgdorferi thrives in nature in an intricate tick-mammal infection cycle (Fig. 2). Given the lack of transovarial transmission, the pathogen must be acquired at one of the vector life stages when ticks engorge on infected mammals, primarily wild rodents (Munderloh and Kurtti 1995; Pal and Fikrig 2003). Spirochetes disseminate along with the blood meal from the infected host to the tick and colonize the gut. They remain in the gut until the next blood meal, when a fraction of the spirochetes exit the gut, invade the salivary glands, and transmit to a new host (de Silva and Fikrig 1995). Thus, maintenance of *B. burgdorferi* in the enzootic cycle requires successful persistence in multiple developmental stages of the arthropod as well as coordinated dissemination through tick tissues to the new host. Major phases of the *B. burgdorferi* life cycle in the vector are briefly highlighted below:

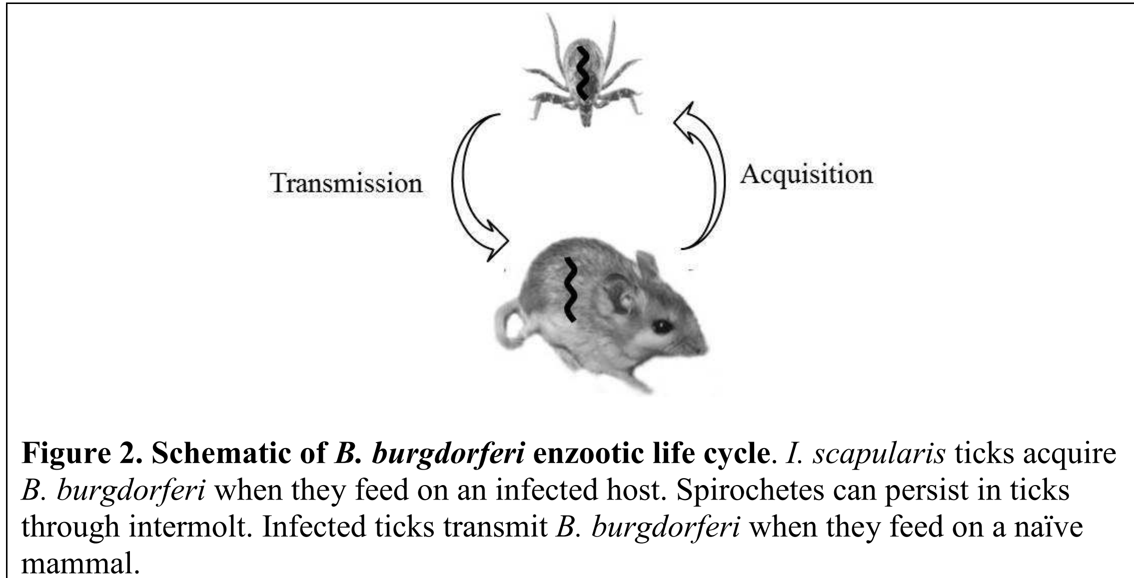


Figure 2. Schematic of *B. burgdorferi* enzootic life cycle. *I. scapularis* ticks acquire *B. burgdorferi* when they feed on an infected host. Spirochetes can persist in ticks through intermolt. Infected ticks transmit *B. burgdorferi* when they feed on a naïve mammal.

Acquisition by feeding ticks

Although a detailed timeline of the molecular events remains unknown, larval or nymphal ticks acquire *B. burgdorferi* through blood during the first 36 hours of attachment and feeding on infected hosts (de Silva, Tyson et al. 2009; Pal and Fikrig 2010), such as *Peromyscus leucopus* (the white-footed mouse) - a common but not the primary reservoir host in the United States (Anderson, Duray et al. 1987; LoGiudice, Ostfeld et al. 2003; Brisson and Dykhuizen 2004; Brisson and Dykhuizen 2006; Hanincova, Kurtenbach et al. 2006; Brisson, Dykhuizen et al. 2008). Spirochetes migrate from the murine dermis, enter the tick, and can be detected by quantitative RT-PCR within one day of tick attachment to the mouse (Promnares, Kumar et al. 2009). *Ixodes* ticks form a transient peritrophic membrane surrounding the blood meal (Pal and Fikrig 2010); however, how this acellular barrier influences spirochete acquisition and colonization of tick gut epithelium remains enigmatic. It is also currently unknown how a certain fraction of *B. burgdorferi* avoids being digested

with the blood meal in the gut and successfully bypasses potential tick innate immune defense mechanisms (Pal and Fikrig 2010). In other vectors, such as mosquitoes, major losses in *Plasmodium* numbers occur during their invasion of the *Anopheles* gut, sometimes resulting in complete elimination of ingested parasites, most likely due to robust innate immune responses mounted by the vector (Sinden 2002; Osta, Christophides et al. 2004).

Persistence in the unfed vector

B. burgdorferi continues to persist in the gut lumen through the next developmental phase of the tick (Pal, Montgomery et al. 2001). This is one of the most remarkable adaptive features of Lyme disease spirochetes, as survival within unfed or intermolt ticks likely poses significant challenges due to several factors, including temperature extremes caused by daily or seasonal changes as well as nutritional stress. The latter results from the fact that the lumen of an unfed gut is poor in nutrients, primarily because gut epithelia store macromolecules within cellular endosomes (Sonenshine 1993), which would be difficult to access by the extracellular pathogen. Notably, mass spectrometry-based proteomics of ticks has shown the persistence of certain mammalian blood components within the tick gut (Wickramasekara, Bunikis et al. 2008).

Transmission from ticks to hosts

For transmission to mammals, spirochetes must first sense events linked to tick-host association and blood meal engorgement. *B. burgdorferi* rapidly multiplies

in the lumen of the gut while the tick is taking a blood meal, generating a phenotypically diverse population of spirochetes (Ohnishi, Piesman et al. 2001), a fraction of which are known to cross the gut epithelial barrier and disseminate to the hemocoel for transmission to the host via the salivary glands (Piesman 1993; Piesman, Schneider et al. 2001). Although the precise mechanism by which *B. burgdorferi* crosses the tissue barrier is unknown and could involve active migration through gut epithelial tissue, the movement of non-motile spirochetes from apical to basal epithelium via adherence to gut epithelial cells has been proposed (Dunham-Ems, Caimano et al. 2009). The identities of tick and borrelial proteins that likely assist in dissemination of spirochetes from the gut to the salivary glands and eventually to the host have also been suggested (Coleman, Gebbia et al. 1997; Pal, Yang et al. 2004; Fingerle, Goettner et al. 2007; Kumar, Yang et al. 2010; Zhang, Zhang et al. 2011). Once the *B. burgdorferi* migrate from the gut to the hemocoelic space, they then rapidly traverse the hemolymph (Dunham-Ems, Caimano et al. 2009), invade the salivary glands, and eventually transfer to the host dermis. More recent studies suggested that RpoS-dependent expression of select genes plays an important role in the transmission of spirochetes by feeding nymphs (Dunham-Ems, Caimano et al. 2012).

1.3 Borrelia burgdorferi

B. burgdorferi is unique among bacteria even compared to related pathogenic spirochetes *Leptospira* and *Treponema* (Subramanian, Koonin et al. 2000; Porcella and Schwan 2001; Nascimento, Verjovski-Almeida et al. 2004). Although described as Gram-negative because of its double membrane, *B. burgdorferi* has unique cellular

organization and membrane composition compared to other diderms (Nava, Guglielmone et al. 2009) (Fig. 3). In particular, its outer membrane contains a low density of membrane-spanning proteins and lacks classical lipopolysaccharides (LPS), which help stabilize and protect bacterial membranes. Rather, the *B. burgdorferi* cell membrane displays a chemically distinct form of peptidoglycan (Schleifer and Kandler 1972), low molecular weight glycolipids, and numerous triacylated lipoproteins, some of the which are differentially regulated *in vivo* and interact with vector or host components (Bergström and Zückert 2010).

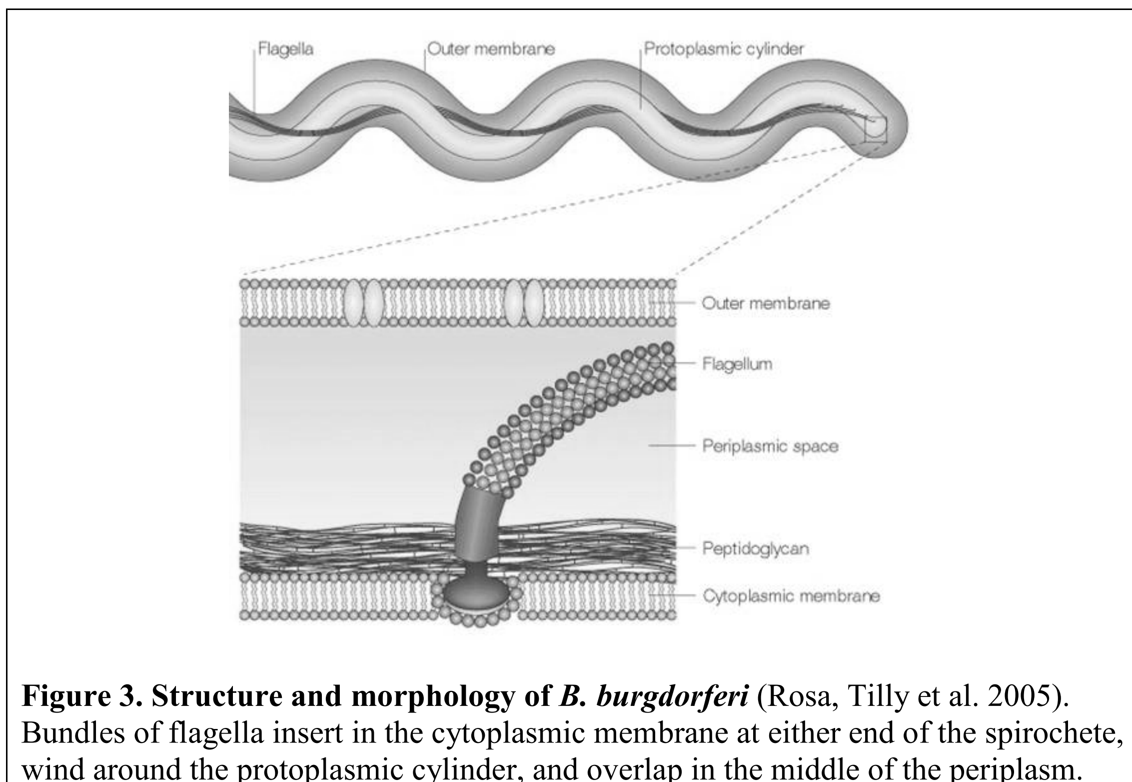


Figure 3. Structure and morphology of *B. burgdorferi* (Rosa, Tilly et al. 2005). Bundles of flagella insert in the cytoplasmic membrane at either end of the spirochete, wind around the protoplasmic cylinder, and overlap in the middle of the periplasm.

1.3.1 Genome

Another distinctive feature of *B. burgdorferi* is its highly segmented and unstable genome (Fraser, Casjens et al. 1997; Casjens, Palmer et al. 2000), which is difficult to genetically manipulate. In addition to a linear chromosome that is fairly conserved and carries most of the housekeeping genes (Fraser, Casjens et al. 1997), *B. burgdorferi* isolates contain up to 21 linear and circular plasmids (Fraser, Casjens et al. 1997; Casjens, Palmer et al. 2000) that were originally named based on their topology (lp or cp) and approximate size (in kb) (Casjens, Mongodin et al. 2012) (Fig. 4).

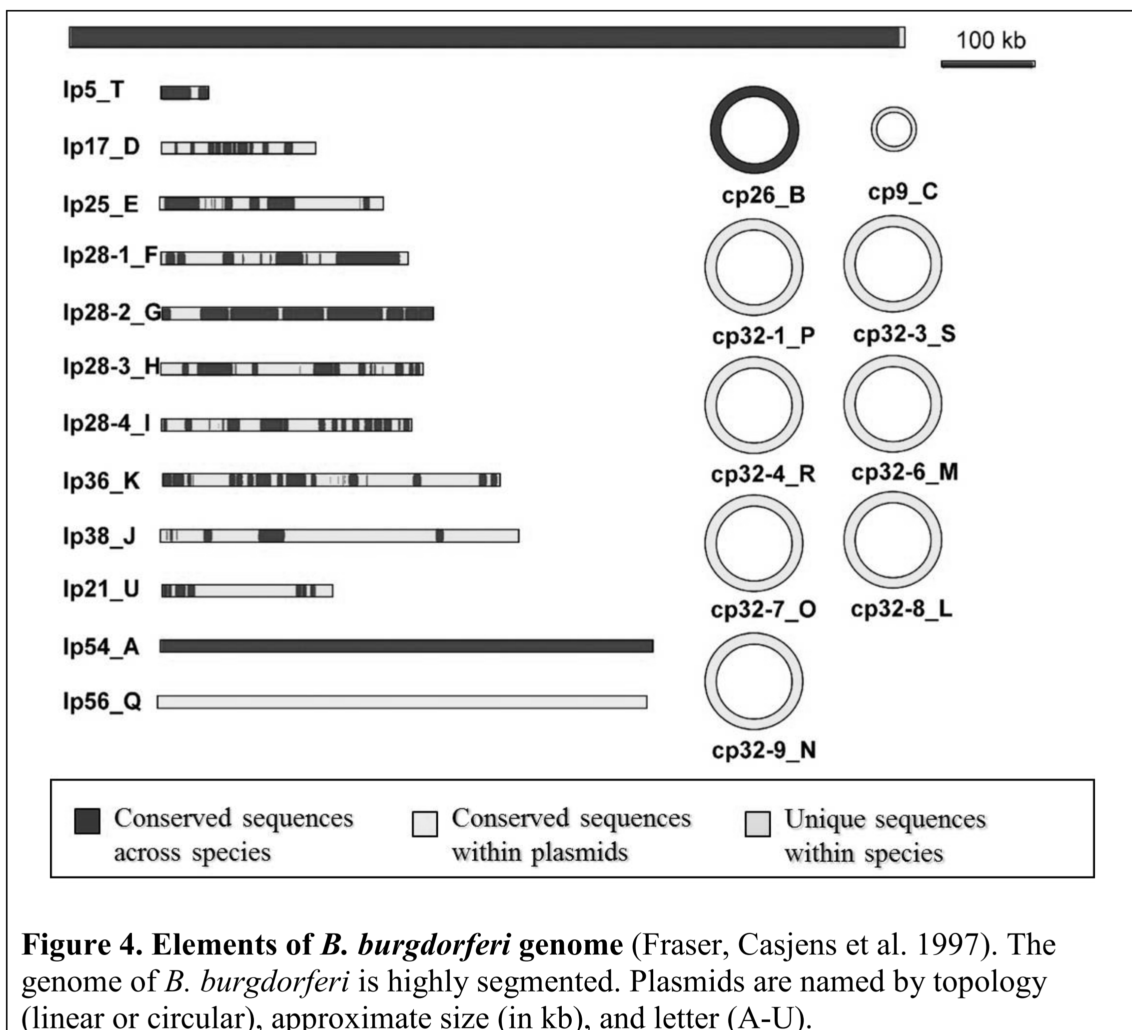


Figure 4. Elements of *B. burgdorferi* genome (Fraser, Casjens et al. 1997). The genome of *B. burgdorferi* is highly segmented. Plasmids are named by topology (linear or circular), approximate size (in kb), and letter (A-U).

The ORFs are designated as follows: The first two letters are the first letter of the genus and species, respectively, and the third letter represents the plasmid compatibility group (Miller, Porcella et al. 2013). Chromosomal and linear plasmid genes are numbered starting from the left end of the molecule (Miller, Porcella et al. 2013). The plasmid ORFs encode numerous proteins without homology to known proteins that potentially perform specific or special functions and respond to different environments during the spirochete's infectious cycle (de Silva, Tyson et al. 2009). Therefore, the biological significance of a vast majority, especially how they support spirochete persistence in the vector or transmission across species, remains enigmatic. A number of studies involving *B. burgdorferi* isolates that lost specific plasmid(s) helped define their essential or redundant functions in spirochete biology and infectivity. The linear plasmid lp25 is shown to be critical for both mouse and tick infection (Purser and Norris 2000; Labandeira-Rey and Skare 2001; Purser, Lawrenz et al. 2003; Grimm, Eggers et al. 2004; Strother, Broadwater et al. 2005; Strother and de Silva 2005). An lp25-deficient strain complemented with *pncA* (for plasmid-encoded nicotinamidase) or *pncA* and *bptA* (for borrelial persistence in ticks), both from this plasmid, was significantly decreased in being acquired by larvae and/or surviving and could not persist through intermolt (Gilmore, Brandt et al. 2014). Another linear plasmid correlated with infectivity, lp28-1 encodes a major surface lipoprotein that undergoes antigenic variation in mammalian hosts via recombination of sequences from silent cassettes into the expressed *vlsE* locus (Zhang, Hardham et al. 1997; Zhang and Norris 1998; Coutte, Botkin et al. 2009; Lin, Gao et al. 2009; Palmer, Bankhead et al. 2009). These intragenic and promiscuous recombination

events are also reported but minimal in feeding ticks (Ohnishi, Piesman et al. 2001; Ohnishi, Schneider et al. 2003). *B. burgdorferi* lacking paralogous plasmid lp28-4 display a reduced ability to survive in ticks and transmit to mice (Strother, Broadwater et al. 2005). Plasmids cp26, cp32-1, -2, and/or 7, cp32-4, -6, -8, -9, lp17, lp28-3, lp36, lp28, and lp54 were consistently present in *B. burgdorferi* B31 clones isolated from needle-inoculated C3H/HeN mice (Purser and Norris 2000). However, cp9, cp32-3, lp21, lp28-2, -4, and lp56 are apparently not required for infection of mice (Purser and Norris 2000).

Remarkably, despite its relatively small size (1.5 Mb), the genome exhibits notable structural and functional redundancy, reflected by the presence of a significant number of paralogous gene clusters (Fraser, Casjens et al. 1997; Casjens, Palmer et al. 2000) as well as experimental evidence that many borrelial genes are not critical for infection (Rosa, Tilly et al. 2005). These sequence similarities lie on the plasmids (Casjens, Palmer et al. 2000). Multiple, highly similar sequences are in the seven cp32s, which are nearly homologous throughout (Casjens, Palmer et al. 2000). Most of the cp32 genes have homologues on every cp32, although there are exceptions (Casjens, Palmer et al. 2000). cp32s may be used for disseminating and controlling the expression of surface antigens and other genes that encode potential host interaction proteins (Guina and Oliver 1997; Gilmore and Mbow 1998). There is evidence that these plasmids could be prophages (Casjens, Palmer et al. 2000). The linear plasmid lp54 contains nine sections homologous to the cp32s, giving 26 of 76 lp54 genes paralogues with DNA insertions and replacements between the cp32-like genes (Casjens, Palmer et al. 2000). Plasmids such as lp36 have been identified to

play nonessential roles in spirochete survival in ticks but are important for mammalian infectivity (Jewett, Lawrence et al. 2007).

1.3.2 Proteome

Unlike many other pathogenic microbes, a substantial portion - nearly 5% of the chromosomal and 15% of the plasmid DNA - produces a diverse set of 130 potential membrane lipoproteins (Fraser, Casjens et al. 1997; Casjens, Palmer et al. 2000), many of which are surface-exposed and may be involved in host-pathogen interactions. Some of these outer surface proteins (Osps) are known to be important for spirochete persistence in and transmission through ticks (de Silva and Fikrig 1997). OspA and OspB, which have been extensively studied since the discovery of the Lyme disease pathogen, are examples of highly abundant proteins expressed by cultured borrelial cells. These two Osps display primarily vector-specific expression *in vivo* and play critical roles in borrelial persistence in the tick gut (Pal, de Silva et al. 2000; Pal, Montgomery et al. 2001; Yang, Pal et al. 2004; Neelakanta, Li et al. 2007). OspA is shown to interact with TROSPA, a tick protein in the gut that is required for spirochete colonization of the gut epithelium (Pal, Li et al. 2004). This highly studied borrelial protein is also reported to bind additional ligands, such as plasminogen (Fuchs, Wallich et al. 1994), and protect spirochetes in the feeding tick gut from host-derived bactericidal antibodies (Battisti, Bono et al. 2008). OspC, another well-studied antigen, is induced in feeding ticks during borrelial transmission to the host (Schwan, Piesman et al. 1995) but not during the intermolt (Gilmore, Mbow et al. 2001). It is also known to bind plasminogen (Lagal, Portnoi et al. 2006;

Onder, Humphrey et al. 2012), which could assist spirochete migration through ticks. While there are conflicting reports on the role of OspC in spirochete dissemination through tick tissues (Grimm, Tilly et al. 2004; Pal, Yang et al. 2004; Fingerle, Goettner et al. 2007; Dunham-Ems, Caimano et al. 2012), it is known that the gene product is required by spirochetes to establish early murine infection. To establish persistent infection, *B. burgdorferi* must downregulate OspC, as the protein represents a target for host acquired immune responses (Liang, Yan et al. 2004; Xu, Seemanapalli et al. 2006). Spirochetes that constitutively express *ospC* are unable to establish chronic infection in immunocompetent mice unless they have very destructive mutations in the gene (Xu, Seemanapalli et al. 2006). Another Osp, annotated as OspD, is shown to be upregulated in a narrow time frame corresponding to tick feeding and plays either a secondary or nonessential role in *B. burgdorferi* persistence in ticks (Neelakanta, Li et al. 2007; Stewart, Bestor et al. 2008).

Other less abundant *B. burgdorferi* outer membrane proteins are targeted by neutralizing host antibodies. BBA52 is only exposed on the microbial surface in the vector and may exist as a homo-oligomer (Kumar, Kaur et al. 2011) that facilitates *B. burgdorferi* dissemination from feeding ticks to mice (Kumar, Yang et al. 2010). Specific antibodies against BBA52 block pathogen transmission from infected ticks to naïve hosts (Kumar, Kaur et al. 2011). *bb0405* is ubiquitously expressed in the infection cycle and encodes a transmembrane protein. *bb0405*-deletion mutants were unable to transmit from ticks to mice (Kung, Kaur et al. 2016). Despite a lack of specific IgM or IgG antibodies during infection, mice immunized with recombinant BB0405 elicited high-titer and remarkably long-lasting antibody responses,

conferring significant host protection against tick-borne infection (Kung, Kaur et al. 2016).

Several *B. burgdorferi* genes are present in stable sections of the genome, conserved across borrelial species, and annotated to encode a putative protein. Gene manipulation studies reveal their essential role(s) in spirochete biology and infectivity. For example, *bb0210* is transcribed at high levels in murine heart, especially during early infection, to produce surface-located membrane protein 1 (Lmp1) (Yang, Coleman et al. 2009). Deleting *lmp1* severely impairs the ability of *B. burgdorferi* to persist in mouse tissues and to induce disease, but the phenotype is restored to wild-type by complementing the mutant with *lmp1* in its native locus (Yang, Coleman et al. 2009). Because of its low homology to known proteins, how Lmp1 contributes to microbial biology and infectivity remains enigmatic (Yang, Lin et al. 2016).

Periplasm protein BB0323 performs critical functions in spirochete outer membrane organization, cell fission, and infectivity (Stewart, Hoff et al. 2004; Zhang, Yang et al. 2009; Kariu, Yang et al. 2013; Kariu, Sharma et al. 2015). More recently, this protein was shown to interact with and mutually stabilize the borrelial protein annotated as BB0238 (Kariu, Sharma et al. 2015), which contains an N-terminal tetratricopeptide repeat (TPR) domain responsible for mediating protein-protein interactions (Groshong, Fortune et al. 2014). Deletion of *bb0238* did not affect growth of *B. burgdorferi in vitro* or survival in ticks but renders the spirochetes unable to persist in mice and transmit from ticks (Groshong, Fortune et al. 2014; Kariu, Sharma et al. 2015).

A number of putative lipoproteins have been implicated in supporting *B. burgdorferi* persistence in the enzootic life cycle; however, in most cases, their function(s) in spirochete biology are unknown. A subsurface membrane protein, LA7 (also annotated p22 or BB0365) supports spirochete survival in the vector (Pal, Dai et al. 2008). LA7-deficient *B. burgdorferi* were severely impaired in their ability to persist in feeding ticks and quiescent ticks during intermolt and transmission (Yang, Hegde et al. 2013). P39 is a specific and highly conserved antigen encoded by two adjacent ORFs, *bmpA* and *bmpB*, also on the chromosome (Simpson, Cieplak et al. 1994). BmpA is an outer surface lipoprotein (Bryksin, Tomova et al. 2010) that plays a significant role in mammalian infection and is important for serodiagnosis of human infection (Verma, Brissette et al. 2009). BmpA antibodies significantly inhibited *B. burgdorferi* adherence to laminin (Verma, Brissette et al. 2009). *dbpA/B* form an operon on lp54 and encode surface-exposed outer membrane lipoproteins (Hagman, Lahdenne et al. 1998). During both early and chronic infection, mice infected with *B. burgdorferi* produced high titers of antibodies against DbpA and B, which may function in host cell adherence and infection (Hagman, Lahdenne et al. 1998; Schmit, Patton et al. 2011). Almost 80% of mice immunized with recombinant DbpA were immune to challenge (Hagman, Lahdenne et al. 1998). Another lp54 locus, *bba57*, encodes a putative outer membrane lipoprotein that is required for early mouse infection and potential transmission (Yang, Qin et al. 2013).

A *B. burgdorferi* protein similar to the DNA-binding protein from starved cells (Dps) family, encoded by *bb0690*, is shown to protect the spirochete against DNA damage and possess a carboxyl-terminal cysteine-rich (CCR) domain that

functions as a copper-binding metallothionein (Wang, Lutton et al. 2012). *dps* expression is low throughout murine infection but increases during tick intermolt (Li, Pal et al. 2007). The protein is important during transmission and for protection of the spirochete against starvation or oxidative stress-induced damage, conditions that are pronounced during the long intermolt period between blood meals (Li, Pal et al. 2007; Wang, Lutton et al. 2012).

B. burgdorferi uses various enzymes to persist in the infection cycle. Two key enzymes integral for purine salvage pathways - IMP dehydrogenase (GuaB) and GMP synthase (GuaA) - are both encoded by genes in the *guaAB* operon (Jewett, Lawrence et al. 2009). The enzymatic activities of the proteins are thought to be essential for *B. burgdorferi* mouse infectivity and provide a survival advantage to spirochetes in the tick (Jewett, Lawrence et al. 2009). The locus *bbe22* on lp25 encodes a nicotinamidase, which is most likely required for nicotinamide adenine dinucleotide (NAD^+) biosynthesis and plays a critical role for spirochete infectivity of mice and ticks (Purser, Lawrenz et al. 2003; Strother and de Silva 2005). A gene located on the plasmid lp36 annotated *bbk17* (*adeC*) encodes an adenine deaminase, which is also shown to contribute to spirochete infectivity in rodents; however, AdeC function may not be important for borrelial persistence in ticks (Jewett, Lawrence et al. 2007). Finally, *bb0728* is dually transcribed by σ_{70} and RpoS to produce a coenzyme A disulphide reductase (CoADR) shown to play important roles during spirochete multiplication in the enzootic cycle (Eggers, Caimano et al. 2011).

1.3.3 Gene expression and regulatory networks

B. burgdorferi, which persist in a complex enzootic infection cycle involving mammalian hosts and arthropod vectors, sense a wide variety of environmental stimuli, including temperature, pH, and chemicals, which allows them to control synthesis of proteins and change their surface antigenic profile (Schwan, Piesman et al. 1995; Stevenson, Schwan et al. 1995). For example, OspC is produced at 32-37°C but not 24°C, and its levels increase when cultures are shifted from 23 to 35°C (Stevenson, Schwan et al. 1995). Also, the protein was decreased at pH 8.0 (Carroll, Garon et al. 1999). Higher levels of OspC are synthesized under anaerobic conditions and decreased with the displacement of CO_2 (Hyde, Trzeciakowski et al. 2007). *B. burgdorferi* couple environmental sensing and gene regulation by two pathways (Samuels 2011; Radolf, Caimano et al. 2012; Caimano, Dunham-Ems et al. 2015).

One involves chromosomally-encoded alternate sigma factors RpoN and RpoS, along with a borrelial two-component system (TCS) Hk2-Rrp2, which globally activate and repress many genes required for different phases of the spirochete infection cycle. For example, genes within the RpoN/RpoS regulon have been shown to promote tick transmission and early mammalian infection, with the pathway being active only in feeding nymphs (Hubner, Yang et al. 2001; Fisher, Grimm et al. 2005; Caimano, Iyer et al. 2007; Boardman, He et al. 2008; Ouyang, Blevins et al. 2008; Ouyang, Narasimhan et al. 2012). RpoN (σ_{54})-dependent transcription of *rpoS* requires the formation of an open promoter complex mediated by an activator of the RpoN/RpoS cascade (Yang, Alani et al. 2003; Boardman, He et al. 2008; Ouyang, Blevins et al. 2008), the response regulator Rrp2 that is phosphorylated by a metabolic intermediate, acetyl phosphate (Xu, Caimano et al. 2010). Attempts to

disrupt *rrp2* have been unsuccessful (Yang, Alani et al. 2003), most likely because Rrp2 is essential for cell growth (Groshong, Gibbons et al. 2012).

Expression of *bbk32* is influenced by environmental factors and regulated by Rrp2 (He, Boardman et al. 2007). *bbk32* transcripts and protein are first detected in engorged ticks. Gene expression increases in mice at the inoculation site and in murine tissues (Fikrig, Feng et al. 2000). Like OspC, BBK32 is produced at higher levels under anaerobic conditions (Hyde, Trzeciakowski et al. 2007). A *bbk32* knockout mutant was significantly attenuated in mice (Seshu, Esteve-Gassent et al. 2006). However, another group found that *B. burgdorferi* lacking BBK32 retained full pathogenicity in mice (Li, Liu et al. 2006). BBK32 binds extracellular matrix component fibronectin (Probert and Johnson 1998) and glycosaminoglycans (GAGs) dermatan sulfate and heparin (Fischer, LeBlanc et al. 2006). Antisera to the protein protect mice from syringe-inoculated and tick-borne *B. burgdorferi*, reducing the number of spirochetes in nymphs that engorged on immunized mice (Fikrig, Feng et al. 2000).

Borrelia oxidative stress regulator (BosR), previously known as Fur, also binds upstream of the *rpoS* promoter and plays an important role in infectivity (Hyde, Shaw et al. 2009; Hyde, Shaw et al. 2009; Ouyang, Kumar et al. 2009; Samuels and Radolf 2009; Ouyang, Deka et al. 2011). *B. burgdorferi* modulates intracellular Mn^{2+} and Zn^{2+} content, and these metals regulate gene expression through influencing BosR (Troxell and Yang 2013). BmtA (BB0219) is likely a metal transporter, as mutants are sensitive to EDTA chelation, and has been shown to be essential to the *B. burgdorferi* infection cycle (Ouyang, He et al. 2009). In addition, *rpoS* expression is

thought to be post-transcriptionally regulated by the RNA-binding protein carbon storage regulator A (CsrA) that plays a critical role in infectivity (Karna, Sanjuan et al. 2011; Sze and Li 2011). Overexpression of CsrA resulted in a significant reduction in FlaB levels (Sanjuan, Esteve-Gassent et al. 2009). As FlaB expression is essential for infection (Motaleb, Corum et al. 2000), regulation by CsrA could be an important factor contributing to *B. burgdorferi* infectivity in mice (Sanjuan, Esteve-Gassent et al. 2009; Karna, Sanjuan et al. 2011) and possibly ticks. Notably, *rpoS* can also be transcribed from an unknown σ_{70}-dependent promoter, and this form of the transcript is subject to small RNA DsrA-dependent post-transcriptional regulation (Lybecker and Samuels 2007). DsrA may act as a temperature-dependent stimulator of translation (Archambault, Linscott et al. 2013). A more recent study shows that RpoS-driven gene expression is especially important for borrelial transmission, as RpoS-deficient *B. burgdorferi* were confined to the tick gut lumen, where they were transformed into an unusual morphology during later stages of the blood meal (Dunham-Ems, Caimano et al. 2012). Inverted repeats (IR) upstream of the *ospC* promoter have been implicated as an operator to regulate its expression (Drecktrah, Hall et al. 2013). *B. burgdorferi* with the palindromic sequence deleted can initiate infection but are unable to persist in immunocompetent mice (Xu, McShan et al. 2007). These spirochetes are cleared by OspC antibody transferred into SCID mice (Xu, McShan et al. 2007). RpoS binds to a minimal promoter that contains a canonical -35/-10 sequence necessary and sufficient for regulation of *ospC* (Yang, Lybecker et al. 2005). Increasing RpoS expression promotes bacterial division before

causing death (Chen, Xu et al. 2013). Induced cells first formed blebs, which were eventually released from dying cells (Chen, Xu et al. 2013).

A putative carbohydrate-responsive protein, BadR (*Borrelia* host adaptation regulator), transcribed from *bb0693*, has been identified as a negative regulator that controls growth phase-dependent induction of *rpoS* and *bosR* (Miller, Karna et al. 2013; Ouyang and Zhou 2015). When *badR* was inactivated, the expression of *rpoS* and *bosR* was induced only during the early stages of bacterial growth, but not during the stationary phase (Ouyang and Zhou 2015). These strains also showed upregulation of other proteins important for host virulence and downregulation of putative tick-specific factors, such as those encoded by lp28-4 (Miller, Karna et al. 2013). Recombinant BadR bound to the promoter of *rpoS* and the regulatory region upstream of *bosR* via AT-rich TAAAATAT motifs (Miller, Karna et al. 2013; Ouyang, Zhou et al. 2014; Ouyang and Zhou 2015). Mutations in this motif and presence of phosphorylated sugars markedly inhibited or abolished rBadR binding (Miller, Karna et al. 2013; Ouyang and Zhou 2015).

Although no report to date demonstrates that Rrp1 is the cognate response regulator for histidine kinase Hk1, the location of *hk1* and *rrp1* on the borrelial chromosome and the phenotype of their mutants suggest the Hk1-Rrp1 pathway (Rogers, Terekhova et al. 2009; Freedman, Rogers et al. 2010; Caimano, Kenedy et al. 2011; He, Ouyang et al. 2011) constitutes a potential TCS essential for *B. burgdorferi* survival during larval and nymphal blood meals. Spirochetes deficient in either protein were virulent in mice and able to migrate out of the bite site during feeding but were killed within the gut following acquisition by ticks (Caimano,

Kenedy et al. 2011; He, Ouyang et al. 2011; Kostick, Szkotnicki et al. 2011). Constitutive expression of the *glp* (glycerol metabolic) operon fully rescued an *rrp1* defect, allowing spirochete survival in BSK-glycerol but only partially restoring persistence in ticks (He, Ouyang et al. 2011). Thus, additional factors for complete restoration of *B. burgdorferi* survival in ticks remain to be identified. These studies suggest that the *glp* operon is a target of a second messenger, cyclic dimeric GMP (c-di-GMP) (He, Ouyang et al. 2011; Pappas, Iyer et al. 2011). On the other hand, spirochetes overexpressing Rrp1 displayed normal motility patterns and chemotactic responses but were noninfectious in mice (Kostick, Szkotnicki et al. 2011); however, the bacteria could persist in ticks and survive a natural blood meal. This response regulator synthesizes c-di-GMP, which regulates tick-specific borrelial infection by modulating the expression and/or activity of gene products required for survival within feeding ticks (Rogers, Terekhova et al. 2009; Caimano, Kenedy et al. 2011; He, Ouyang et al. 2011; Kostick, Szkotnicki et al. 2011). The c-di-GMP-binding protein PlzA positively modulates the production of RpoS, linking Hk1-Rrp1 with Hk2-Rrp2 (He, Zhang et al. 2014). PlzA regulates *rpoS* expression through modulation of BosR at both the transcriptional and the posttranscriptional levels and also independent of Rrp1 (He, Zhang et al. 2014). BB0374, also known as PdeB, specifically hydrolyzes c-di-GMP (Sultan, Pitzer et al. 2011). *pdeB* mutants exhibited significantly increased flexing motion and reduced ability to survive in ticks and were subsequently unable to transmit to the host (Sultan, Pitzer et al. 2011). All together, these results indicate that Rrp1 and c-di-GMP are not required for murine infection but are important for *B. burgdorferi* colonization of ticks.

A purine-rich element binding (PUR) domain is found in critical regulatory proteins of higher eukaryotes and in certain species of bacteria (Jutras, Chenail et al. 2013). The borrelial PUR domain protein, Bpur, binds with high affinity to the 5' region of its own mRNA and dsDNA adjacent to the *erp* promoter and enhances the effects of the *erp* repressor protein (Jutras, Chenail et al. 2013; Jutras, Jones et al. 2013).

1.3.4 Genetic manipulation

Insertional mutagenesis and other genetic techniques in *B. burgdorferi* can be used for detailed studies, such as of transcriptional regulation and protein function (Hardham and Rosey 2000). Circular heterologous DNA can be introduced into the *B. burgdorferi* genome by a single-crossover event and maintained (Stevenson, Bono et al. 1998). Several antibiotic markers are available for spirochetal genetic manipulation. *B. burgdorferi* transformed with *kan* expressed from the *flaB* and *flgB* promoter are resistant to high levels of kanamycin, allowing direct selection of mutants (Bono, Elias et al. 2000). The gene that confers high level gentamicin resistance when expressed from the same promoters shows no cross-resistance with the kanamycin resistance cassette (Elias, Bono et al. 2003). The shuttle vector pKFSS1 carries *aadA* fused to the *B. burgdorferi flgB* promoter, which together confers resistance to spectinomycin and streptomycin in *E. coli* and *B. burgdorferi* (Frank, Bundle et al. 2003).

1.4 Experimental models

Various species have been tested as animal models of Lyme disease. Rats, mice, hamsters, and rabbits develop multisystemic infection as well as arthritis and carditis (Moody, Barthold et al. 1990). The disease is more severe in rats and mice inoculated with pathogenic *B. burgdorferi* (Moody, Barthold et al. 1990). Several strains of mice inoculated with *B. burgdorferi* develop severe polyarthritis, with C3H/He(J) and SWR mice the most severely affected (Barthold, Beck et al. 1990; Yang, Weis et al. 1994). Carditis occurs in C3H/He and BALB/c mice (Barthold, Beck et al. 1990). *B. burgdorferi* DNA can be detected in many tissues of C3H/HeJ mice as early as a week after infection (Yang, Weis et al. 1994). Heart, ear, and ankle joint are heavily infected, although spirochete DNA is present in spleen, liver, brain, kidney, bladder, uterus, and lymph nodes (Yang, Weis et al. 1994). *B. burgdorferi* induces progressive polyarthritis and carditis in SCID but not in normal C.B-17 mice, and infectious spirochetes can be isolated from blood and joints of inoculated SCID mice, making it an attractive model to study the role of the immune system in *B. burgdorferi* infection (Schaible, Kramer et al. 1989). Several studies have used dialysis membrane (diffusion) chambers (DMC) to generate "mammalian host-adapted" spirochetes (Brooks, Hefty et al. 2003). *B. burgdorferi* 297 cultivated in DMCs express proteins not expressed *in vitro* (Akins, Bourell et al. 1998).

1.5 Research objectives

This work focuses on the gene product of *bbd18*, which is interesting for several reasons. The gene is conserved among *Borrelia* species and differentially

expressed *in vivo*, specifically upregulated in ticks during transmission to mice. A number of *B. burgdorferi* proteins have already been shown to affect virulence and/or persistence in a phase-specific manner. While identification of new borrelial virulence determinants is an important area of research, to the best of our knowledge, other studies have not explored in-depth the biological significance of unorthodox genetic regulators like BBD18. Notable discoveries have contributed to understanding the regulation accounting for the temporal and spatial expression of *B. burgdorferi* genes during distinct phases of the life cycle. The prior work on BBD18 uncovered more questions regarding its function in *B. burgdorferi* biology as part of gene regulatory networks and also its role in infectivity. These research directions will enrich our knowledge of vector-borne infections and contribute towards the development of preventative strategies against Lyme disease.

Chapter 2: Assess role(s) of BBD18 in tick-mammal infection cycle

2.1 Introduction

B. burgdorferi adapt to changes in temperature, pH, nutrients, and host immune responses by altering their transcriptome (Schwan, Piesman et al. 1995; Carroll, Garon et al. 1999; Narasimhan, Santiago et al. 2002; Ojaimi, Brooks et al. 2002; Revel, Talaat et al. 2002; Brooks, Hefty et al. 2003; Tokarz, Anderton et al. 2004; Fisher, Grimm et al. 2005). About 8.6% of the genome or 150 genes are differentially expressed *in vitro* in response to growth conditions (Revel, Talaat et al. 2002). To establish infection and colonize diverse tissues in ticks and mammals, *B. burgdorferi* undergoes reciprocal changes in gene expression (Schwan, Piesman et al. 1995; Hubner, Yang et al. 2001; Narasimhan, Santiago et al. 2002; Narasimhan, Caimano et al. 2003; Pal, Yang et al. 2004; Boardman, He et al. 2008; Tilly, Rosa et al. 2008) mediated at least partly by regulatory pathways such as one involving Rrp2-RpoN/RpoS (Zhang, Hardham et al. 1997; Yang, Alani et al. 2003; Caimano, Iyer et al. 2007; Coutte, Botkin et al. 2009; Rogers, Terekhova et al. 2009; Iyer, Caimano et al. 2015). Gene deletion studies (Rosa, Tilly et al. 2005) confirmed that some of the differentially-expressed gene products support spirochete infectivity. For example, *bbk32*, *dbpA/B* and *bmpA/B* are selectively expressed in mammals and facilitate infection of the murine host (Seshu, Esteve-Gassent et al. 2006; Pal, Wang et al. 2008; Shi, Xu et al. 2008). In contrast, *ospA*/*B, bb0365* and *bb0690* are highly expressed during specific stages of *B. burgdorferi* persistence in ticks and support the spirochete life cycle in the arthropod (Yang, Pal et al. 2004; Li, Pal et al. 2007; Pal, Dai et al. 2008). Despite phase-specific expression, proteins could be functionally

redundant and nonessential for infectivity. Therefore, identification of additional virulence genes that have significant impact on *B. burgdorferi* survival *in vivo* and pathogenesis is important for the developing strategies to prevent Lyme disease.

Genome-wide screens can reveal the differential expression of many genes. Microarray analysis of transcript levels in spirochetes grown at 23°C and shifted to 35°C to partly mimic conditions in the tick gut identified many genes that are differentially expressed, including *bbd18*, which was downregulated with the addition of blood or in mammalian conditions (Brooks, Hefty et al. 2003; Tokarz, Anderton et al. 2004). qPCR validated that *bbd18* transcripts were detected in unfed but not fed nymphs (Tokarz, Anderton et al. 2004). *bbd18* expression is high in infected ticks but low in murine tissues (Fig. 5), which suggests importance of its gene product in the tick phases or during tick-to-mammal transmission and establishment of early mammalian infection. Our studies expanded on these observations of microbial persistence *in vivo*.

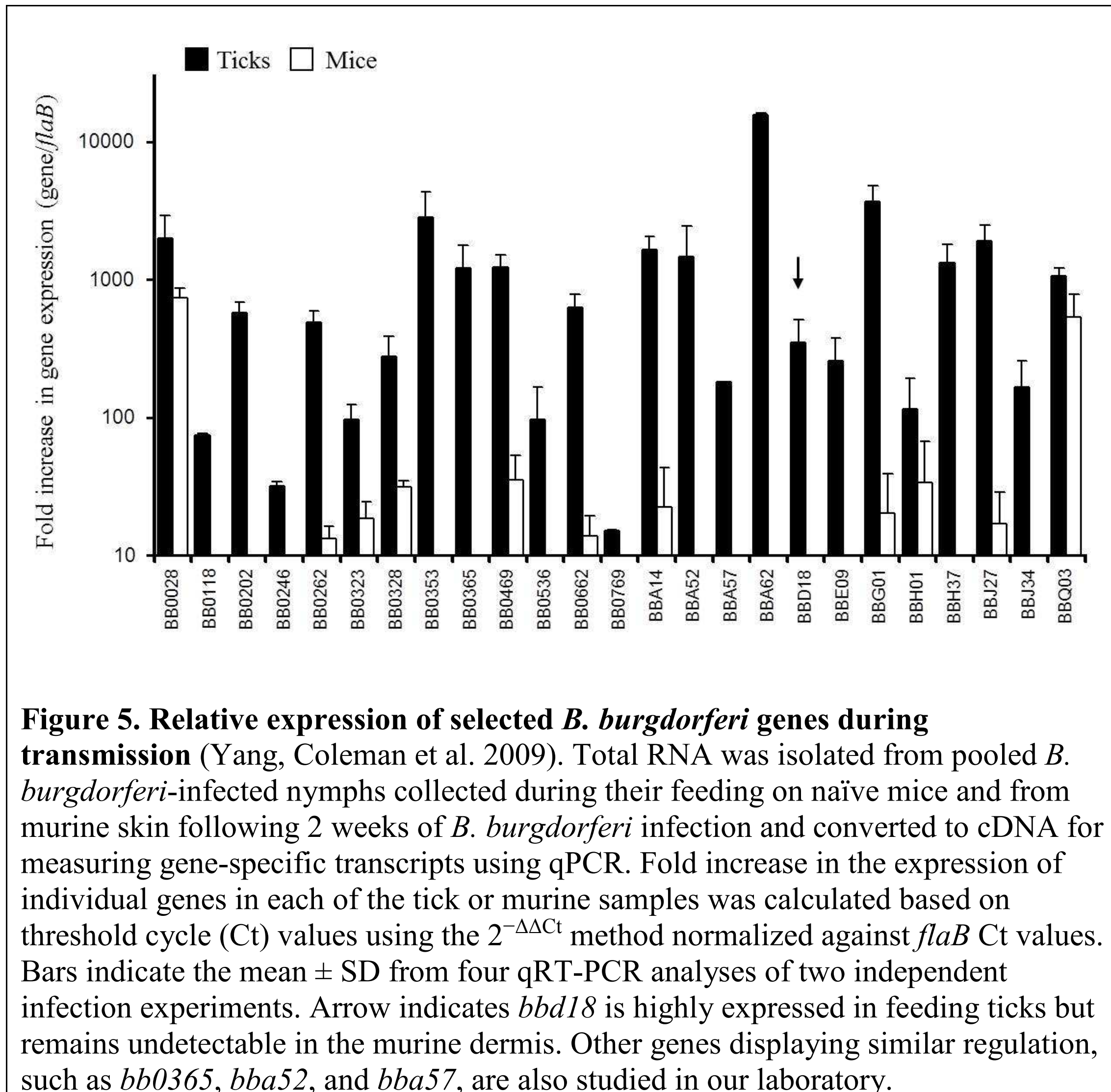


Figure 5. Relative expression of selected *B. burgdorferi* genes during transmission (Yang, Coleman et al. 2009). Total RNA was isolated from pooled *B. burgdorferi*-infected nymphs collected during their feeding on naïve mice and from murine skin following 2 weeks of *B. burgdorferi* infection and converted to cDNA for measuring gene-specific transcripts using qPCR. Fold increase in the expression of individual genes in each of the tick or murine samples was calculated based on threshold cycle (Ct) values using the $2^{-\Delta\Delta Ct}$ method normalized against *flaB* Ct values. Bars indicate the mean ± SD from four qRT-PCR analyses of two independent infection experiments. Arrow indicates *bbd18* is highly expressed in feeding ticks but remains undetectable in the murine dermis. Other genes displaying similar regulation, such as *bb0365*, *bba52*, and *bba57*, are also studied in our laboratory.

bbd18 is located on a stable genetic element. Although once annotated as the eighteenth gene of more than 20, *bbd18* is currently recognized by the NCBI (www.ncbi.nlm.nih.gov/) as the tenth of 14 genes on lp17, which at 17 kb is the second smallest linear plasmid. The G+C content of this plasmid is very low (23%), possibly because it does not encode many functional proteins but rather contains pseudogenes (Casjens, Palmer et al. 2000). Other than *bbd001* and *bbd10*, which code

for lipoproteins, and *bbd15*, which encodes a membrane protein, the other 11 genes, including *bbd18*, produce hypothetical proteins.

Database searches reveal that BBD18 is well conserved (85% identity) among diverse *B. burgdorferi* sensu lato strains, which cause Lyme disease (Fig. 6), and that related sequences are present in some relapsing-fever *Borrelia* strains but do not identify any homologs in other organisms (Sarkar, Hayes et al. 2011).

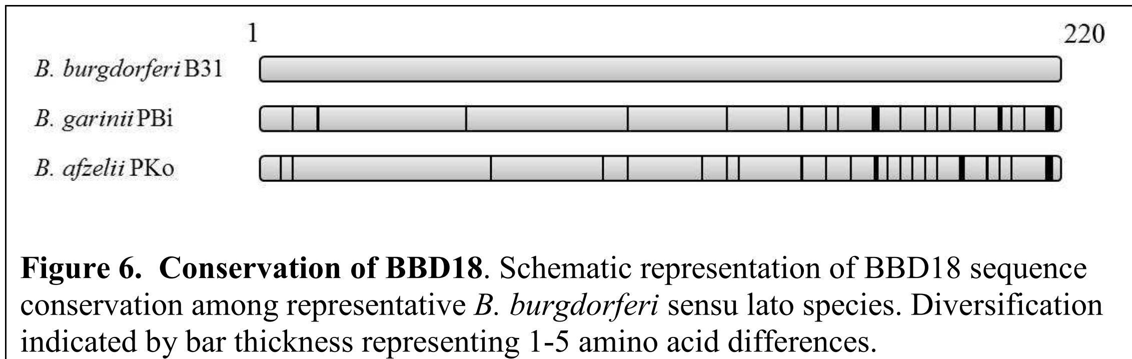


Figure 6. Conservation of BBD18. Schematic representation of BBD18 sequence conservation among representative *B. burgdorferi* sensu lato species. Diversification indicated by bar thickness representing 1-5 amino acid differences.

2.2 Materials and methods

B. burgdorferi, mice, and ticks

An infectious isolate of *B. burgdorferi*, B31 A3, was used. Cultures were kept at 34°C for the duration of growth or subcultured and placed at 37°C. For *in vitro* growth analysis, spirochetes were diluted to a density of 10^5 cells/ml, grown until stationary phase (~10^8 cells/ml), and counted by dark-field microscopy using a Petroff-Hausser cell counter.

Four to six-week-old C3H/HeN mice were purchased from the National Institutes of Health. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee protocols R-09-11, R-10-76, and R12-33 and the Institutional Biosafety Committee. An *Ixodes scapularis* colony was

derived from adult ticks collected from sites in Connecticut and maintained in the laboratory.

Polymerase chain reaction

The oligonucleotide sequences for each of the primers used in specific PCR reactions are listed in Table 1. The primers were designed using OligoPerfect Designer software (Thermo Fisher Scientific, Waltham, MA). The cycling conditions were as follows: initial denaturation at 95°C for 5 min and 35 to 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 1 min/kb and a final extension at 72°C for 5 min.

Table 1. Oligonucleotide primers for checking plasmid content of genetically-modified *B. burgdorferi* isolates.

Sequence (5' - 3')	Purpose
GCACAAAAGGTGCTGAG	Forward primer for lp54
TTTTAAAGCGTTTTTAAGC	Reverse primer for lp54
AATAATTCAGGGAAAGATGGG	Forward primer for cp26
AGGTTTTTTTGGACTTTCTGCC	Reverse primer for cp26
CAAACTTATCAAATAGCTTATG	Forward primer for lp17
ACTGCCACCAAGTAATTTAAC	Reverse primer for lp17
ATGGGTAAAATATTATTTTTTGGG	Forward primer for lp25
AAGATTGTATTTTGGCAAAAAATTTTC	Reverse primer for lp25
ATGAACAAAAAATTTTCTATTTC	Forward primer for lp28-1
GTTGCTTTTGCAATATGAATAGG	Reverse primer for lp28-1
TCCCTAGTTCTAGTATCTACTAGACCG	Forward primer for lp28-2
TTTTTTTTGTATGCCAATTGTATAATG	Reverse primer for lp28-2
GATGTTAGTAGATTAAATCAG	Forward primer for lp28-3
TAATAAAGTTTGCTTAATAGC	Reverse primer for lp28-3
AGGCCGGATTTTAATATCGA	Forward primer for lp28-4
TTTATATTTTGACACTATAAG	Reverse primer for lp28-4
AAATTCTATGGAAGTGATG	Forward primer for lp38
TTTATCTTTATTTTTAGGC	Reverse primer for lp38
AAGTTTATGTTTATTATTGC	Forward primer for lp36
ATTGTTAGGTTTTTCTTTTCC	Reverse primer for lp36
GAAGATTTAAACAAAAAAATTGCG	Forward primer for cp32-8
GTAATCACTTCTTTTTTACCATCG	Reverse primer for cp32-8
GACTTTACATAGTATAAATGCTTTTGG	Forward primer for cp32-6
TCTCGTTATTATAAAATAAGTAGG	Reverse primer for cp32-6
AATAGAAAGTAGGAATGGTGCCGAAC	Forward primer for cp32-9
GTATACGCAAATTTAGTTACACC	Reverse primer for cp32-9
GGAATGTATTAATTGATAATTCAG	Forward primer for cp32-7
GCGAACTAAATAGTGCCTTATGGG	Reverse primer for cp32-7
ACGATAGGGTAATATCAAAAAAGG	Forward primer for cp32-1
AGTTCATCTAATAAAAATCCCGTG	Reverse primer for cp32-1
AAGATTGATGCAACTGGTAAAG	Forward primer for lp56
CTGACTGTAACTGATGTATCC	Reverse primer for lp56
AGATCCTCAAAATAGTTTAACCAG	Forward primer for cp32-4
TTAATATTGGCAGAGAGTCTACAG	Reverse primer for cp32-4
GCAAGTTCCCACGATAACACACCCGTAT	Forward primer for cp32-3
TTTTCATATCCCCTCCTAGCTTTATTGCC	Reverse primer for cp32-3

CTTGCTTTAAGCCCTATTTCAC	Forward primer for lp5
GCACACTACCCATTTTTGAATC	Reverse primer for lp5
AGTAAAGGAGTTCTGCAAAAATT	Forward primer for lp21
GTTGTCACCTCGTGTAATATG	Reverse primer for lp21

RNA isolation, cDNA synthesis, and qPCR

Groups of three C3H/HeN mice were sacrificed two weeks after infection (10^5 spirochetes/mouse) and skin, heart, joint, and bladder isolated. Larval and nymphal ticks that fed on two-week-infected mice (20 ticks/mouse) were collected after repletion. For *bbd18* expression analyses during spirochete acquisition and persistence in ticks, larval and nymphal ticks that fed on mice infected for two weeks (25 ticks/mouse) were analyzed at three days of feeding or as freshly molted infected unfed nymphs. For analyses of *bbd18* gene expression in feeding ticks during transmission, naturally infected nymphs were allowed to engorge on three naïve mice (20 ticks/mouse). The nymphs were collected at 12, 24, and 48 h after attachment and pooled. Mouse tissues and ticks were frozen with liquid nitrogen and crushed by mortar and pestle. Total RNA was isolated first by adding TRIzol reagent (Thermo Fisher Scientific). To perform phase separation, chloroform was added to the samples, which were then vigorously mixed and centrifuged. The colorless upper aqueous phase was transferred to another tube and mixed with isopropanol to precipitate the RNA. Samples were incubated at -20°C for at least 1 h. After centrifuging, the supernatant was removed and the RNA pellet washed with 75% ethanol and dried. To reduce the amount of contaminating DNA, samples were resuspended in UltraPure distilled water (Invitrogen, Carlsbad, CA) with RNase-free DNase I (New England Biolabs, Ipswich, MA), using a reaction condition of 37°C for

10 to 15 min and inactivation of 75°C for 10 min. The RNA was reverse transcribed to cDNA using a SuperScript VILO MasterMix (Thermo Fisher Scientific). The reaction mix was incubated at 25°C for 10 min and 42°C for 60 min before the reaction was terminated at 85°C for 5 min. Undiluted or diluted cDNA was used for qPCR analyses. The oligonucleotide sequences for each of the primers used are listed in Tables 2 and 3. The primers were designed using OligoPerfect Designer software (Thermo Fisher Scientific) based on the *B. burgdorferi* B31 M1 genomic sequence (Fraser, Casjens et al. 1997; Casjens, Palmer et al. 2000). Primer pairs (Table 2-4) have annealing temperatures of 60°C and span 100 to 300 bp of their target gene. Parameters were modified if no primer pairs could be designed. Amplification was performed using FastStart Universal SYBR Green Master (Rox) reaction mix (Roche) and 96-well PCR plates with duplicate wells in an iQ5 real-time or C1000 Touch thermal cycler (Bio-Rad, Hercules, CA). The cycling conditions were as follows: initial denaturation at 95°C for 10 min and 40 to 45 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 min. For melt curve analysis to determine efficiency and specificity of primers, plates were subjected to 65°C for 30 seconds and a temperature gradient starting at 65°C and increasing 0.5°C/cycle for 60 cycles. Expression of *bbd18* was analyzed by measuring amount of *bbd18* transcripts. The levels of *B. burgdorferi bbd18* transcript in tick and mouse samples were normalized against *flaB* transcripts using the $-2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Two independent experiments used the same parameters to ensure the reproducibility of the assay.

Table 2. Oligonucleotide primers for evaluating *B. burgdorferi* infectivity.

Sequence (5' - 3')	Purpose
TTGCTGATCAAGCTCAATATAACCA	Forward primer for *flaB*
TTGAGACCCTGAAAGTGATGC	Reverse primer for *flaB*
AGAGGGAAATCGTGCGTGAC	Forward primer for mouse *β-actin*
CAATAGTGATGACCTGGCCGT	Reverse primer for mouse *β-actin*
GGTATCGTGCTCGACTC	Forward primer for tick *β-actin*
ATCAGGTAGTCGGTCAGG	Reverse primer for tick *β-actin*

Table 3. Oligonucleotide primers for evaluating expression of potentially regulated *B. burgdorferi* genes.

Sequence (5' - 3')	Purpose
TCTGTGAGCGAAAAAGTTGG	Forward primer for *bb0038*
ATCTCGCTTGATTGCCTTTC	Reverse primer for *bb0038*
CAGGACTTGCAACGGGTTAT	Forward primer for *bb0241*
CCAGGATTTTGCTTTTCCAA	Reverse primer for *bb0241*
TCCTTTGCATTTTGATGTGAA	Forward primer for *bb0292*
GCCTTCACACCTCTCTCAACA	Reverse primer for *bb0292*
GAGCAGAGCCAAGCAGTCTT	Forward primer for *bb0329*
TGCAAGTCCCGGTTTATTTC	Reverse primer for *bb0329*
GGGCTATGTGTCTTCTGAGCA	Forward primer for *bb0502*
TTTGGCAGAAATCACACCTG	Reverse primer for *bb0502*
GCCAAGCCCAATTAGAGTGT	Forward primer for *bb0602*
TGCTCCCCAGTTACAGTTCC	Reverse primer for *bb0602*
AAAAATTGCTGAGGCAATGG	Forward primer for *bb0649*
CAAGAGCATCCCCCTCAATA	Reverse primer for *bb0649*
GAAGTGCTCAAAACGGCATT	Forward primer for *bb0730*
GATCTTTTCCCCATCAAGCA	Reverse primer for *bb0730*
CCAGAAAATGCAAAAGAAAAA	Forward primer for *bb0741*
ACAGCAGCTCCTGCGTATTT	Reverse primer for *bb0741*
ACAAGCAGATGAGGGAGTGC	Forward primer for *bb0800*
TTTTACAACCCGGACAGGAG	Reverse primer for *bb0800*
CGCTCCAGGCGAAATAATAA	Forward primer for *bba10*
TTTAAATCCCGCGTGCTTAG	Reverse primer for *bba10*
CTAACGGGGTGCGTACAAAT	Forward primer for *bba11*
TTTCCTTGCCACCAAATAGG	Reverse primer for *bba11*
AACTCTGGCATTGACGCTTT	Forward primer for *bba13*
TGCTTGCATCTCTCTCATCA	Reverse primer for *bba13*
TCAAACTAGCAAAGGCTTTTTACA	Forward primer for *bba44*
TCCTGGGTTTTTCAATGCTT	Reverse primer for *bba44*
GCGATCCGATTGATGAGACT	Forward primer for *bba46*
CATTCAAGCCCTGCTGTGTA	Reverse primer for *bba46*
TGGCTAAAGCAAGCACAGAA	Forward primer for *bba47*
TCTCGTAAAATTGCTCGTTGC	Reverse primer for *bba47*
TGGGACAGCGTTTTAGGATT	Forward primer for *bba48*
CCGGGCTTTTTAAAACCTTC	Reverse primer for *bba48*
TGAATAAGCAAGAGATTGCGACT	Forward primer for *bba49*
CAAGCTCCTTGTAGCCCATT	Reverse primer for *bba49*

ACAAAATCGCTTTGCCTGAT	Forward primer for *bba50*
TTCTTGCAAATCCCCAAAAG	Reverse primer for *bba50*
GCCCGTGCAGTAATTGATTT	Forward primer for *bba51*
GCTTACCACCTCGTGAAACC	Reverse primer for *bba51*
GAGCCAATCAAAGCACGAAC	Forward primer for *bba67*
AAAAATACAAATTGGCGTTTAC	Reverse primer for *bba67*
TGGGATTTTAATGTGTGAGTTTTT	Forward primer for *bbe11*
CAAGCTTGTGCATATTCTTCAAA	Reverse primer for *bbe11*
TGAAAAAGAATACAAAAAGTAAGAGAA	Forward primer for *bbe27*
TCTTTCAAACCATCTTCAAGACA	Reverse primer for *bbe27*
AAAAGGAACTTCCCTGGATCA	Forward primer for *bbg11*
GCCCCTATTAGGCAAGCTG	Reverse primer for *bbg11*
GTCTCAAGTTAATGGCAATGAAGG	Forward primer for *bbh15*
TTAAACAATCAAAATAACCTTGCAC	Reverse primer for *bbh15*
TTTTTCAACAAGCATAAAACAGATT	Forward primer for *bbi15*
GGTGTTCTTCAGATTTCCAACC	Reverse primer for *bbi15*
AAGATCTGCGGCTTTCTTTTT	Forward primer for *bbj08*
CCATGGCCAAACCTCTTTTA	Reverse primer for *bbj08*
CATGCAAATGAAGCATTTAAGG	Forward primer for *bbj13*
AAATTCAGTCTTTATGTCTCGCAAT	Reverse primer for *bbj13*
TCTCTTTTTAAAATATAAAGTGCAGCAT	Forward primer for *bbj40*
CTAAACCAAGCTGATTAATCTAAA	Reverse primer for *bbj40*
GGGAATTCACAACGCTCAGT	Forward primer for *bbm42*
TCTTGCCCGCATCTTAGTCT	Reverse primer for *bbm42*
TGTATTTCTTGCGGAGTCAGAA	Forward primer for *bbq01*
AAACTCCAACATATAAAAATCCAAATG	Reverse primer for *bbq01*
TGCGAACTGCAAAAATCAAC	Forward primer for *bbq18*
AATCCCCTGTCGACAAACCT	Reverse primer for *bbq18*
TGGTGATTTTGTTGCTTTTGA	Forward primer for *bbq20*
TTAGCAACCAGCTCATTGGA	Reverse primer for *bbq20*
GCTAACGAATTTATGAGGGGTCT	Forward primer for *bbs43*
TTGTCTTGTTGATATCTATTGACCAC	Reverse primer for *bbs43*
GCAAAAATAAGCTATCTTCACACTT	Forward primer for *bbt02*
CGGCGTATAAACCCCCTATT	Reverse primer for *bbt02*

Generation of *bbd18* mutant and complement isolates of *B. burgdorferi*

BBD18-deficient *B. burgdorferi* were generated by homologous recombination, replacing the 663-base pair (bp) *bbd18* gene with a kanamycin resistance cassette. DNA fragments flanking the *bbd18* open reading frame (ORF) were PCR-amplified and inserted into the plasmid pXLF10601. This plasmid was sequenced to confirm its identity and electroporated into wild-type *B. burgdorferi* A3, and transformants were selected for growth in the presence of kanamycin. To verify the desired genomic arrangements in the *bbd18* mutant clones, a series of PCRs were performed. The plasmid profile of the mutant *B. burgdorferi* was assessed for maintenance of wild-type plasmids. A construct for complementing *bbd18* was generated by reinsertion of a wild-type copy of the *bbd18* gene at the native locus. The intergenic promoter sequence and *bbd18* were amplified and cloned into pKFSS1, which contains a streptomycin resistance cassette (*aadA*). The plasmid construct was sequenced to confirm its identity, and 25 μg of the DNA was electroporated into the *bbd18* mutant.

Phenotypic analysis of *bbd18* mutant and complement isolates

To examine and compare the phenotypes *in vivo*, wild-type, mutants, and complement *B. burgdorferi* were separately inoculated into groups of mice (3/group, 10^5 spirochetes/mouse). Skin, joint, heart, and bladder samples from the infected mice were isolated at day 7 and 14 after challenge. *B. burgdorferi* burdens were measured by quantitative RT-PCR analyses of *flaB* mRNA and then normalized against murine *β-actin* gene. One microgram of RNA per tissue sample was used for qPCR analyses. Portions of skin and spleen from the mice were cultured in BSK media for the

presence of viable *B. burgdorferi*. For transmission studies, infected nymphs were generated by microinjecting nymphs with 10^9 wild-type, *bbd18* mutant, or complemented *B. burgdorferi*. Infected ticks were fed on naïve mice (5 ticks/mice, 3 mice/group). The engorged ticks were subjected to qPCR analyses to assess the infection. At day 10 following tick feeding, all the mice were sacrificed, and the tissues were isolated and assessed for the spirochete burden by qPCR. Portions of skin and spleen tissues were cultured in BSK media.

Growth within diffusion chambers implanted in mice

Mixed cellulose ester membranes (0.05 µm pore size; Millipore) were adhered to lucite diffusion chamber rings (weight–0.4 g, diameter–14 mm, width–5 mm; Millipore (Colombo, Abraham et al. 2011) (Fig. 7). Diffusion chambers were loaded with 200 µl of WT or Mut 1 *B. burgdorferi* in BSK and sealed (Fig. 7). Mice were anesthetized with 3% isofluorane (Colombo, Abraham et al. 2011). With asceptic technique, chambers were implanted in a subcutaneous pocket formed by an incision of approximately 1.5 cm in the dorsal-anterior skin of each mouse (Colombo, Abraham et al. 2011) (Fig. 7). After one week, the chambers were removed and the contents collected. Bacteria were enumerated by dark-field microscopy.

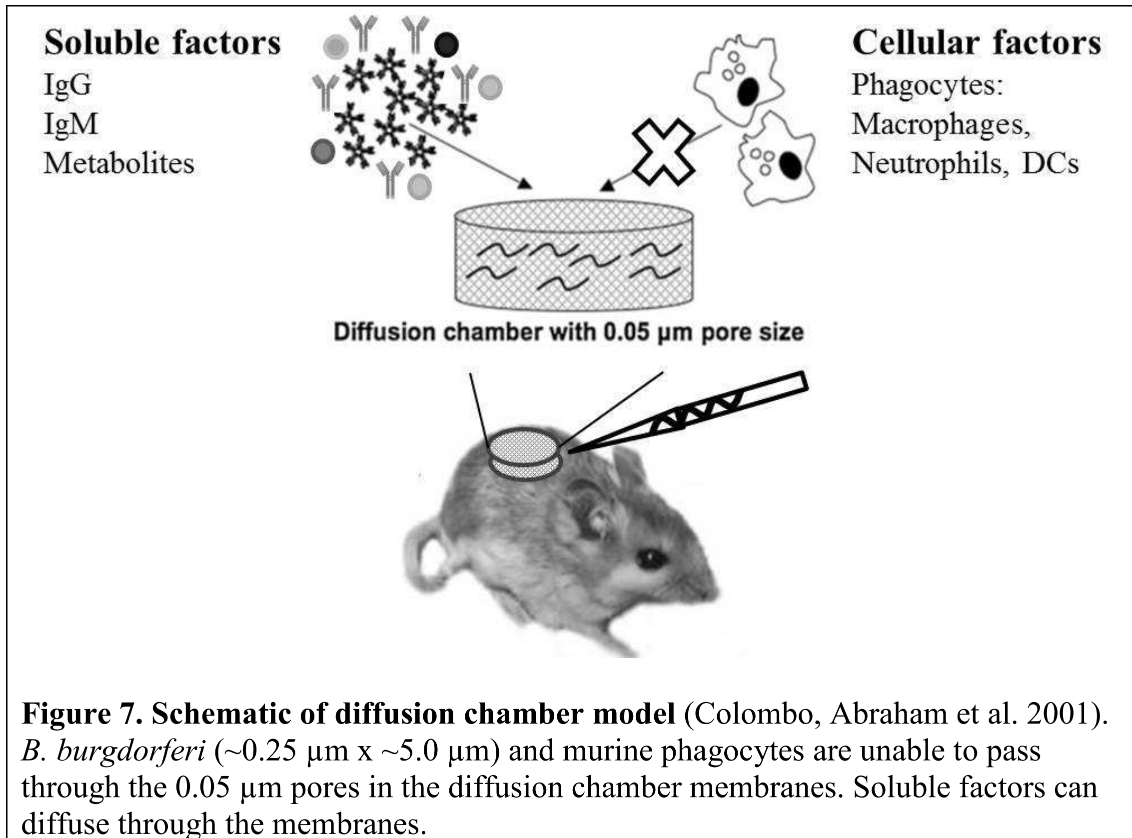


Figure 7. Schematic of diffusion chamber model (Colombo, Abraham et al. 2001). *B. burgdorferi* (~0.25 µm x ~5.0 µm) and murine phagocytes are unable to pass through the 0.05 µm pores in the diffusion chamber membranes. Soluble factors can diffuse through the membranes.

2.3 Results

Construction and characterization of mutant and complement of *bbd18*, a gene upregulated in ticks

bbd18 is differentially expressed *in vivo*, and thus, the protein may be important in a particular environment. We assessed its expression *in vitro* during representative phases of the *B. burgdorferi* infection cycle by qPCR. A temperature of 25°C simulates the tick environment, while mammals have a body temperature of 37°C. An immediate temperature of 34°C is commonly used for culturing *Borrelia*. Skin and joint were collected from mice infected for seven or 21 days. Larval ticks were fed on *B. burgdorferi*-infected mice, and some were allowed to molt into nymphs. The results show that *bbd18* is upregulated during early and persistent tick

infection but has low expression *in vitro* at various temperatures and in murine skin and joint (Fig. 8), suggesting that the gene may also be important for survival in the vector.

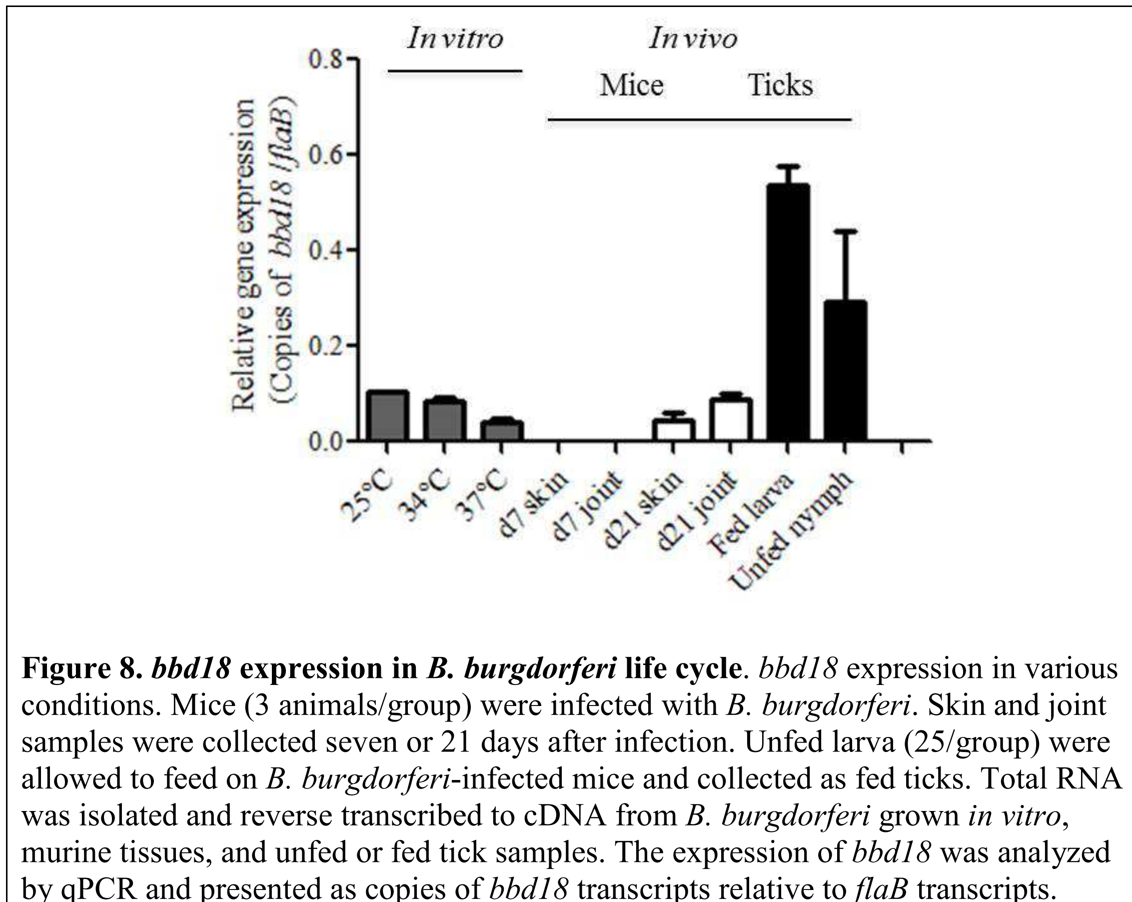


Figure 8. *bbd18* expression in *B. burgdorferi* life cycle. *bbd18* expression in various conditions. Mice (3 animals/group) were infected with *B. burgdorferi*. Skin and joint samples were collected seven or 21 days after infection. Unfed larva (25/group) were allowed to feed on *B. burgdorferi*-infected mice and collected as fed ticks. Total RNA was isolated and reverse transcribed to cDNA from *B. burgdorferi* grown *in vitro*, murine tissues, and unfed or fed tick samples. The expression of *bbd18* was analyzed by qPCR and presented as copies of *bbd18* transcripts relative to *flaB* transcripts.

To further assess the role of the gene product in *B. burgdorferi* survival and infectivity, we then created *bbd18*-knockout spirochetes. Infectious isogenic mutants were generated by replacing the *bbd18* ORF with a kanamycin resistance cassette via homologous recombination (Fig. 9A). Two kanamycin-resistant *B. burgdorferi* transformants were obtained. The plasmid content of these two mutants were analyzed and compared to that of the wild-type parental clone using PCR. B31 carries 20 linear and circular plasmids but not cp9 or plasmid 'C'. One clone (Mut 2) was

missing cp32-6 (Fig. 10), while the other lacked lp28-1, which contains the *vls* antigenic variation locus important for infectivity. A gentamicin cassette was cloned into a copy of lp28-1 and transformed into the latter mutant (Mut 1). The plasmid profile was then identical to that of wild type (Fig. 10). To verify the desired genomic arrangements in the *bbd18* mutants, a series of PCRs were performed. Primer combinations generated different sized DNA fragments between the wild-type strain and *bbd18* mutants, consistent with the replacement of the gene with the 1,248-bp kanamycin cassette (Fig. 9B). PCRs with primer combinations were used to further ensure that the antibiotic cassette was appropriately inserted. Collectively, the PCR results show that a double-crossover event had occurred in the mutants. An RT-PCR analysis demonstrated that while *bbd18* mRNA was undetectable in the mutants, it is present in wild-type and complement isolates (Fig. 9C). Compared to parental isolates, the *bbd18* mutant displayed slower *in vitro* growth at 34°C (Fig. 9H), as was observed for *B. burgdorferi* lacking *bbd16* to *bbd25* (Casselli, Tourand et al. 2012). To show that the phenotype observed in the mutant spirochetes was due to the loss of the specific gene product and not aberrant changes in the genome, we attempted to generate *bbd18*-complemented isolates by reinserting a wild-type copy of the gene at the native locus or into the chromosome of Mut 1 spirochetes (Fig. 9D). Clones that grew in the presence of both kanamycin and streptomycin were selected for further study. PCR (Fig. 9E) and RT-PCR (Fig. 9F) analyses confirmed the intended recombination events in one of the tested isolates that also retained the endogenous set of plasmids. This *bbd18*-complemented isolate was used in subsequent experiments. From SDS-PAGE followed by Coomassie Brilliant Blue staining, all

four strains (WT, Mut 1, Mut 2, and Com) had similar overall protein profiles (Fig. 9G), although Mut 1 and Com produced increased amounts of a high-molecular-weight protein compared to wild type. The complemented spirochetes did not exhibit a growth defect (Fig. 9H).

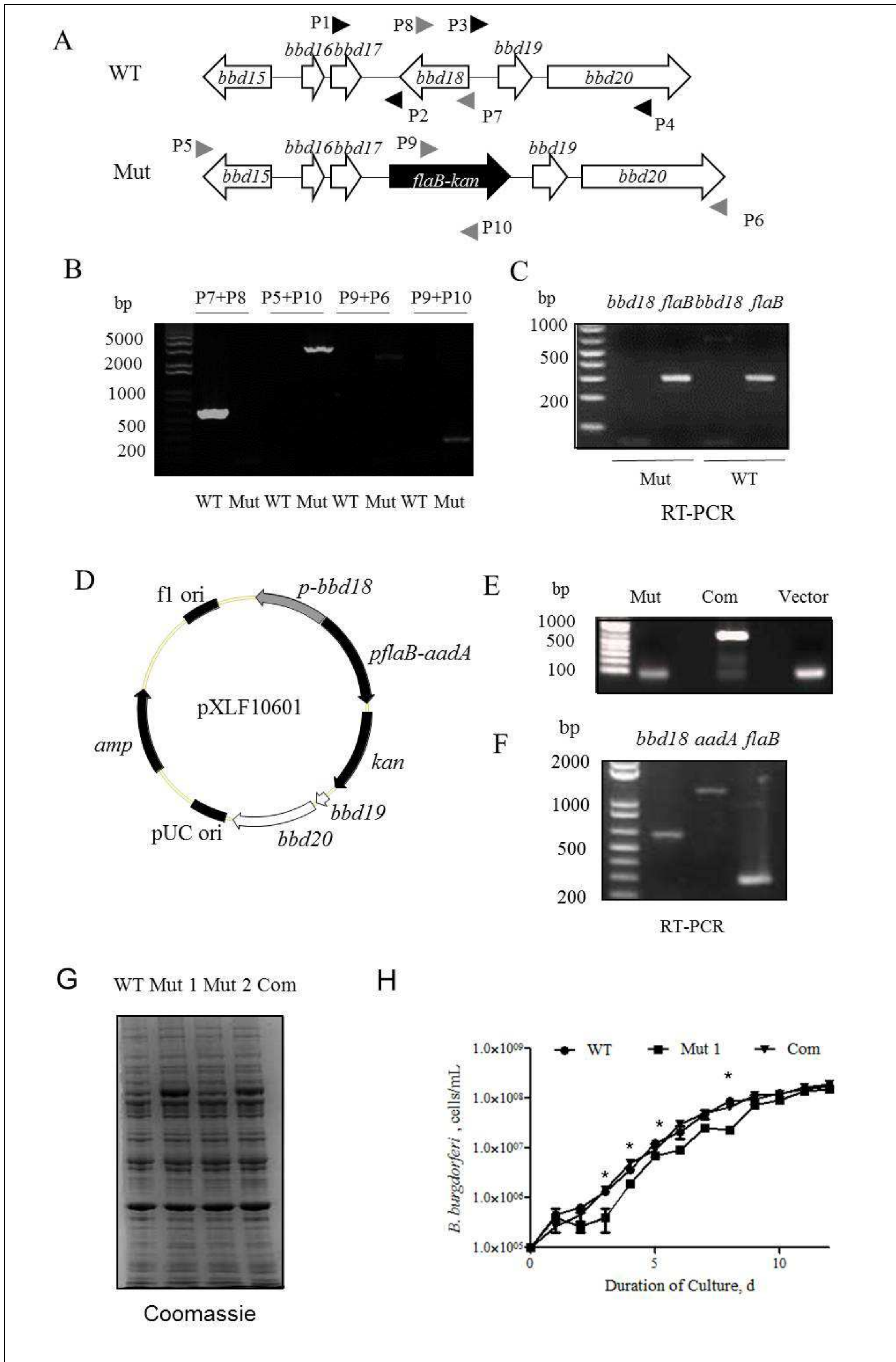

A
WT
P1
P8
P3
bbd15
bbd16
bbd17
bbd18
bbd19
bbd20
P2
P7
P4
Mut
P5
P9
flaB-kan
P10
P6
B
bp
P7+P8
P5+P10
P9+P6
P9+P10
5000
2000
1000
500
200
WT Mut WT Mut WT Mut WT Mut
C
bp
bbd18 flaB bbd18 flaB
1000
500
200
Mut
WT
RT-PCR
D
f1 ori
p-bbd18
pflaB-aadA
pXLF10601
amp
kan
bbd19
pUC ori
bbd20
E
bp
Mut
Com
Vector
1000
500
100
F
bp
bbd18 aadA flaB
2000
1000
500
200
RT-PCR
G
WT Mut 1 Mut 2 Com
Coomassie
H
WT
Mut 1
Com
B. burgdorferi , cells/mL
Duration of Culture, d

Figure 9. Generation of *bbd18* mutant and genetically complemented isolates of *B. burgdorferi*.
(A) Schematic representation of WT spirochetes and *bbd18* mutants at the *bbd18* locus. Regions flanking *bbd18* upstream and downstream were amplified using primers P1-P4 (*black arrowheads*) and ligated on either side of the *flaB-kan* cassette to obtain the mutagenic construct.
(B) Integration of *flaB-kan* in the *bbd18* locus. Combinations of primers P5-P10 (*gray arrowheads* in (A)) were used for PCR analyses of DNA isolated from WT and *bbd18*-mutant *B. burgdorferi* and the samples subjected to gel electrophoresis. Migration of DNA ladder in base pairs is shown on the left.
(C) RT-PCR analyses of *bbd18* transcripts. Total RNA was isolated from wild-type *B. burgdorferi* and *bbd18* mutant, converted to cDNA, and used to amplify *flaB* and *bbd18*. Samples were visualized on an agarose gel.
(D) Construction of *bbd18* complementation construct. The native promoter, open reading frame of *bbd18*, and streptomycin resistance cassette (*pflaB-aadA*) were cloned into pXLF10601 for recombination into the *bbd18* locus by allelic exchange.
(E) Integration *bbd18* in its native locus. *bbd18* primers were used for PCR analysis of DNA isolated from *bbd18*-mutant and complement *B. burgdorferi* and the samples subjected to gel electrophoresis.
(F) RT-PCR analysis of *bbd18* expression. Total RNA was isolated from *bbd18* mutant and complemented *B. burgdorferi*, converted to cDNA, and subjected to PCR analyses with *bbd18*, *aadA*, and *flaB* primers, and then run on an agarose gel.
(G) Protein analysis. *B. burgdorferi* lysates were separated by SDS-PAGE. Samples were stained with Coomassie Brilliant Blue.
(H) Growth analysis of *B. burgdorferi* isolates. WT spirochetes (*circles*), *bbd18* mutants (*squares*), and *bbd18*-complemented isolates (*inverted triangles*) were diluted to 10^5 cells/ml and grown at 34°C in BSK media. Triplicate samples were counted under a dark-field microscope with a Petroff-Hausser cell counter. Asterisks indicate significant difference between WT and mutant ($p<0.05$) during log phase.

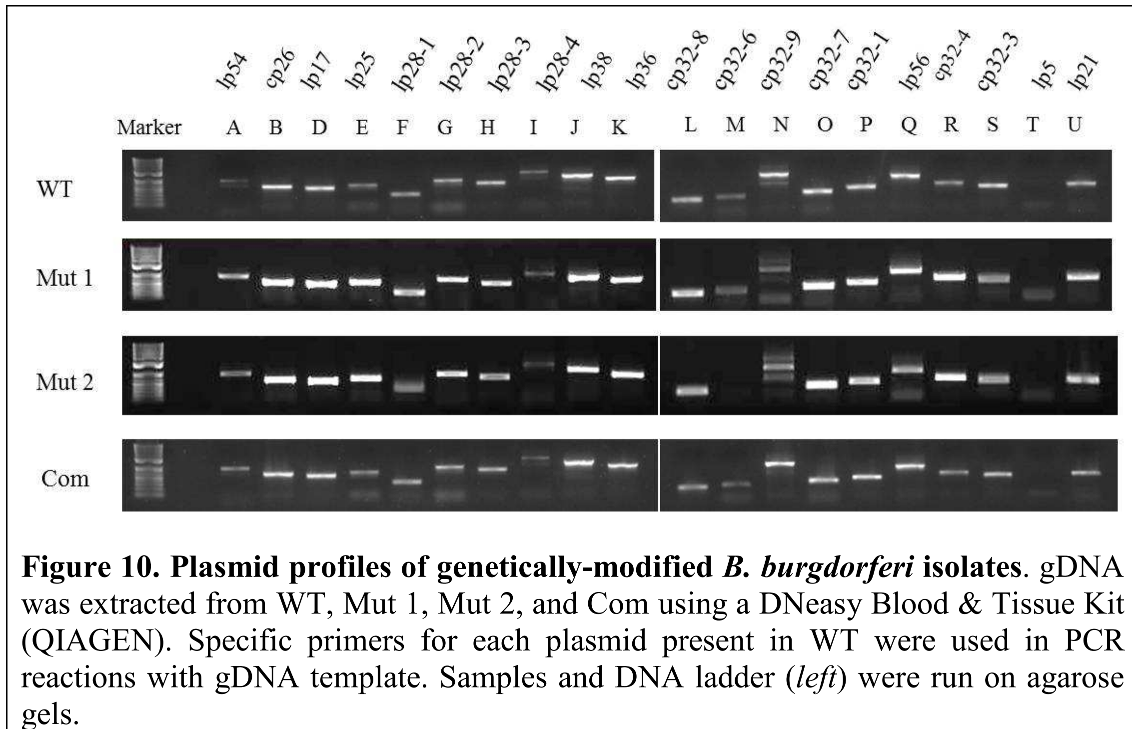


Figure 10. Plasmid profiles of genetically-modified *B. burgdorferi* isolates. gDNA was extracted from WT, Mut 1, Mut 2, and Com using a DNeasy Blood & Tissue Kit (QIAGEN). Specific primers for each plasmid present in WT were used in PCR reactions with gDNA template. Samples and DNA ladder (*left*) were run on agarose gels.

Loss of *bbd18* significantly reduces infectivity in mice but not ticks

To determine whether the lack of *bbd18* influences *B. burgdorferi* infectivity *in vivo*, groups of C3H/HeN mice (3/group) were inoculated intradermally with equal numbers of wild-type, *bbd18* mutant, or *bbd18* complement *B. burgdorferi* (10^5 spirochetes/mouse). Infection was assessed by quantitative RT-PCR analyses of pathogen burden in skin, joint, heart, and bladder samples at 7 days post-infection and by culture of tissue biopsies. The qPCR results indicated that although wild-type spirochetes persisted in mice, *bbd18* mutants were significantly reduced or undetectable (Fig. 11). As seen by Hayes et al., their genetically-complemented isolates behaved surprisingly like the mutants (Hayes, Dulebohn et al. 2014). Wild-type spirochetes were isolated by culture of infected skin, whereas attempts to isolate viable *bbd18* mutants and complement remained unsuccessful (Table 4). Further,

mice infected with wild-type *B. burgdorferi* showed seroconversion, which was absent in mice infected with *bbd18*-mutant or complemented *B. burgdorferi*.

Table 4. Culture positivity of skin from *B. burgdorferi*-injected mice.

Mouse	WT	Mut 1	Mut 2	Com
1	+	-	-	-
2	+	-	-	-
3	+	-	-	-

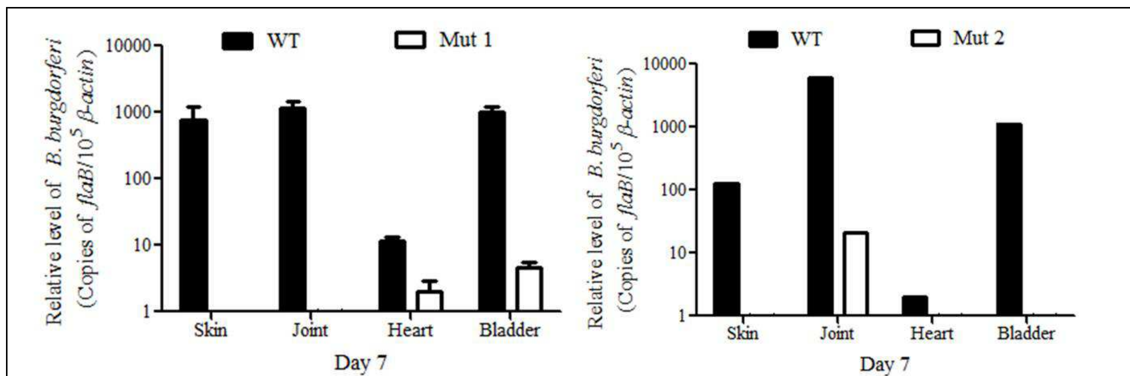


Figure 11. Characterization of *bbd18* mutant in immunocompetent mice. *bbd18* deletion impairs ability of *B. burgdorferi* to persist in mice. Animals were infected with wild type (WT, *black bars*) or *bbd18* mutant (Mut 1 or Mut 2, *white bars*). Pathogen levels were analyzed in skin, heart, joint, and bladder samples by measuring copies of *B. burgdorferi flaB* transcripts at 7 days of infection by qPCR, and data were normalized against murine *ß-actin* levels. *bbd18* mutants were either undetectable or persisted at levels lower than corresponding wild-type pathogens.

We then examined whether a larger challenge inoculum of mutant and complement could enhance survival against host immune defenses and thus detection, as we previously showed for *bba57* mutant joint colonization (Yang, Qin et al. 2013). Higher doses could allow WT-level loads if deleting *bbd18* diminished the ability of the spirochetes to escape the immune system rather than caused a change in their inherent survival in a host environment. Mice were individually challenged with 10^6, 10^7, or 10^8 *bbd18* mutant. Two weeks after challenge, pathogen levels in skin, joint, heart, and bladder were determined by qPCR (Fig. 12). Even a 1000-fold higher initial inoculum was undetectable, suggesting deleting *bbd18* affects the ability of spirochetes to establish infection in mice.

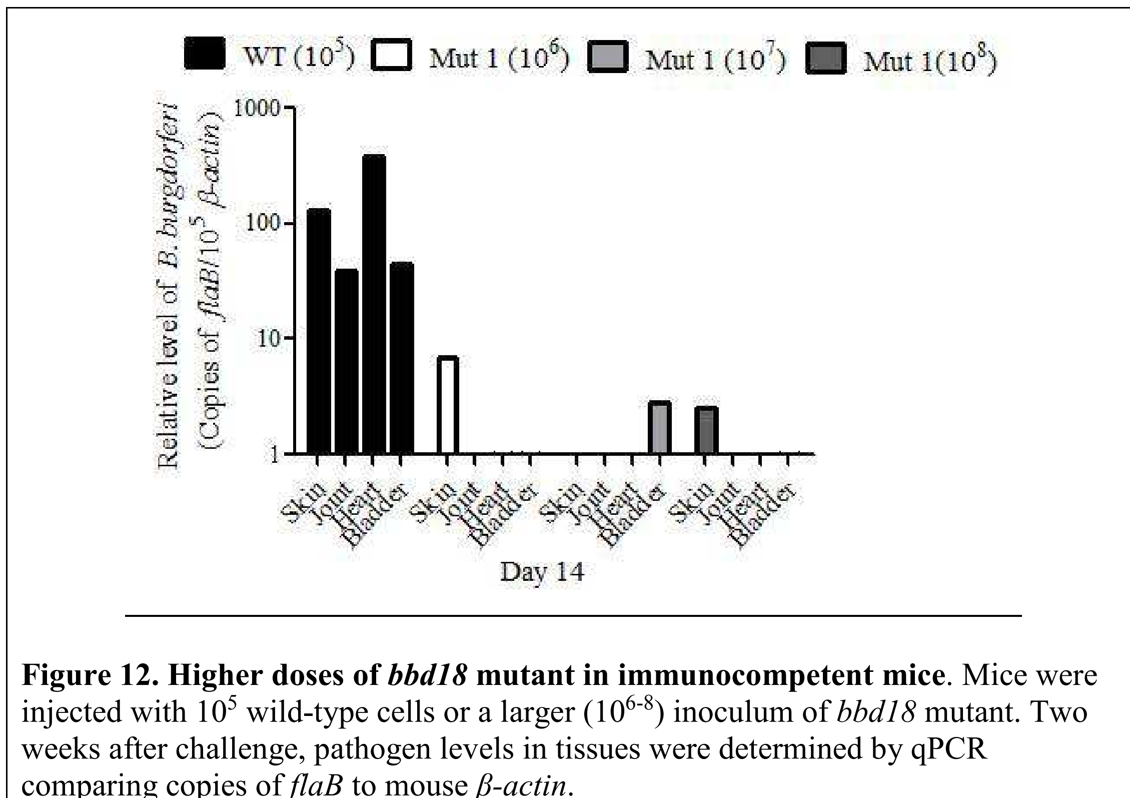


Figure 12. Higher doses of *bbd18* mutant in immunocompetent mice. Mice were injected with 10^5 wild-type cells or a larger (10^{6-8}) inoculum of *bbd18* mutant. Two weeks after challenge, pathogen levels in tissues were determined by qPCR comparing copies of *flaB* to mouse *β-actin*.

The low levels of mutant and complement *B. burgdorferi* may be due to killing mediated by B and/or T cells. To protect spirochetes from these host immune cells, DMCs loaded with WT or Mut 1 *B. burgdorferi* were implanted under the skin of mice and removed after one week. WT spirochetes could be regrown from the chambers, while Mut 1 could not (Table 5). As soluble factors, such as antibodies and metabolites, can diffuse through the membrane pores, but the chambers are impermeable to phagocytes, the attenuated murine infectivity of the mutant is likely not due to host cellular immune response. Thus, BBD18 appears to be required for *B. burgdorferi* to evade other immune defenses and establish infection in mice.

Table 5. Culture positivity of *B. burgdorferi* from diffusion chambers.

Mouse	WT	Mut 1
1	+	+/-
2	+	+/-

Additionally, we injected SCID mice with 10^5 of wild-type, mutant, or complement spirochetes. The bacterial burden was low in the mutant and complement-injected SCID mice (Fig. 13), further indicating that the adaptive immune system is not responsible for clearing the manipulated spirochetes.

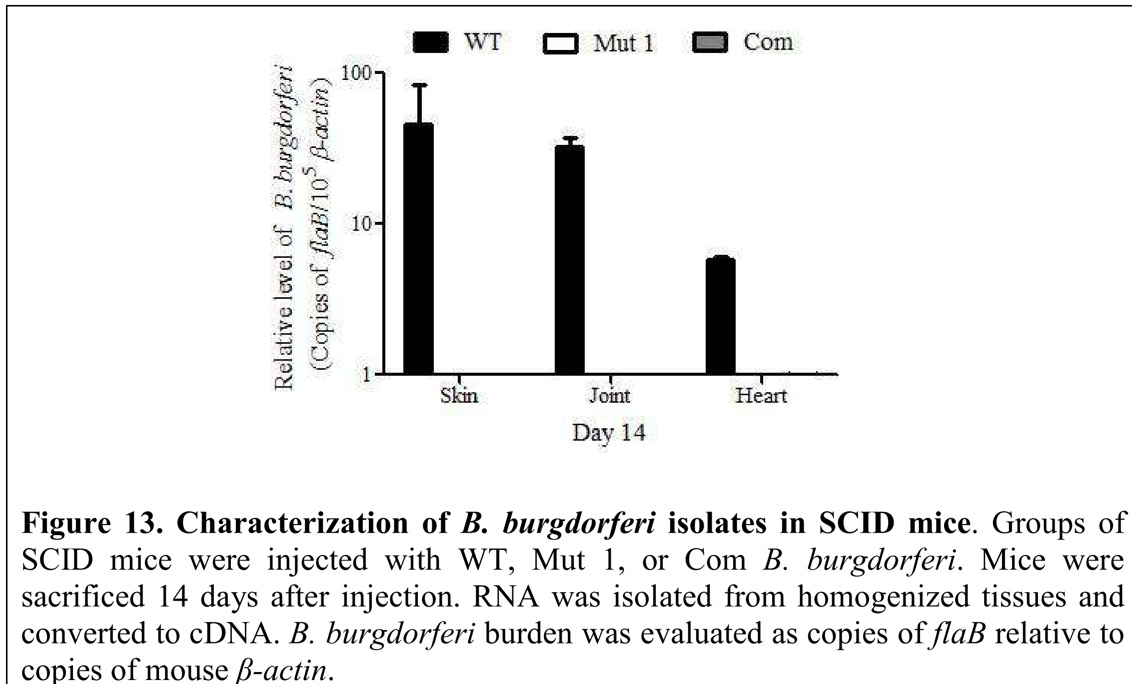


Figure 13. Characterization of *B. burgdorferi* isolates in SCID mice. Groups of SCID mice were injected with WT, Mut 1, or Com *B. burgdorferi*. Mice were sacrificed 14 days after injection. RNA was isolated from homogenized tissues and converted to cDNA. *B. burgdorferi* burden was evaluated as copies of *flaB* relative to copies of mouse *β-actin*.

Because *bbd18* is induced in fed ticks and survives in ticks but not mice, it was possible that the gene is involved in the transmission of spirochetes. We performed the following study to determine whether the loss of *bbd18* expression in the mutants had any adverse effects on their ability to transmit from infected ticks to naïve mice that could be restored by complementation of the gene: Separate groups of nymphs that were microinjected with wild-type or mutant isolates were allowed to feed on naïve C3H mice (3/group) and collected as fully engorged ticks. Spirochete burdens in the ticks were assessed by qPCR analyses. After 14 days of feeding, mouse infection was assessed by culture of skin and spleen samples and by qPCR analyses of skin and joint tissues. Results indicated that the burden of *bbd18* mutants was lower in fed ticks (Fig. 14A), and little to no *B. burgdorferi* was detected in tissues of mice parasitized by mutant-injected ticks (Fig. 14B), further supporting the result that the mutants have a defect in their ability to survive *in vivo*.

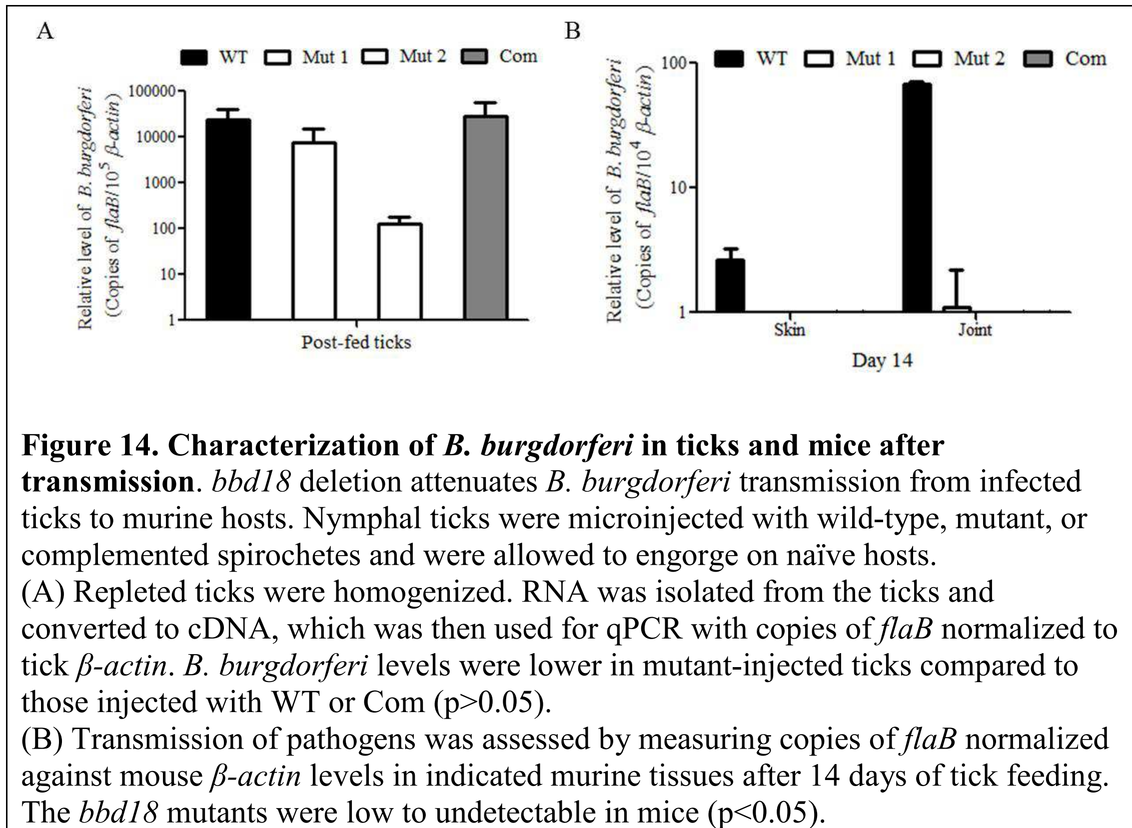


Figure 14. Characterization of *B. burgdorferi* in ticks and mice after transmission. *bbd18* deletion attenuates *B. burgdorferi* transmission from infected ticks to murine hosts. Nymphal ticks were microinjected with wild-type, mutant, or complemented spirochetes and were allowed to engorge on naïve hosts.
(A) Repleted ticks were homogenized. RNA was isolated from the ticks and converted to cDNA, which was then used for qPCR with copies of *flaB* normalized to tick *β-actin*. *B. burgdorferi* levels were lower in mutant-injected ticks compared to those injected with WT or Com ($p>0.05$).
(B) Transmission of pathogens was assessed by measuring copies of *flaB* normalized against mouse *β-actin* levels in indicated murine tissues after 14 days of tick feeding. The *bbd18* mutants were low to undetectable in mice ($p<0.05$).

2.4 Discussion

Compared to wild type, both *bbd18* mutant isolates generated in our laboratory were less infectious in mice when inoculated by needle or transmitted via tick bite, suggesting that BBD18 facilitates spirochete persistence *in vivo*, specifically in mice. Complementing the gene at the native locus allowed production of a wild-type level of *bbd18* transcripts but did not restore murine infectivity, as otherwise observed previously with most other genes involved in mammalian infectivity (Hayes, Dulebohn et al. 2014). To further investigate these unusual observations, we could complement *bbd18* mutant with a larger or the entire sequence of lp17. If the

defect is in the plasmid and not only *bbd18*, we expect that such complementation would completely restore the WT phenotype.

We also examined and record that larger (up to 1000-fold higher) inoculums of *bbd18* mutant still could not establish infection. 5A4 strain *B. burgdorferi* lacking *bbd16* to *bbd25* were deficient in early tissue colonization of SCID mice (Casselli, Tourand et al. 2012). Our *bbd18* mutant and complement could not infect such mice lacking functional adaptive immune systems, suggesting that the *bbd18* region is important for spirochete infection of mice. A better understanding of the role of BBD18 for *B. burgdorferi* persistence in the infection cycle could contribute to the development of new strategies to combat Lyme borreliosis.

Chapter 3: Determine function(s) of BBD18 in *B. burgdorferi* biology

3.1 Introduction

bbd18 may encode a protein that plays important role(s) in infectivity of globally prevalent spirochetes. We discovered that *bbd18*-deletion mutant *B. burgdorferi* persist in unfed ticks at wild-type levels, but these spirochetes cannot survive as well in mice. Thus despite low expression, the gene product of *bbd18* appears to play significant role(s) in mammalian phases of the infection cycle. As these and many other *B. burgdorferi* gene products bear unique sequences, their functions are currently unknown. BBD18 is predicted to be a protein of 220 amino acids with molecular mass of 25.7 kDa. This protein contains fairly high percentages of both charged (29%) and aromatic (11%) residues and is very basic, having a pI of 9.39 (Sarkar, Hayes et al. 2011) (Fig. 15). Structural predictions suggest predominantly alpha helices with a few beta sheets connected by a number of coiled regions (Sarkar, Hayes et al. 2011) (bioinf.cs.ucl.ac.uk/psipred/).

```
Conf:
Pred:
Pred: CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCEECEEEE
  AA: MQKEITINYNEYTIGGIKGTFFGNSQYEANYRAKINGFII
                10        20        30        40

Conf:
Pred:
Pred: ECCCCCHHHHHHCCCCEEECCCCCCCCCCHHHHHCCCHHE
  AA: DFFKIPISLKKKYELNIKALSDPNFSSTNIAMNCINTFKL
                50        60        70        80

Conf:
Pred:
Pred: EEEEECCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHH
  AA: IVDIVNMQTGENYDYDTFITKTDTEKMLKYGTKIIAALAR
                90        100       110       120

Conf:
Pred:
Pred: HHHHCCCCCCCHHHHHHHCCCCCCCCCCCCCHHHHHHHHH
  AA: HFDEQNKTNFNESYYEWEKGWIDKKWINYEPTAEEIKEIQ
                130       140       150       160

Conf:
Pred:
Pred: HHHHCCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHH
  AA: IMNQKLNPLKLKHRKKNLNNGQIRLLQAINKIEDQSQQQA
                170       180       190       200

Conf:
Pred:
Pred: CCCCCCCCCCCCCCCCCCCC
  AA: TKSNSKTKKLKGNHGEKTKI
                210       220

Legend:
  - helix     Conf: - confidence of prediction
                    -    +
  - strand    Pred: predicted secondary structure
  - coil      AA: target sequence
```

Figure 15. Predicted secondary structure of BBD18 (bioinf.cs.ucl.ac.uk/psipred/). PSIPRED (Psi-blast based secondary structure prediction) was used to investigate the structure of BBD18.

BBD18 has yet to be characterized for its function in microbial biology. Phyre^2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) results give up to 23.5% confidence for oxidoreductase function of the C-terminus. A previous report (Hayes, Dulebohn et al. 2014) and our preliminary data indicate that the protein may be involved in regulating the expression of several genes. Although the general characteristics and predicted structure of BBD18 suggest involvement in DNA interaction, analysis of *bbd18* sequence using the Pfam database (Finn, Bateman et al. 2014) does not reveal homology with known DNA binding domains or proteins or transcriptional regulators. The goal was to determine the specific function(s) of BBD18 and its importance in *B. burgdorferi* biology or at least further characterize the protein.

3.2 Materials and methods

B. burgdorferi

An infectious isolate of *B. burgdorferi*, B31 A3, was used. Cultures were kept at 34°C for the duration of growth or subcultured and placed at 37°C. For *in vitro* growth analysis, spirochetes were diluted to a density of 10^5 cells/ml, grown until stationary phase (~10^8 cells/ml), and counted by dark-field microscopy using a Petroff-Hausser cell counter.

RNA isolation, cDNA synthesis, and qPCR

Total RNA from *B. burgdorferi*, reverse transcribed, and subjected to qPCR analyses with primers in Table 6, as described in Section 2.2.

Table 6. Oligonucleotide primers for evaluating expression of lp17 genes.

Sequence (5' - 3')	Purpose
TCCAAATGGGAATATTTCTCG	Forward primer for *bbd01*
TCGACATGAATATGCGAGTCTT	Reverse primer for *bbd01*
AAAACAAGTGCTCAGAAATAGAAAAA	Forward primer for *bbd02*
AGCAGGCATACCCCAGTAAA	Reverse primer for *bbd02*
TTGCGATCTACCAACAATGAA	Forward primer for *bbd03*
CGAAACACTTTAAGGGGGATT	Reverse primer for *bbd03*
ATACGCCGTGCGTTTACTTT	Forward primer for *bbd04*
TTTGTGTAACATATTTTTAAAGGATAATTG	Reverse primer for *bbd04*
TCCATAGTGCAAATAAAAATTATGCT	Forward primer for *bbd05*
AACAGGGTTTTAGAATCAACTTCA	Reverse primer for *bbd05*
GGGGGTAAATTCGGGTACAG	Forward primer for *bbd06*
CCAATTGTACACTACCCATTTTTG	Reverse primer for *bbd06*
TTTATGGCTAATTATAAAAACATAGCA	Forward primer for *bbd08*
TCATGCAATTCTAAATCATTAATACTC	Reverse primer for *bbd08*
CGCTAATGAATGCGGAATCT	Forward primer for *bbd09*
AGCAAAACTCTCAATTGCTTG	Reverse primer for *bbd09*
TTTGAGGCTAAAGGAGAGTTGG	Forward primer for *bbd10*
CATTTCCCAAGGCAACAAAT	Reverse primer for *bbd10*
TGAGAATGGGTTGGGAGGTA	Forward primer for *bbd11*
GGGGTTACATTTGAAGCTTGG	Reverse primer for *bbd11*
TCAAGGATGTTAGGTTATATGTTGAA	Forward primer for *bbd12*
TTTTGGAAAGACAAATTACTGCAA	Reverse primer for *bbd12*
GCGCAATCCATGTAGACAAC	Forward primer for *bbd13*
TGTTTTGTAGATTTTCGGCTCA	Reverse primer for *bbd13*
GCTGGGCAATTGACCTTAAA	Forward primer for *bbd14*
AAAGCGGTGTTTCCAAAGATT	Reverse primer for *bbd14*
CAAGATAGATGGGGCTTCTCA	Forward primer for *bbd15*
TTTGAAACATTAGATACTCTGGAAGAA	Reverse primer for *bbd15*
ATACAATTGGGGGCATAA	Forward primer for *bbd18*
TTTCTGTGTCTGTTTTGGTAATAA	Reverse primer for *bbd18*
TGTAAGTAAATTCTGGTGATAAATGAA	Forward primer for *bbd19*
AATGCCAACCTTCTCTTTGC	Reverse primer for *bbd19*
GTGTGCCGAGTGGAACTTTT	Forward primer for *bbd21*
GCTCCCAAAGCTCAAAACTG	Reverse primer for *bbd21*
TCAAAAATGACGATCAAGCAA	Forward primer for *bbd22*
AACCAGAAGCTGTTAATCCCTTT	Reverse primer for *bbd22*

GCCGATAAAGTTTGTAAAAACAGA	Forward primer for *bbd23*
GCGAGGCTTTAATCAATCCA	Reverse primer for *bbd23*
AAATTTTTATGACAGCAATAATCGTG	Forward primer for *bbd24*
ATTTTGTGAAAAATGTTTTTAGTTGC	Reverse primer for *bbd24*
TGAGAGTGTTTTTAAATGCCTTTTT	Forward primer for *bbd25*
GAATGCCCATTAAATGTAACAAGA	Reverse primer for *bbd25*

Whole genome microarray analysis

Oligonucleotides representing all annotated ORFs and pseudogenes of *B. burgdorferi* B31MI in the TIGR and/or NCBI genome databases were synthesized by QIAGEN-Operon (Alameda, CA) according to an algorithm that optimizes the melting temperatures within 5°C of each other. All oligonucleotides were 70 nucleotides and at least 40 nucleotides from the 3′ end of the ORF (except for some short ORFs). Random sequences were synthesized as negative controls (Terekhova, Iyer et al. 2006).

The oligonucleotides were printed on UltraGAPS gamma amino propyl silane slides (Corning Inc., Corning, NY) using a Bio-Rad VersArray arrayer. Each oligonucleotide was printed in triplicate on each array, with spot-to-spot separation of 350 µm. Several quality control and quality assurance tests were performed for each array print run. Following printing, the arrays were crosslinked with 600 mJ of UV by using a Stratalinker crosslinker (Terekhova, Iyer et al. 2006).

RNA from the *B. burgdorferi* strains were fluorescently labeled with cyanine-5-dUTP (Cy5) or Cy3 dye (Perkin-Elmer, Boston, MA) by reverse transcription. Microarray hybridizations were performed as described with cDNA prepared from the cells with two technical replicates (dye swap). Cy3- and Cy5-labeled DNA were combined and

further purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Purified DNA were dried and resuspended in 45 µl of nuclease-free water. A reagent mixture with the DNA was applied to arrays. After application of the labeled DNA sample, slides were incubated overnight at 42°C, washed several times, and immediately dried by centrifugation at 2,700 rpm for 3 min (Terekhova, Iyer et al. 2006).

The hybridized microarray slides were scanned on a GenePix 4000B scanner (Axon Instruments, Union City, CA) at 10-µm resolution. The resulting dual-color images were analyzed by GenePix Pro software (Axon Instruments). The data were inputted to Microsoft Excel for background correction, normalization, and filtering. Means and standard deviations of the fluorescence intensities were calculated for all blank control spots in Cy3 and Cy5 channels separately; the mean plus two standard deviations was considered to be background and subtracted from each intensity value in the appropriate channel. Spots yielding signals below background for B31MI DNA hybridization were excluded from analysis. The background-adjusted intensity values were normalized across all arrays. The resultant values were used to calculate $\log_2(Cy3/Cy5)$ for each spot, and six $\log_2(Cy3/Cy5)$ values for each ORF from separate microarrays were averaged to yield a final value (Terekhova, Iyer et al. 2006). Significance of differential expression was determined by two-tailed, unpaired Student's *t*-test at $p<0.02$ and fold comparison greater than two.

Generation of recombinant BBD18 and production of antiserum

The *bbd18* ORF was cloned into pET-46 using the Ek/LIC Vector Kit (EMD Millipore). Recombinant protein was produced in *E. coli*. Purification was performed as described: Pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) with lysozyme, incubated at RT for 30 min with intermittent vortexing, sonicated, and then centrifuged for 30 min at 10,000 x g. The resulting pellets were resuspended in urea running buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 8 M urea, 5 mM βME, pH 8.0) and incubated at RT for 60 min with occasional vortexing and centrifuged at 10,000 x g, 4°C for 30 min. His Bind resin (Novagen) was added to a Poly-Prep chromatography column (Bio-Rad) and rinsed with running buffer and charge buffer (0.1 M $NiSO_4$). The sample was loaded into the column and rinsed with running buffer, and recombinant BBD18 was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 8 M urea, 5 mM βME, pH 8.0). The eluted protein was dialyzed O/N at 4°C against Refolding Buffer A (4 M urea, 20 mM ethanolamine, pH 11.7). Half the buffer was removed and replaced with Refolding Buffer B (20 mM ethanolanime, cystine, pH 11.7) and used for dialysis for 8 h at 4°C. Recombinant BBD18 was then dialyzed with Refolding Buffer C (20 mM Tris, 2 mM cysteine, 0.2 mM cystine, pH 10.7) O/N at 4°C and finally into PBS O/N at 4°C.

To generate murine antiserum against BBD18, C3H mice were immunized with recombinant protein mixed with an equal amount of Complete Freund's adjuvant and boosted twice at ten-day intervals with protein in Incomplete Freund's adjuvant. Ten days after the final immunization, serum was collected, and immunoblot analyses showed BBD18 antibodies from the mice.

Two-dimensional SDS-PAGE and immunoblotting

Two-dimensional (2D) SDS-PAGE was performed as detailed. Samples were solubilized in rehydration solution (7 mM urea, 2 M thiourea, 0.5% v/v Triton X-100, 0.5% v/v IPG buffer pH 3-10, and 60 mM DTT). Immobiline DryStrips (GE Healthcare) were placed in rehydration solution overnight. After isoelectic focusing, the strips were incubated in equilibrium buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 29.3% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue, and 0.1% DTT) and resolved by SDS-PAGE. Proteins were separated on 12 or 15% polyacrylamide gels at 90 to 190V. Gels were stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane. Immunoblotting was performed as described. Membranes were blocked with 5% milk in PBS-T for at least 1 h. Then, they were incubated with mouse sera or specific primary antibodies in PBS-T for at least 40 min and the respective secondary antibody conjugated to HRP for at least 50 min. Equal volumes of peroxide solution and enhanced luminol solution (Thermo Fisher Scientific) were added to the blots, and proteins were detected by film imaging.

Native/nondenaturing PAGE

Nondenaturing gel solution was made by mixing 10x TBE, 30% acrylamide/bisacrylamide, and deionized water. TEMED and 10% APS was added and thoroughly mixed. The solution was pipetted in between gel plates. Combs were added before allowing the solution to polymerize. Binding reactions were prepared by gently mixing deionized water and 2 µl of 5x binding buffer (750 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 50 mM Tris, pH 7.4) with protein and incubating at room

temperature. At the end of the incubation, 2 µl of 6x gel-loading solution was added for each 10 µl reaction mix and mixed gently but thoroughly. For electrophoresis, the lower reservoir of the tank was filled with 1x TBE. Then, the gel plates were clamped to the top of the tank and the upper reservoir filled with 1x TBE. Gels were run at 5 V/cm. The gels were incubated in SYPRO Ruby stain with TCA with continuous, gentle agitation for at least 3 h in the dark and washed twice with deionized water for ~10 min each. Gels were destained in 10% methanol and 7% acetic acid for at least 60 min and washed twice. Stained proteins were visualized using 300 nm UV transillumination.

Generation of *kan* insertion isolates of *B. burgdorferi*

DNA fragments flanking the *bbd18* open reading frame (ORF) were PCR-amplified (Table 7) and inserted into the plasmid pXLF10601. This plasmid was sequenced to confirm its identity and electroporated into wild-type *B. burgdorferi* A3, and transformants were selected for growth in the presence of kanamycin. To verify the desired genomic arrangements in the *kan* insertion clones, a series of PCRs were performed. The plasmid profile of the *B. burgdorferi* was assessed for maintenance of wild-type plasmids.

Table 7. Oligonucleotide primers for generating *kan* downstream insertion isolate of *B. burgdorferi*.

Name	Sequence (5' - 3')	Purpose
P1	GG***GAGCTC***GCCCCATCTATCTTGTTTCG	Forward primer of left arm for *kan* D
P2	AA***TCTAGA***TAAGATCGGGGGTAGGGACT	Reverse primer of left arm for *kan* D
P3	GG***GTCGAC***GGGTGTTGGGGACTATTGGT	Forward primer of right arm for *kan* D
P4	GC***GGTACC***AATTTAGGCGGGCAATTCTT	Reverse primer of right arm for *kan* D
P6	AA***TCTAGA***AAAGTATTTCTTTTGGTTGGTGTT	Reverse primer of left arm for *kan* U2.
*kan*F	GGTTGCATTCGATTCCTGTT	Kanamycin internal forward primer
*kan*R	ATTCCGACTCGTCCAACATC	Kanamycin internal reverse primer
*kan*R2	GCCTGAGCGAGACGAAATAC	Kanamycin internal reverse primer
*kan*R3	AACAGGAATCGAATGCAACC	Kanamycin internal reverse primer
*Kan*check	TTTGAGAATTTTTAGCCTCAGACTC	Forward primer to check *kan* insertion

Phenotypic analysis of *kan* insertion isolates

To examine and compare the phenotypes *in vivo*, *kan* U and D *B. burgdorferi* were separately inoculated into groups of mice (3/group, 10^5 spirochetes/mouse). Skin, joint, heart, and bladder samples from the infected mice were isolated at day 7 and 14 after challenge. *B. burgdorferi* burdens were measured by quantitative RT-PCR analyses of *flaB* mRNA and then normalized against murine *β-actin* gene. One microgram of RNA per tissue sample was used for qPCR analyses. Portions of skin and spleen from the mice were cultured in BSK media for the presence of viable *B. burgdorferi*.

3.3 Results

Effect of *bbd18* deletion on other lp17 genes

Manipulating a locus could affect DNA topology and/or the transcription of other genes on the same molecule. In the first microarray, only one gene (*bbd10*) on the same plasmid as *bbd18* was downregulated, while two lp17 genes (*bbd01* and *bbd16*) were upregulated. When Com was included in the experiment, other plasmid 'D' genes appeared to be almost unaffected in the mutants and complement (Table 11 & 12). The difference between the arrays suggests that the observed changes in transcription are due to variation in the samples and not from *bbd18* deletion. qPCR results show that other than *bbd19* (p=0.0430), which may be a pseudogene, the expression of lp17 genes is not significantly affected by manipulations in the *bbd18* locus (Fig. 16).

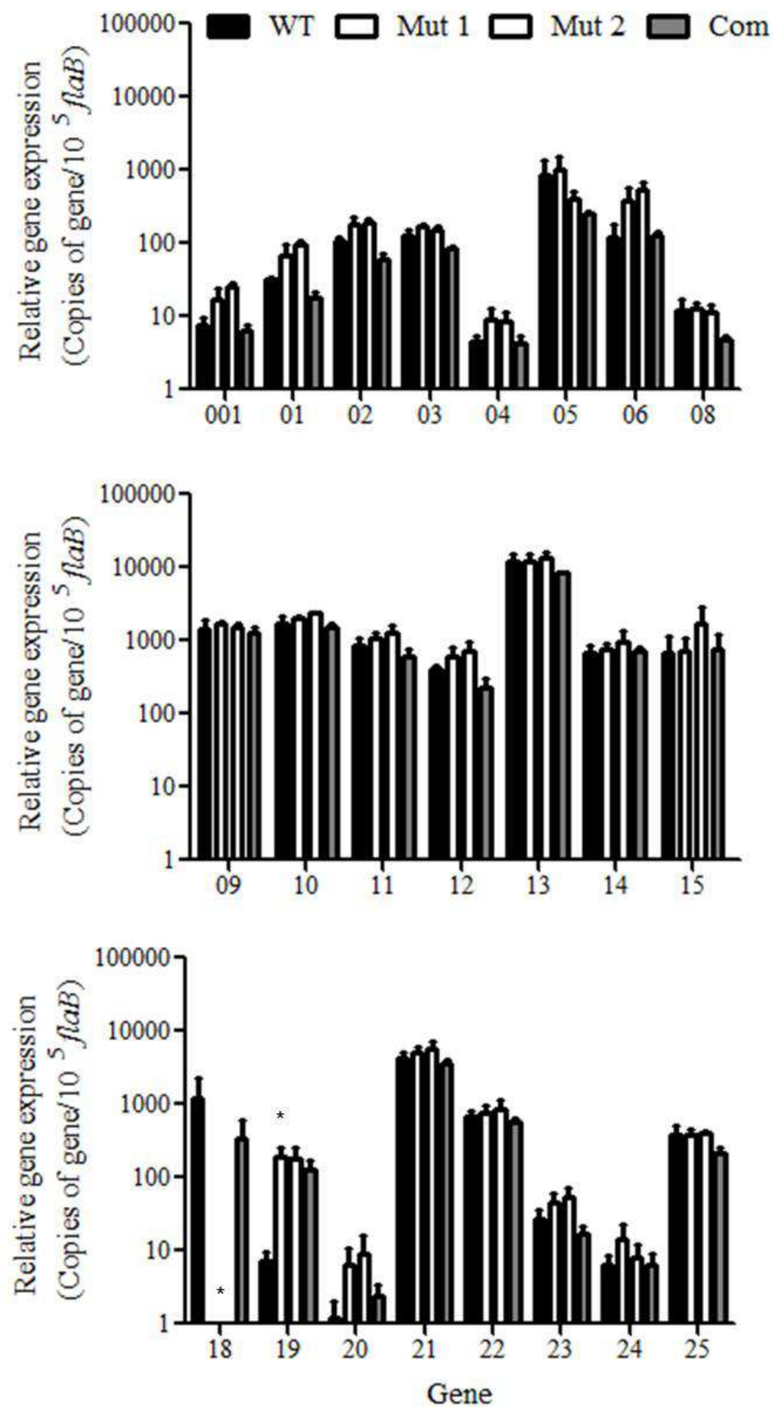


Figure 16. Expression of lp17 genes in various *B. burgdorferi* isolates. Total RNA was isolated and reverse transcribed to cDNA from *B. burgdorferi* grown *in vitro*. The expression of lp17 genes was analyzed by qPCR and presented as copies of gene transcripts relative to *flaB* transcripts. Increase in *bbd19* expression in the mutants and complement was significant (p=0.0430).

***bbd18* deletion influences expression of several other genes**

We sought to detect changes in *B. burgdorferi* gene transcription that could be responsible for the noninfectious phenotype from *bbd18* deletion. Microarray analyses were first used to compare wild-type and *bbd18*-mutant isolates that had been grown at 37°C to simulate mammalian host conditions. Although *bbd18* had low expression in the wild-type *B. burgdorferi*, its absence in *bbd18*-mutant spirochetes influenced the expression of many genes. There was a similar number of genes downregulated (82) in the mutant or expressed more in wild type compared to upregulated (90) or expressed more in the mutant. However, the level of change in gene expression was very different. Almost a third of downregulated genes (25) were greater than 10-fold, with about half (12) of those genes more than 100-fold. The most severely downregulated gene was *ospD* (*bbj09*), which was more than 1000-fold. On the other hand, only five of the upregulated genes were greater than 10-fold and none more than 20-fold. Many of the downregulated genes are in the essential genome and located on the chromosome or plasmid lp54 or 'A'. Several genes in paralogous family 54 were expressed less in the mutant, including *bba69*, *bbi36* and *bbi38*, which are 99% identical, and *bbi39* and *bbj41*, which also have 99% identity (Gilmore, Howison et al. 2007). *bbd10*, which encodes a lipoprotein, was found to be among the highly downregulated genes at more than 200-fold. *bbd01* codes for a hypothetical protein and *bbd16* may be a pseudogene. Both had slightly less than half the amount of transcript in WT versus Mut 1.

Table 8. *B. burgdorferi* genes downregulated more than 2-fold in Mut 1 from microarray experiment 1.

Gene	WT/Mut 1	Gene	WT/Mut 1	Gene	WT/Mut 1
bbj09	1677	*bba40*	5	*bb0336*	3
bbg11	506	*bb0194*	4	*bb0278*	3
bbj41	283	*bb0377*	4	*bb0276*	3
bba50	243	*bba05*	4	*bb0700*	3
bbd10	208	*bba60*	4	*bb0825*	3
bba11	194	*bbf12*	4	*bbb06*	3
bba48	189	*bb0401*	4	*bb0309*	2
bba13	180	*bb0834*	4	*bb0385*	2
bba46	167	*bbb04*	4	*bb0402*	2
bbi36	148	*bba54*	4	*bba61*	2
bbi39	140	*bba03*	3	*bbf05*	2
bbh37	122	*bb0588*	3	*bb0170*	2
bba24	66	*bb0640*	3	*bba15*	2
bba25	55	*bb0772*	3	*bba39*	2
bbe27	36	*bb0335*	3	*bb0019*	2
bbi38	33	*bb0581*	3	*bb0236*	2
bba44	31	*bb0334*	3	*bb0183*	2
bbj21	17	*bba43*	3	*bb0271*	2
bbj08	15	*bb0553*	3	*bb0151*	2
bba47	13	*bba74*	3	*bb0639*	2
bba49	13	*bbj20*	3	*bb0843*	2
bba69	12	*bb0337*	3	*bb0269*	2
bba51	11	*bb0350*	3	*bb0566*	2
bb0241	10	*bba38*	3	*bb0230*	2
bba10	10	*bba17*	3	*bba16*	2
bba41	9	*bb0034*	3	*bb0282*	2
bbg22	6	*bb0118*	3	*bb0304*	2
bba53	5				

Table 9. *B. burgdorferi* genes upregulated more than 2-fold in Mut 1 from microarray experiment 1.

Gene	Mut 1/WT	Gene	Mut 1/WT	Gene	Mut 1/WT
bbh10	19	*bbu09*	4	*bbj19*	3
bbh10.1	17	*bbe12*	4	*bbl12*	3
bbq11	15	*bbo37*	4	*bb0459*	3
bbh03	12	*bbn30*	4	*bbg14*	3
bbn37	10	*bbm19*	4	*bbm10*	3
bbi04	9	*bbp21*	4	*bbs19*	3
bbp01	9	*bbg16*	4	*bba73*	3
bbh06	9	*bbp20*	4	*bbp19*	3
bbh09	9	*bbj06*	4	*bb0803*	3
bbh05	7	*bbf06*	3	*bbn35*	3
bbu03	6	*bbh01*	3	*bbc07*	3
bbr28	6	*bbo29*	3	*bbm28*	3
bbq28	5	*bbm11*	3	*bbu05*	3
bbh04	5	*bbh26*	3	*bbo28*	3
bbr36	5	*bbs32*	3	*bb0707*	2
bbo30	5	*bbs21*	3	*bbr19*	2
bbo03	5	*bbq27*	3	*bbn33*	2
bbl27	5	*bbg34*	3	*bbs38*	2
bbq43	5	*bbl11*	3	*bbn32*	2
bbt07	4	*bbr20*	3	*bbn11*	2
bbr21	4	*bbm30*	3	*bbh27*	2
bbp02	4	*bbr33*	3	*bbd16*	2
bb0814	4	*bbl10*	3	*bb0384*	2
bbg17	4	*bbs20*	3	*bbp24*	2
bbm27	4	*bbo44*	3	*bbd01*	2
bbl26	4	*bbo22*	3	*bbr34*	2
bbd15	4	*bb0586*	3	*bbl04*	2
bbn29	4	*bbs23*	3	*bbm14*	2
bbp35	4	*bbo43*	3	*bbf30*	2
bbm12	4	*bbf29*	3	*bb0455*	2

As *bbd18* deletion affected the expression of many other genes, we wanted to determine if the phenotype could be restored to wild type by complementing *bbd18.* Microarray analyses were performed with the addition of Com. *ospD* expression was also reduced in the mutant this time and almost completely restored to wild-type levels by complementation. There were several differences between the two experiments. Fewer genes were highly regulated in the second array, although the number of genes upregulated in Mut 1 (90) was similar. This time another lp54 gene, *bba67* was expressed at a much lower level in the mutant and partially complemented. *ospC* (*bbb19*) and *bbj40* were also downregulated, but the former gene also had reduced expression in the complemented isolate. Surprisingly, some genes had differential expression in the complement but not mutant, resulting in more up (44) than downregulated (8) genes. None of the genes were more than 10-fold higher in the complemented strain.

Table 10. *B. burgdorferi* genes downregulated more than 2-fold in Mut 1 from microarray experiment 2.

Gene	**WT/Mut 1**
bbj09	269
bba67	112
bbb19	30
bbj40	20
bbe04	8

Table 11. *B. burgdorferi* genes upregulated more than 2-fold in Mut 1 from microarray experiment 2.

Gene	Mut 1/WT	Gene	Mut 1/WT	Gene	Mut 1/WT
bbf23	8	*bbs20*	3	*bbs26*	3
bbf19	6	*bbp25*	3	*bbh27*	3
bbo10	5	*bb0197*	3	*bbi02.2*	3
bbq76	5	*bbg08*	3	*bb0159*	3
bbq11	5	*bb0398*	3	*bb0157*	3
bbh28	5	*bbm01*	3	*bb0177*	3
bbp17	4	*bbl21*	3	*bbd11*	2
bb0158	4	*bbr10*	3	*bbp22*	2
bbi29	4	*bbn24*	3	*bbs11*	2
bbm23	4	*bbp23*	3	*bb0012*	2
bbn06	4	*bbq31*	3	*bb0686*	2
bbp19	4	*bbs19*	3	*bb0664*	2
bbm19	4	*bbo25*	3	*bbq26*	2
bbk35	4	*bbp21*	3	*bbd13*	2
bbq29	4	*bba52*	3	*bbr34*	2
bbk23	4	*bb0186*	3	*bb0112*	2
bba22	4	*bb0795*	3	*bbs21*	2
bbq07	4	*bbn21*	3	*bb0149*	2
bba54	4	*bbm25*	3	*bb0444*	2
bb0198	4	*bbd15*	3	*bbq30*	2
bbf16	4	*bbq27*	3	*bbr27*	2
bbf20	4	*bb0769*	3	*bb0068*	2
bbr33	3	*bbo26*	3	*bbk22*	2
bbo07	3	*bbg06*	3	*bbq32*	2
bbe17	3	*bb0446*	3	*bb0799*	2
bbj19	3	*bbg07*	3	*bbo27*	2
bbh26	3	*bbn23*	3	*bbn27*	2
bbs22	3				

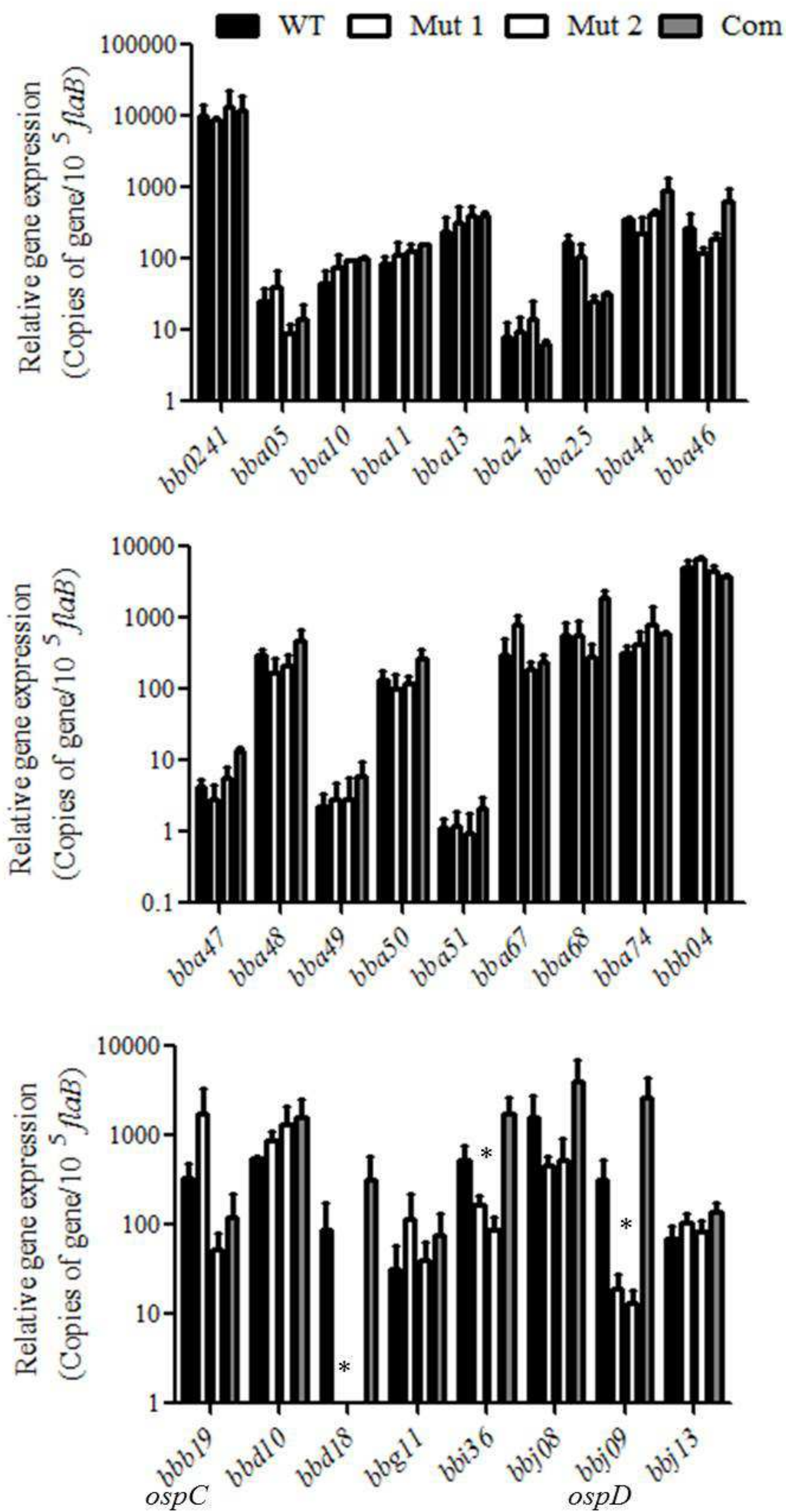
Table 12. *B. burgdorferi* genes downregulated more than 2-fold in Com from microarray experiment 2.

Gene	WT/Com
bbb19	36
bba67	11
bb0323	9
bbq01	5
bbk54	4
bb0562	4
bbj09	4
bb0188	3

Table 13. *B. burgdorferi* genes upregulated more than 2-fold in Com from microarray experiment 2.

Gene	Com/WT	Gene	Com/WT	Gene	Com/WT
bb0002	7	*bb0101*	4	*bbt06*	3
bb0148	6	*bb0754*	4	*bb0307*	3
bb0077	6	*bb0126*	4	*bbq34*	3
bbi13	6	*bb0517*	4	*bb0027*	3
bbg19	6	*bbg07*	4	*bb0124*	3
bb0075	5	*bbe17*	4	*bb0047*	3
bb0071	5	*bbi23*	4	*bb0654*	3
bbb23	5	*bb0592*	4	*bba18*	3
bb0031	4	*bb0145*	3	*bbp34*	3
bb0171	4	*bb0179*	3	*bb0149*	3
bb0100	4	*bb0518*	3	*bbh26*	3
bb0178	4	*bb0569*	3	*bb0751*	3
bbn16	4	*bb0455*	3	*bb0115*	2
bba19	4	*bb0230*	3	*bb0012*	2
bb0587	4	*bba41*	3		

To verify the microarray results, qPCR analyses were performed for the highly regulated genes. Most importantly, *bbd18* transcripts could be detected in wild-type and complement but not mutant *B. burgdorferi* (Fig. 15 & 16). qPCR revealed two groups of genes in *bbd18* mutants: expression of one set could be complemented, while that of the other could not. The first group includes *bbj09*/*ospD*, two *Borrelia* paralogous family 54 genes - *bba68* and *bbi36*, as well as several linear plasmid lp54 genes (Fig. 17). Downregulation of *ospD* (p=0.0457 and 0.0424) and *bbi36* (p=0.0410 and 0.0191) was significant. We speculate that the product of the *bbd18* ORF indirectly or directly regulates the expression of the first group of genes, while disruption of an element on lp17 accounts for the decreased transcription of genes that could not be complemented. Expression of the first group was significantly downregulated in our *B. burgdorferi* isolates without *bbd18* but restored to WT levels in complemented spirochetes. The expression of other regulated genes was similar among the manipulated isolates, including mutants and complement, once the *bbd18* locus was disrupted.



WT
Mut 1
Mut 2
Com
Relative gene expression
(Copies of gene/10 5 flaB)
100000
10000
1000
100
10
1
bb0241
bba05
bba10
bba11
bba13
bba24
bba25
bba44
bba46
Relative gene expression
(Copies of gene/10 5 flaB)
10000
1000
100
10
1
0.1
bba47
bba48
bba49
bba50
bba51
bba67
bba68
bba74
bbb04
Relative gene expression
(Copies of gene/10 5 flaB)
10000
1000
100
10
1
*
*
*
bbb19
ospC
bbd10
bbd18
bbg11
bbi36
bbj08
bbj09
ospD
bbj13

Figure 17. Potentially regulated genes in various *B. burgdorferi* isolates. Total RNA was isolated and reverse transcribed to cDNA from *B. burgdorferi* grown *in vitro*. The expression of various genes was analyzed by qPCR and presented as copies of gene transcripts relative to *flaB* transcripts. Some genes that are downregulated in the mutants, including *bbi36* (p=0.0410 and 0.0191) and *bbj09*/*ospD* (p=0.0457 and 0.0424), are restored to WT levels in the complement.

Deletion of *bbd18* modifies *B. burgdorferi* protein profile

As BBD18-deficient spirochetes have displayed decreased ability to survive in mice and have altered expression of outer surface protein-encoding and other genes, we wanted to evaluate whether the proteome was affected. Immunoblot analyses using serum against *B. burgdorferi* lysate or from wild type-infected mice showed that *bbd18* mutants produced less of several proteins, as could be expected from a decrease in gene transcription (Fig. 18A). Two-dimensional gel electrophoresis results more clearly reflected differential proteomes of WT, Mut 1, and Com (Fig. 18B). Some proteins produced at lower levels in Mut 1 were restored to wild-type amounts in the *bbd18* complement (*white circles*), while others were not (*black circles*). Based on the microarray data as well as estimating pI and mass, immunoblotting with specific antibodies was performed. Downregulation of *bbi39* and *ospD* in both mutants translated to decreased production of both proteins (Fig. 19A). Mutants produced less BBI39 and OspD than WT or Com. As seen previously and also with the microarray, *ospC* regulation is more complex and affected by disrupting the *bbd18* locus. OspC is produced in WT and Mut 1 *B. burgdorferi* cultured at 34°C or 37°C. The protein can be detected in Mut 2 but not in Com (Fig. 19B). The absence of cp32-6 or plasmid 'M' in Mut 2 may play a part in the difference between the mutants, although the plasmid has several paralogs that could

at least partially compensate for any loss of function. Levels of enolase, encoded by nonregulated gene *bb0337* (Fig. 19C), and loading control flaB were similar in all isolates (Fig. 19). Based on these data, we assume that phenotypic changes from manipulating the *bbd18* region are caused by altered gene expression affecting amount of protein produced.

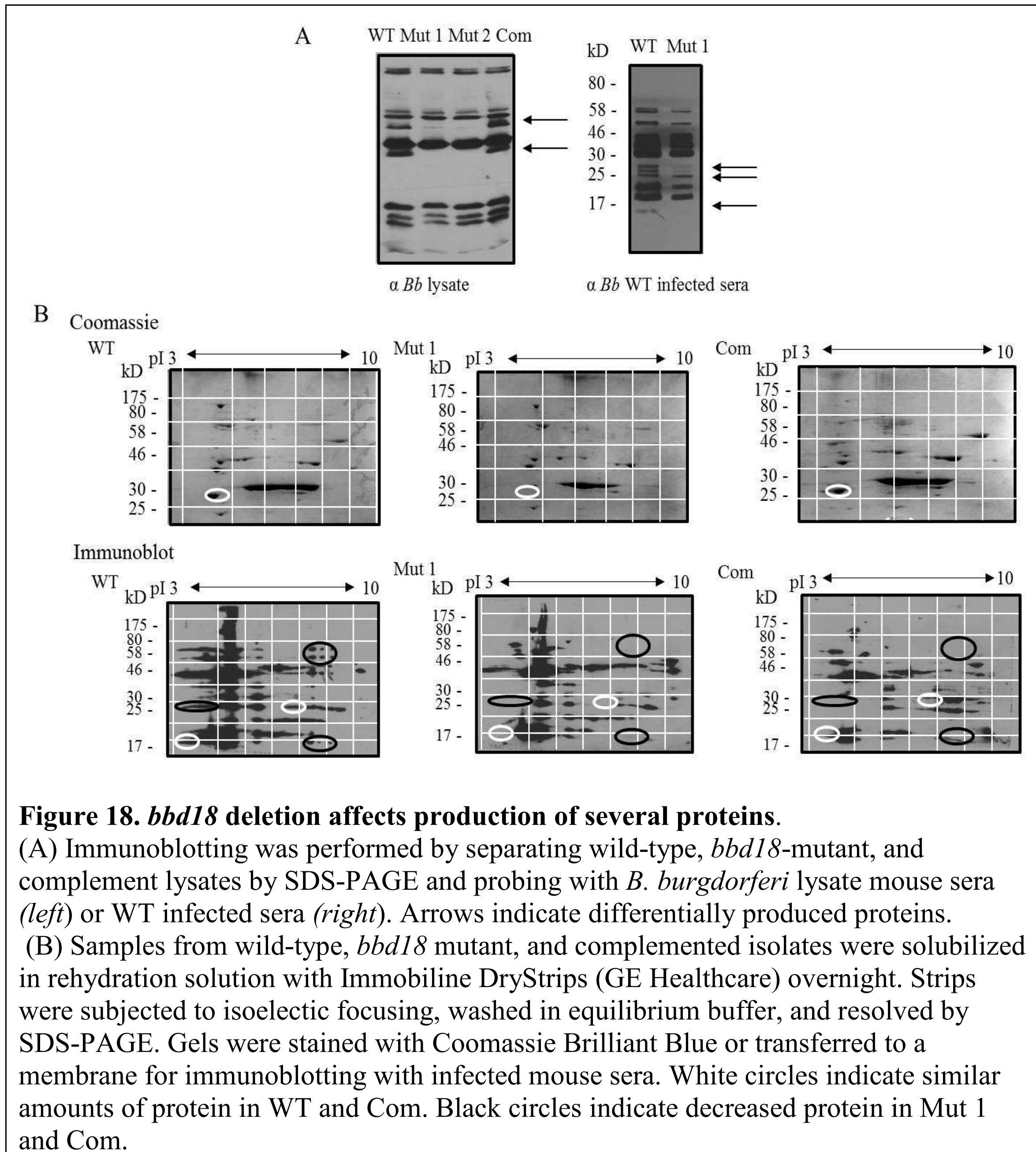


Figure 18. *bbd18* deletion affects production of several proteins.
(A) Immunoblotting was performed by separating wild-type, *bbd18*-mutant, and complement lysates by SDS-PAGE and probing with *B. burgdorferi* lysate mouse sera *(left)* or WT infected sera *(right)*. Arrows indicate differentially produced proteins.
(B) Samples from wild-type, *bbd18* mutant, and complemented isolates were solubilized in rehydration solution with Immobiline DryStrips (GE Healthcare) overnight. Strips were subjected to isoelectic focusing, washed in equilibrium buffer, and resolved by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue or transferred to a membrane for immunoblotting with infected mouse sera. White circles indicate similar amounts of protein in WT and Com. Black circles indicate decreased protein in Mut 1 and Com.

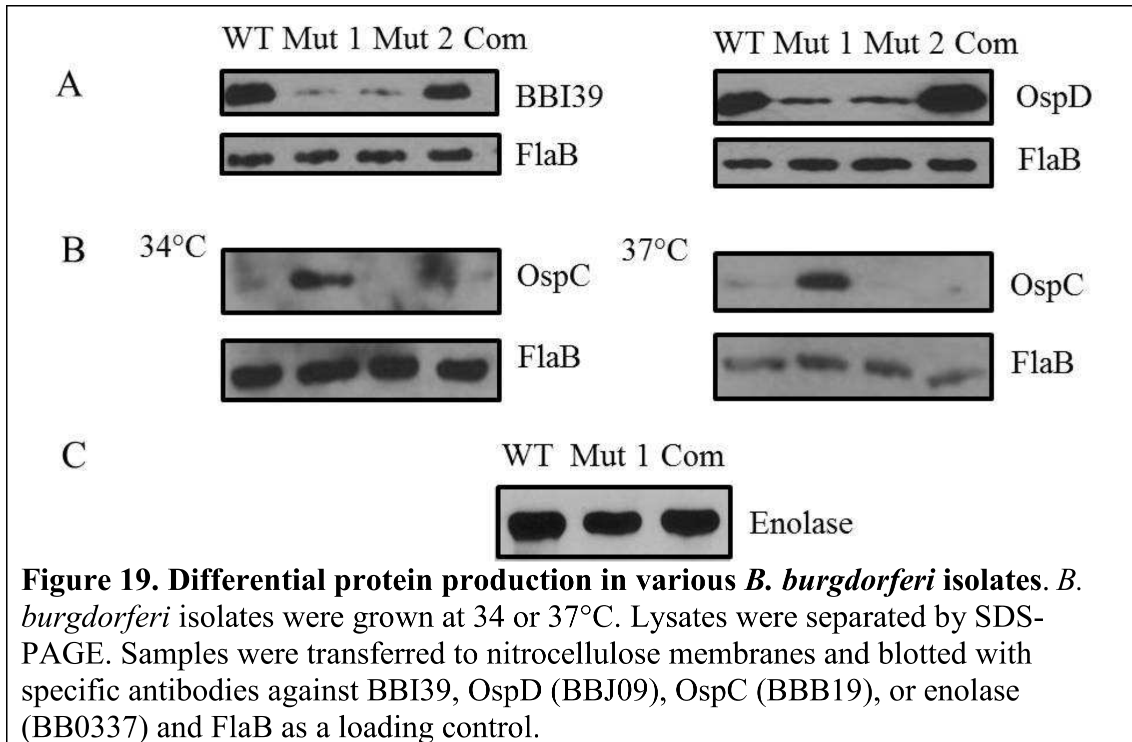


Figure 19. Differential protein production in various *B. burgdorferi* isolates. *B. burgdorferi* isolates were grown at 34 or 37°C. Lysates were separated by SDS-PAGE. Samples were transferred to nitrocellulose membranes and blotted with specific antibodies against BBI39, OspD (BBJ09), OspC (BBB19), or enolase (BB0337) and FlaB as a loading control.

Recombinant BBD18 is stable and exhibits properties of basic protein

Recombinant BBD18 was stable and did not degrade when kept at room temperature for up to an hour (Fig. 20).

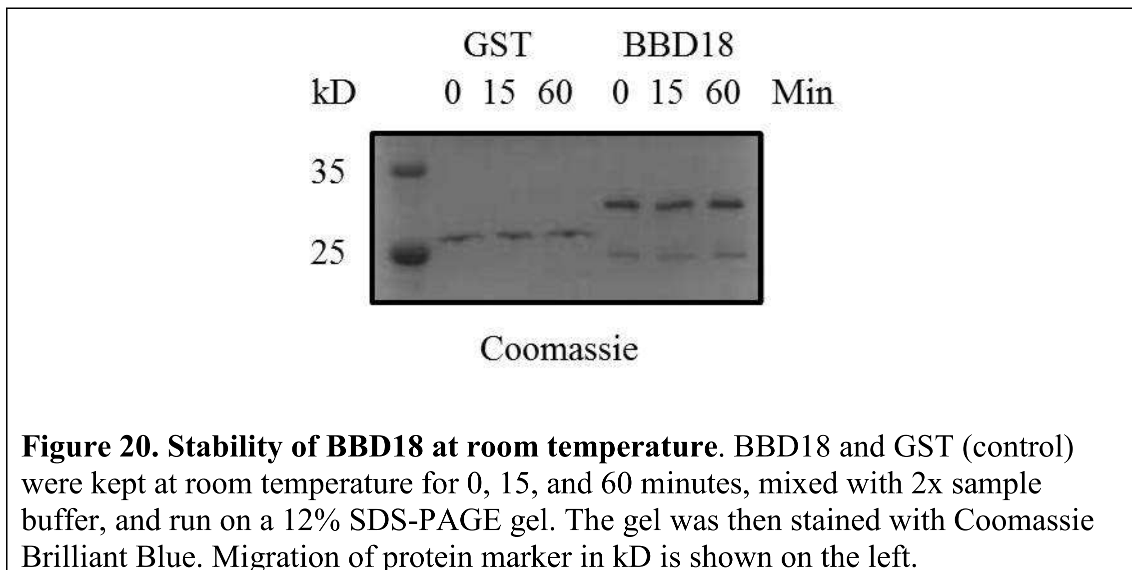


Figure 20. Stability of BBD18 at room temperature. BBD18 and GST (control) were kept at room temperature for 0, 15, and 60 minutes, mixed with 2x sample buffer, and run on a 12% SDS-PAGE gel. The gel was then stained with Coomassie Brilliant Blue. Migration of protein marker in kD is shown on the left.

However, even 5 µg of the protein could not be visualized in a 5% TBE gel that showed increasing amounts of GST from 1 to 10 µg (Fig. 21). As a basic protein without negative charge from SDS, BBD18 may not enter the native gel but rather remain in the well or even migrate in the opposite direction out of the gel.

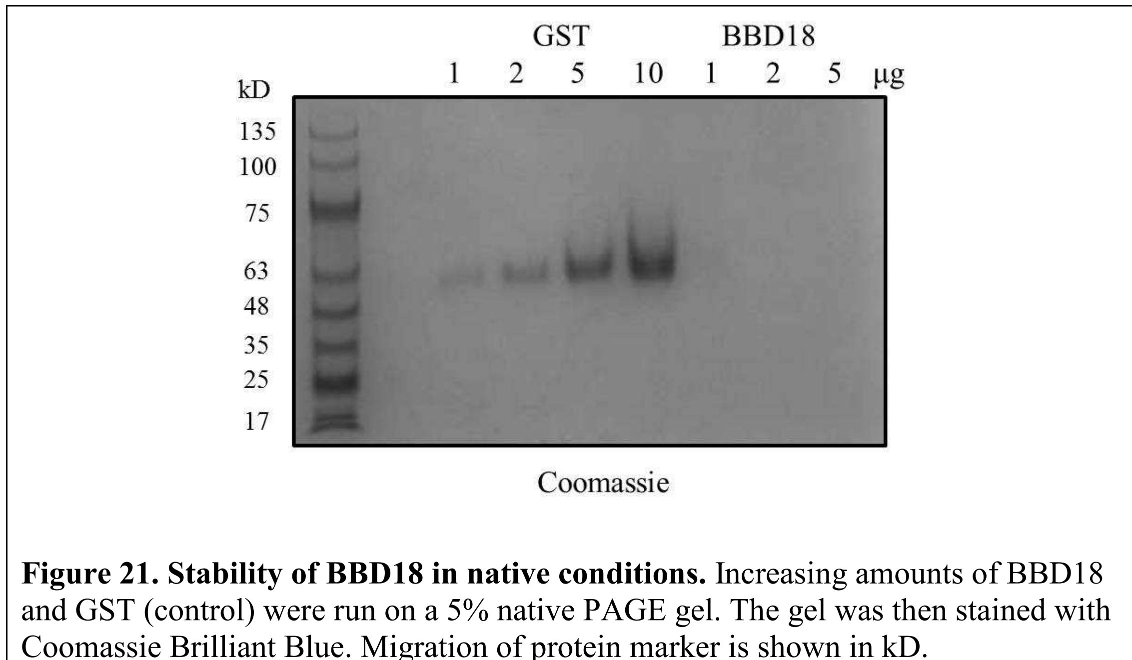


Figure 21. Stability of BBD18 in native conditions. Increasing amounts of BBD18 and GST (control) were run on a 5% native PAGE gel. The gel was then stained with Coomassie Brilliant Blue. Migration of protein marker is shown in kD.

Construction and characterization of *kan* insertion isolates

As gene complementation could not completely restore the WT phenotype, we hypothesized that disrupting the loci surrounding *bbd18* was sufficient to render *B. burgdorferi* noninfectious. We generated constructs inserting only *kan* upsteam (*kan* U) or downstream (*kan* D) of *bbd18* (Fig. 22A & 23A, Table 6). In the downstream insertion, 18 bp were deleted. The plasmid content of *kan* D was analyzed, compared to that of the wild-type parental clone using PCR, and found to be the same (Fig. 24). A series of PCRs were also performed to verify that *kan* was inserted in the desired

location and orientation. With *kan* U, fragments were generated similar to the positive vector control (Fig. 23B). *kan* primers alone (*left*) and in combination with primers upstream or downstream of the insertion (*right*) showed the presence of the cassette in *kan* D but not WT (Fig. 23B). WT and *kan* D had similar overall protein profiles and antibody production against tick-transmitted *B. burgdorferi* (Fig. 23C). Compared to parental isolates, *kan* D did not display an *in vitro* growth defect at 34°C (Fig. 23D).

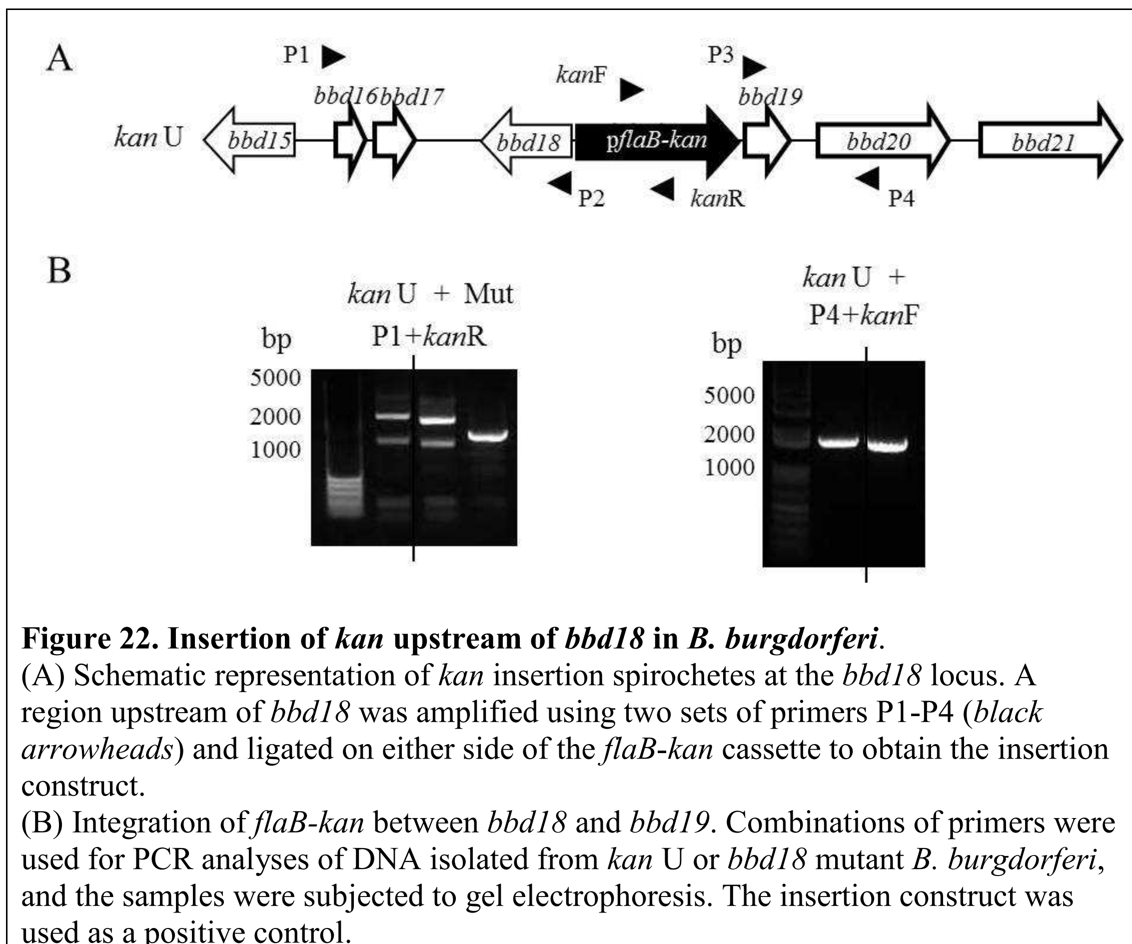


Figure 22. Insertion of *kan* upstream of *bbd18* in *B. burgdorferi*.
(A) Schematic representation of *kan* insertion spirochetes at the *bbd18* locus. A region upstream of *bbd18* was amplified using two sets of primers P1-P4 (*black arrowheads*) and ligated on either side of the *flaB*-*kan* cassette to obtain the insertion construct.
(B) Integration of *flaB*-*kan* between *bbd18* and *bbd19*. Combinations of primers were used for PCR analyses of DNA isolated from *kan* U or *bbd18* mutant *B. burgdorferi*, and the samples were subjected to gel electrophoresis. The insertion construct was used as a positive control.

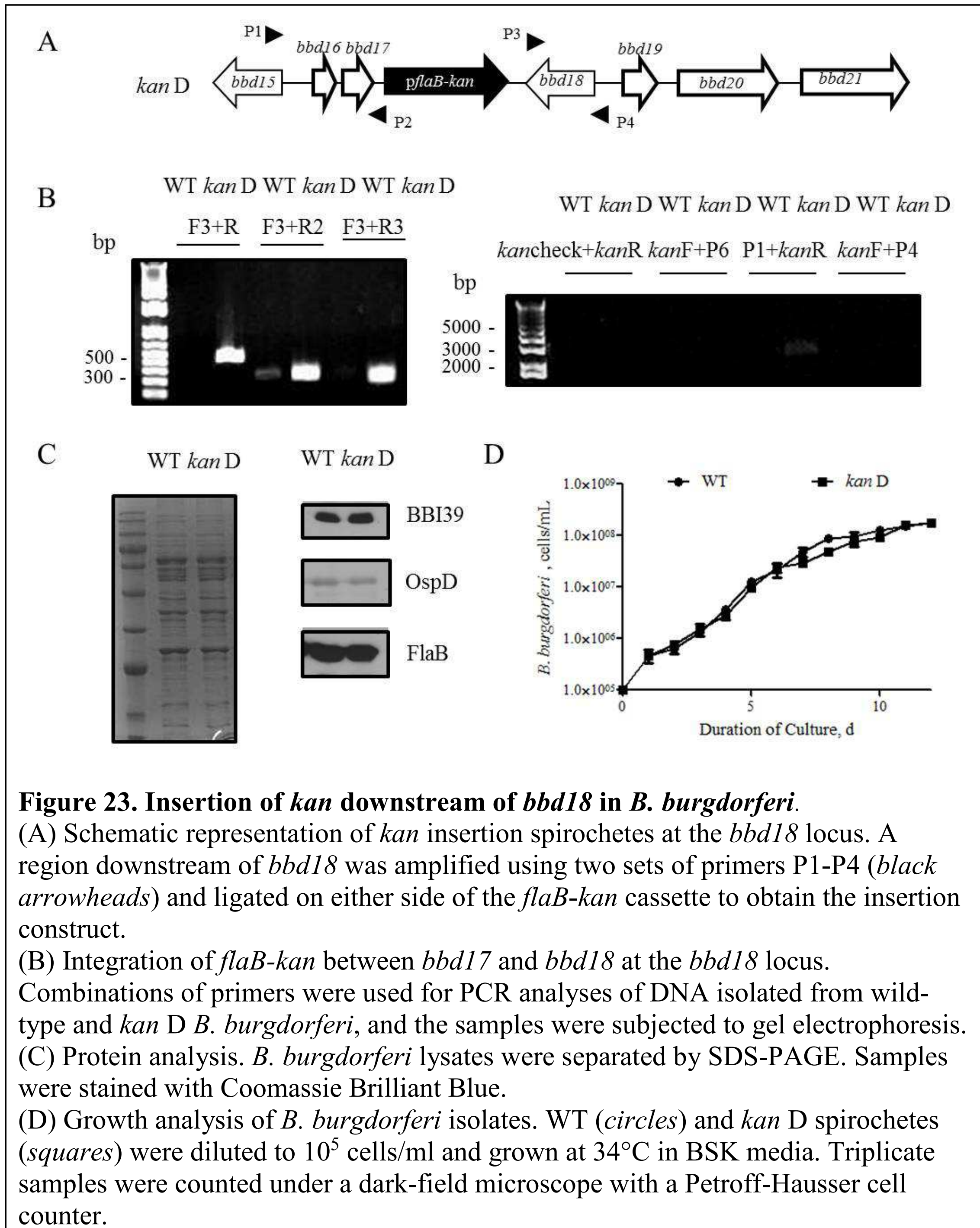


Figure 23. Insertion of *kan* downstream of *bbd18* in *B. burgdorferi*.
(A) Schematic representation of *kan* insertion spirochetes at the *bbd18* locus. A region downstream of *bbd18* was amplified using two sets of primers P1-P4 (*black arrowheads*) and ligated on either side of the *flaB-kan* cassette to obtain the insertion construct.
(B) Integration of *flaB-kan* between *bbd17* and *bbd18* at the *bbd18* locus. Combinations of primers were used for PCR analyses of DNA isolated from wild-type and *kan* D *B. burgdorferi*, and the samples were subjected to gel electrophoresis.
(C) Protein analysis. *B. burgdorferi* lysates were separated by SDS-PAGE. Samples were stained with Coomassie Brilliant Blue.
(D) Growth analysis of *B. burgdorferi* isolates. WT (*circles*) and *kan* D spirochetes (*squares*) were diluted to 10^5 cells/ml and grown at 34°C in BSK media. Triplicate samples were counted under a dark-field microscope with a Petroff-Hausser cell counter.

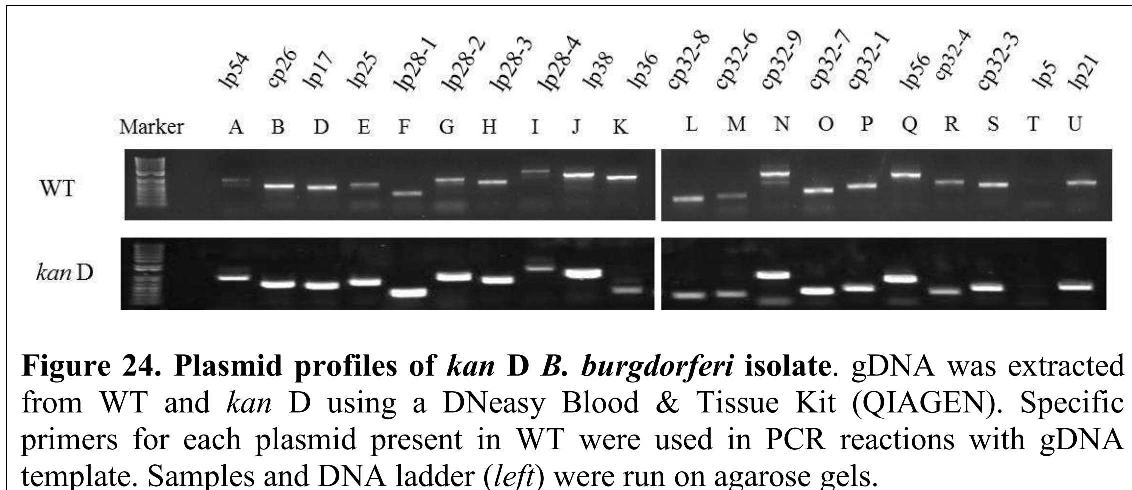


Figure 24. Plasmid profiles of *kan* D *B. burgdorferi* isolate. gDNA was extracted from WT and *kan* D using a DNeasy Blood & Tissue Kit (QIAGEN). Specific primers for each plasmid present in WT were used in PCR reactions with gDNA template. Samples and DNA ladder (*left*) were run on agarose gels.

Disrupting locus downstream of *bbd18* attenuates *B. burgdorferi* persistence in mice

To determine whether insertion of only *kan* influences *B. burgdorferi* infectivity *in vivo*, groups of C3H/HeN mice (3/group) were inoculated intradermally with equal numbers of wild-type or either *kan* insertion *B. burgdorferi* (10^5 spirochetes/mouse). After 14 days, mice infected with wild-type showed seroconversion, which was absent in mice infected with *kan* D (Fig. 25A). Infection was assessed by quantitative RT-PCR analyses of pathogen burden in skin, joint, heart, and bladder samples at 7 and 14 days of infection. *Kan* U *B. burgdorferi* survived similar to wild type (Table 14). However, *kan* D spirochetes were significantly reduced or undetectable (Fig. 25B). Thus, the region downstream of *bbd18* appears to be important for *B. burgdorferi* to establish infection in mice.

Table 14. Infectivity of *kan* upstream insertion isolate of *B. burgdorferi*.

Mouse	WT	*kan* U
1	+	+
2	+	+
3	+	+

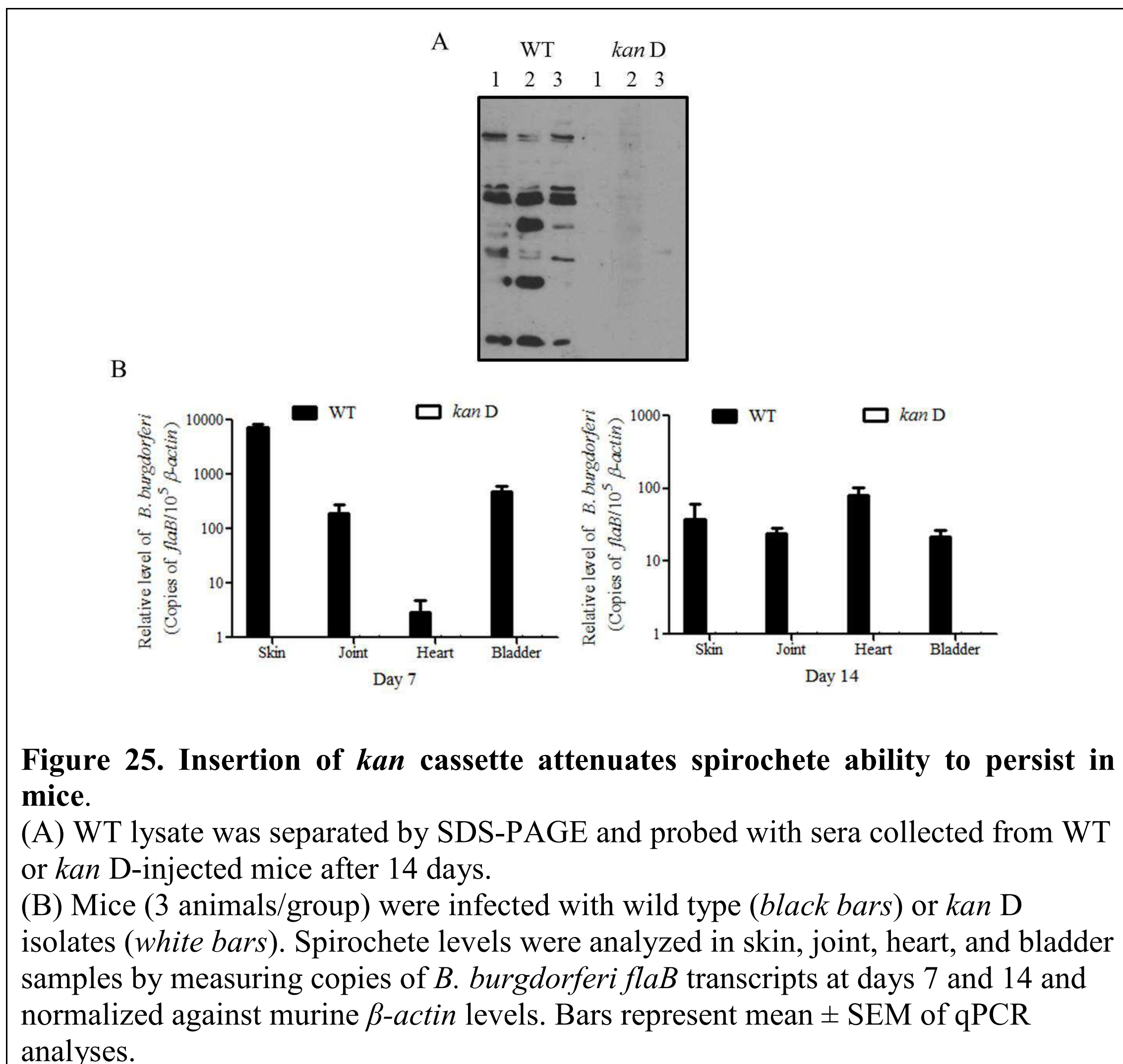


Figure 25. Insertion of *kan* cassette attenuates spirochete ability to persist in mice.
(A) WT lysate was separated by SDS-PAGE and probed with sera collected from WT or *kan* D-injected mice after 14 days.
(B) Mice (3 animals/group) were infected with wild type (*black bars*) or *kan* D isolates (*white bars*). Spirochete levels were analyzed in skin, joint, heart, and bladder samples by measuring copies of *B. burgdorferi flaB* transcripts at days 7 and 14 and normalized against murine *β-actin* levels. Bars represent mean ± SEM of qPCR analyses.

3.4 Discussion

Changes in infectivity would likely be attributed to differential gene expression and/or protein production. To identify genes regulated by only mammal-specific signals and not temperature, microarrays were hybridized with cDNA generated either from *B. burgdorferi* shifted from 23°C to 37°C *in vitro* or those cultivated in DMCs (Brooks, Hefty et al. 2003). A total of 125 genes were expressed at significantly different levels in the mammalian host, with almost equal numbers of genes being regulated by *B. burgdorferi* in s compared to those that underwent the temperature shift (Brooks, Hefty et al. 2003). Strikingly, 11 of the 25 most downregulated genes were encoded by lp54, although they did not have conserved upstream promoter regions (Brooks, Hefty et al. 2003). Four ORFs from different paralogous gene families - *bba62* (*lp6.6*), *bba74* (*oms28*), *bbd18*, and *bbj09* (*ospD*) - appear to share a highly conserved 35-bp region that overlaps the -35 hexamer, a *cis*-acting element known to play a key role in RNA polymerase binding (Brooks, Hefty et al. 2003). This homologous region also encompasses a smaller 17-bp motif that is found four times in the 100 bps upstream of *ospD*, the most highly downregulated gene (Brooks, Hefty et al. 2003). Interestingly, while the 100 bps upstream were found to share an average of 61% identity, the genes averaged only 27% identity (Brooks, Hefty et al. 2003). A prior analysis of the *ospD* upstream region showed that the motif is present seven times in the upstream 200-bp region (Norris, Carter et al. 1992; Marconi, Samuels et al. 1994). Given the magnitude of the downregulation of these four genes, combined with the conserved sequences and motifs found in their promoters, a *trans*-acting repressor, potentially BBD18, may bind these promoters

during DMC cultivation and regulate transcription. Genes in family 54, including *bbi36*, *bbi39*, and *bbj41*, were downregulated in the presence of blood (Tokarz, Anderton et al. 2004). These genes possess a 24-bp inverted repeat sequence downstream of their ends and may encode lipoproteins (Tokarz, Anderton et al. 2004), which are important for infectivity.

To assess whether BBD18 binds these promoters or other DNA, we could attempt various experiments, including an electrophoretic mobility shift assay (EMSA), DNase footprinting or protection assay, and nitrocellulose filter-binding (Hellman and Fried 2009). Bound BBD18 would migrate slower or less distance than free protein or DNA and likely prevent the DNA from being cleaved by enzymes. In order to perform EMSA studies, we would require recombinant BBD18 to be in its We could also a reporter assay to provide a real-time *in vivo* readout (Blevins, Revel et al. 2007). The reporter gene, such as for luciferase, would only produce enzyme when the fused promoter is activated (Blevins, Revel et al. 2007).

The *kan* cassette was inserted downstream of *bbd18* in the opposite direction. Without a strong transcription terminator, the RNA polymerase may be reading through to *bbd18* and generating antisense RNA, which could hybridize with and silence *bbd18* transcripts. Having observed an *in vivo* phenotype with this orientation of *kan*, we could switch the direction to prevent the formation of complementary RNA. We should assess the expression of genes regulated by BBD18 in this and the other *kan* insertion isolates.

Chapter 4: Summary and Perspectives

BBD18 function has been implicated in the regulation of the lipoprotein OspC, which is a virulence factor required for *B. burgdorferi* to establish infection in mammals. Only strains with sequences hybridized by total [lp17] DNA were those that did not produce major proteins the size of OspC (Sadziene, Wilske et al. 1993). However, Sarkar et al. suggest an indirect mechanism for BBD18 repression of *ospC* in *B. burgdorferi* (Sarkar, Hayes et al. 2011). BBD18 could either repress an activator or induce a repressor (Sarkar, Hayes et al. 2011). In their *lacZ* reporter study, some colonies turned blue, meaning there may have been a mutation in the putative *ospC* repressor or another regulator (Sarkar, Hayes et al. 2011). The plasmids lost by strain B312 may encode factors that contribute to *ospC* and/or *bbd18* regulation (Sarkar, Hayes et al. 2011). Synthesis of OspC correlated with the loss of lp17 and that *ospC* expression was highest in clones lacking lp17 and lp54 (Sadziene, Wilske et al. 1993). lp54 had the most number of differentially expressed genes when *B. burgdorferi* were incubated with blood for 48 hours (Tokarz, Anderton et al. 2004). *ospC* and several family 54 genes on lp54 exhibited the highest induction (Tokarz, Anderton et al. 2004). Most of the upregulated genes encode lipoproteins (Tokarz, Anderton et al. 2004), which play important roles in pathogenesis, such as for adhesion and dissemination. We observed downregulation of several lp54 genes in a *bbd18* deletion mutant, possibly indicating interaction of elements from these two linear plasmids. We found the production of other proteins to be affected as well, which could account for the loss of infectivity.

While BBD18 function has been linked to the regulation of OspC, *B. burgdorferi* encodes additional regulators other than sigma factors that are involved in regulation of Osps. For example, BosR is shown to be required for repression of OspA and OspD in the mammal (Wang, Dadhwal et al. 2013). The regulator binds directly to sequences upstream of the *ospAB* operon and the *ospD* gene through recognition of palindromic motifs similar to those recognized by other Fur homologues (Wang, Dadhwal et al. 2013). Putative BosR binding sites have been identified upstream of 156 *B. burgdorferi* genes (Wang, Dadhwal et al. 2013). Some of these genes share the same expression pattern of *ospA* and *ospD*. Most notably, 12 of the 18 genes previously identified in a genome-wide microarray study (Brooks, Hefty et al. 2003) to be most significantly repressed in the mammal are among the putative BosR regulon (Wang, Dadhwal et al. 2013).

B. burgdorferi appears to need an exact amount of BBD18, as both overexpression and deletion of the gene reduced the production and/or stability of several other proteins of a wide range of sizes, including RpoS (Dulebohn, Hayes et al. 2014). BBD18 production led to a decrease in RpoS transcription and an increase in RpoD transcription (Dulebohn, Hayes et al. 2014). Further, these strains were less infectious in mice (Dulebohn, Hayes et al. 2014). Infected ticks could transmit *B. burgdorferi* only after the shuttle plasmid to constitutively express *bbd18* was lost (Hayes, Dulebohn et al. 2014).

One explanation for the loss of murine infectivity in the genetically-manipulated strains is a change in folding or supercoiling from the insertion and deletion of DNA. Changes in DNA supercoiling as a response to the environment,

such as osmotic (Cheung, Badarinarayana et al. 2003) or thermal (Tse-Dinh, Qi et al. 1997) stress, are known to modulate global transcription patterns (Dorman 1996). As temperature increases, negative supercoiling should decrease and repress transcription initiation (Tse-Dinh, Qi et al. 1997; Lopez-Garcia and Forterre 2000; Travers and Muskhelishvili 2005). A decrease in superhelicity in *E. coli* (Peter, Arsuaga et al. 2004) and *Haemophilus influenzae* (Gmuender, Kuratli et al. 2001) results in changes in transcription of a large number of genes. DNA topology may be involved in global transcriptional regulatory networks in *B. burgdorferi* (Beaurepaire and Chaconas 2007) as well. Changes in topology along with copy number can result in a global change in transcription up to 160-fold (Beaurepaire and Chaconas 2007). DNA supercoiling also influences bacterial transcription of specific genes (Beaurepaire and Chaconas 2007). GroEL is a major heat shock protein (HSP) that can inhibit the DNA gyrase encoded by *gyrB* (Alverson and Samuels 2002). Both its mRNA and protein levels were upregulated in *gyrB* mutants and greater than those in experimentally heat-shocked cultures of wild type (Alverson and Samuels 2002). Circular DNA in the *gyrB* mutants was more relaxed than in wild-type *B. burgdorferi*, although *groEL* is on the linear chromosome (Alverson and Samuels 2002). This may have been the first evidence, although indirect, for the effect of DNA topology on gene expression from linear DNA in a bacterium. Inhibitory activity of GroEL against DNA gyrase could be tested *in vitro* in a "cleavable complex" assay by adding a denaturing agent (Barrett, Bernstein et al. 1993). The level of supercoiling of a circular plasmid in *B. burgdorferi* affects expression of *ospA/B* and *ospC* (Alverson, Bundle et al. 2003; Yang, Pal et al. 2004). Genetic complementation may be difficult if the native

topological state of the gene plasmid and vector are not the same (Beaurepaire and Chaconas 2007).

There is evidence that topology is important for plasmid processes. Chaconas et al. showed that linearizing circular plasmids decreased copy number five-fold (Chaconas, Stewart et al. 2001). Circularization of linear plasmids may also be detrimental. Previous data suggest that lp17 replication occurs preferentially on a linear rather than circular DNA molecule (Beaurepaire and Chaconas 2005). Transcription of *Borrelia* plasmid genes may also be optimized for a particular topologic state (Beaurepaire and Chaconas 2007). Frequent reorganization of genetic information on linear plasmids (Terekhova, Iyer et al. 2006) is believed to occur by dimerization or circularization (Chaconas 2005; Kobryn and Chaconas 2005). Spontaneous dimerization has been reported (Marconi, Casjens et al. 1996) but circularization has not. Level of P*flgB kan* mRNA was much higher from a circular versus linear shuttle vector, whereas expression of *bbd14* replication initiator was higher per gene copy on a linear versus circular plasmid (Beaurepaire and Chaconas 2007). No recognizable difference was observed in sequence pattern, which if present, could account for the topology-dependent transcriptional control (Beaurepaire and Chaconas 2007). Topology-dependent transcription of genes may help block topological interconversions (Beaurepaire and Chaconas 2007).

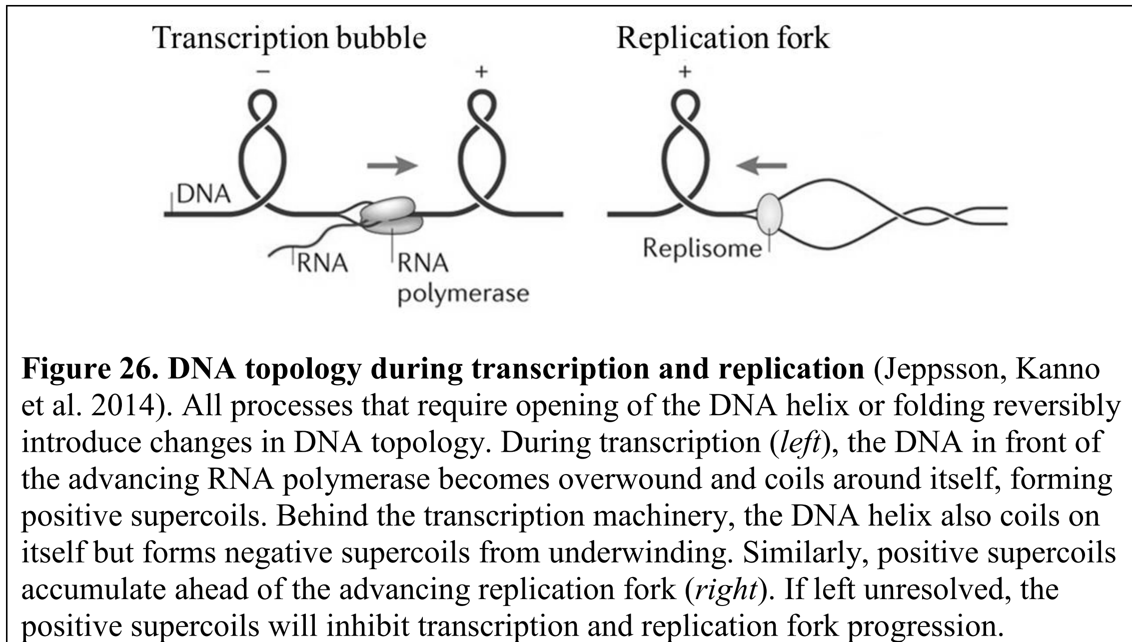


Figure 26. DNA topology during transcription and replication (Jeppsson, Kanno et al. 2014). All processes that require opening of the DNA helix or folding reversibly introduce changes in DNA topology. During transcription (*left*), the DNA in front of the advancing RNA polymerase becomes overwound and coils around itself, forming positive supercoils. Behind the transcription machinery, the DNA helix also coils on itself but forms negative supercoils from underwinding. Similarly, positive supercoils accumulate ahead of the advancing replication fork (*right*). If left unresolved, the positive supercoils will inhibit transcription and replication fork progression.

Another possibility is that the insertion of *kan* may disrupt non-protein-coding region(s), such as small RNAs (sRNAs), and prevent their function. sRNAs can be antisense, affecting translation or degradation of target mRNA, or bind to and sequester proteins (Storz, Opdyke et al. 2004). They have recently been shown to control several regulatory pathways (Ostberg, Bunikis et al. 2004). In many cases, their function depends on RNA-binding protein Hfq (Ostberg, Bunikis et al. 2004). The Hfq protein stimulates duplex formation between the sRNA and its target mRNA. Hfq may also protect the sRNA against RNase E digestion (Folichon, Arluison et al. 2003; Moll, Afonyushkin et al. 2003). Sequence analyses of the putative *B. burgdorferi* Hfq, encoded by *bb0268*, revealed only modest similarity with the *E. coli* Hfq (Lybecker, Abel et al. 2010). However, experimental evidence shows the protein to be a functional homologue (Lybecker, Abel et al. 2010). RpoS and OspC are not upregulated in an *hfq* mutant, which as a result is not infectious by needle inoculation in the murine model (Lybecker, Abel et al. 2010). The *B.*

burgdorferi genome encodes a small number of sRNAs (Ostberg, Bunikis et al. 2004). Two have been found on lp17 - one near the *bbd01* locus, and the other surrounding *bbd08* and *bbd09* (Ostberg, Bunikis et al. 2004). Although neither sRNA is adjacent to the *bbd18* locus, insertion of exogenous DNA may still affect these elements or an unidentified sRNA. New sRNAs can be detected using microarrays, RNA-Seq (RNA sequencing), RNase protection, or primer extension (Aravin and Tuschl 2005; Sharma and Vogel 2009). Once discovered, these sequences can be overexpressed or deleted using similar protocols as for ORFs to characterize them and/or understanding their function(s). Northern blot analysis and qPCR could be used to detect changes in mRNA level from sRNA regulation (Sharma and Vogel 2009). A two-plasmid system has been developed to verify targets *in vivo* (Sharma and Vogel 2009). Many sRNAs associate with and will bind to cellular proteins in gel-shift assays and/or can be co-purified (Sharma and Vogel 2009).

Based on the current data presented in our work, we speculate that DNA topology and/or function of an element immediately downstream of *bbd18* is critical for maintaining spirochete infectivity. A targeted deletion of endogenous DNA or introduction of exogenous DNA downstream of the *bbd18* locus introduces nonreversible and deleterious change(s) in the spirochete genome, which ultimately interfere with the ability of *B. burgdorferi* to infect mammalian hosts. We have provided new information about the regulatory effect of BBD18 as well as an important region of lp17. Further studies to understand the biological significance of *bbd18* and its surrounding DNA, including those mentioned above, will enrich our

understanding of the unusual biology of spirochetes and contribute developing new strategies to combat infection.

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