Delineating Key Genetic Components On Linear Plasmid 36 That Contribute To Its Essential Role In Borrelia Burgdorferi Mammalian Infectivity.

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DELINEATING KEY GENETIC COMPONENTS ON LINEAR PLASMID 36 THAT CONTRIBUTE TO ITS ESSENTIAL ROLE IN *BORRELIA BURGDORFERI* MAMMALIAN INFECTIVITY.

by

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ABSTRACT

The spirochete *Borrelia burgdorferi* is the etiologic agent of Lyme disease. This pathogen has a complex enzootic life cycle that involves passage between the tick vector (*Ixodes scapularis*) and various vertebrate hosts with humans being inadvertent hosts. There is a pressing need to study the genetic aspects of the *B. burgdorferi* infectious cycle and particularly spirochete genes involved in mammalian infectivity so as to develop novel therapeutic and diagnostic strategies to combat Lyme disease. The *B. burgdorferi* genome is fragmented and comprised of a single 900 kb linear chromosome and multiple linear and circular plasmids. It has been observed that plasmids are lost during serial passage and manipulation *in vitro* and the loss of some of the plasmids has been shown to be related to the loss of infectivity and persistence in the host. One such plasmid is linear plasmid 36 (lp36). lp36 is approximately 36kb in size and carries 56 putative open reading frames a majority of which have no predicted function. *B. burgdorferi* lacking lp36 show no deficiency in survival in ticks; however, these mutant spirochetes are highly attenuated for mammalian infectivity. The genetic components of this plasmid that contribute to its function in mammalian infectivity have yet to be clearly defined.

Using an *in vivo* expression technology (IVET) based genetic screen the lp36-encoded gene *bbk46* was identified as a candidate *B. burgdorferi* gene that is expressed during mammalian infection. Herein we present evidence that *bbk46* is required for *B. burgdorferi* persistent infection of immunocompetent mice. Our data

support a molecular model of immune evasion by which *bbk46* functions as an RNA to regulate expression of the antigenic variation protein VlsE. These data represent the first demonstration of a regulatory mechanism critical for controlling *vlsE* gene expression. Moreover these findings further define the critical role of linear plasmid 36 in *Borrelia burgdorferi* pathogenesis.

I dedicate this thesis to my family, my parents whose patient and unwavering support gave me strength through all of life's challenges and my husband who helped me find happiness and laughter on the bleakest of days.

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CHAPTER ONE: INTRODUCTION

The following chapter provides an introduction to Lyme disease, its causative agent *Borrelia burgdorferi* and the unique genetic features of this pathogen. It also introduces the fundamental basis of this study, which was aimed at elucidating the genetic components of linear plasmid 36 that make a contribution toward the essential role of this plasmid in *B. burgdorferi* mammalian infectivity.

1.1: *Borrelia burgdorferi* Is The Causative Agent Of Lyme Disease.

In 1975, a group of children in Lyme, Connecticut diagnosed with an unusual form of juvenile arthritis sparked off an investigation that led to the discovery of a disease that is known today as Lyme borreliosis or Lyme disease [1]. It is now considered to be the most prevalent arthropod-borne bacterial disease in the world. According to the Centers for Disease Control and Prevention (CDC) an average of about 20,000 to 30,000 cases are reported every year in the United States with the major prevalence in the northeastern states [2]. Though Lyme disease has never been reported as fatal it can be severely debilitating if left undiagnosed and untreated. The early symptoms include the tell-tale "bull’s eye" skin rash termed erythema migrans accompanied by aches, fever and nausea. Antibiotic treatment at this, early localized, stage is effective in eliminating the infection. However, untreated infections lead to a complex disease affecting several organs including the skin, joints, heart and nervous system [1].

The etiologic agent of this disease was identified as the spirochete *Borrelia burgdorferi* by Dr. William Burgdorfer in 1982 [1]. Spirochetes are a phylum of Gram-negative bacteria that are characterized by their spiral shape and axial, periplasmic flagella [3]. The genus *Borrelia* includes several medically relevant species causing diverse diseases including Lyme disease, syphilis, leptospirosis, relapsing fever and periodontitis. The species associated with Lyme disease are *Borrelia burgdorferi*, *B. garinii* and *B. afzelii*, together called *Borrelia burgdorferi* sensu lato [1, 4, 5].

Borrelia burgdorferi has a complex enzootic life cycle that involves passage between the tick vector (*Ixodes scapularis*) and various vertebrate hosts (Fig. 1) [6]. The tick larvae can acquire spirochetes when they feed on infected animals, usually small mammals such as mice and squirrels. The spirochetes survive within the tick midgut as the ticks overwinter and then are transferred to other, larger, mammalian hosts when the ticks take their next blood meal. Humans become infected with *B. burgdorferi* via the bite of an infected *Ixodes* tick and are incidental hosts of these spirochetes. Although the spirochetes can be transferred to humans through tick bites, naive ticks cannot acquire spirochetes from an infected human. As a result, humans are termed as “dead end hosts” for *B. burgdorferi* and are not part of the natural enzootic life cycle of the spirochete. [1].

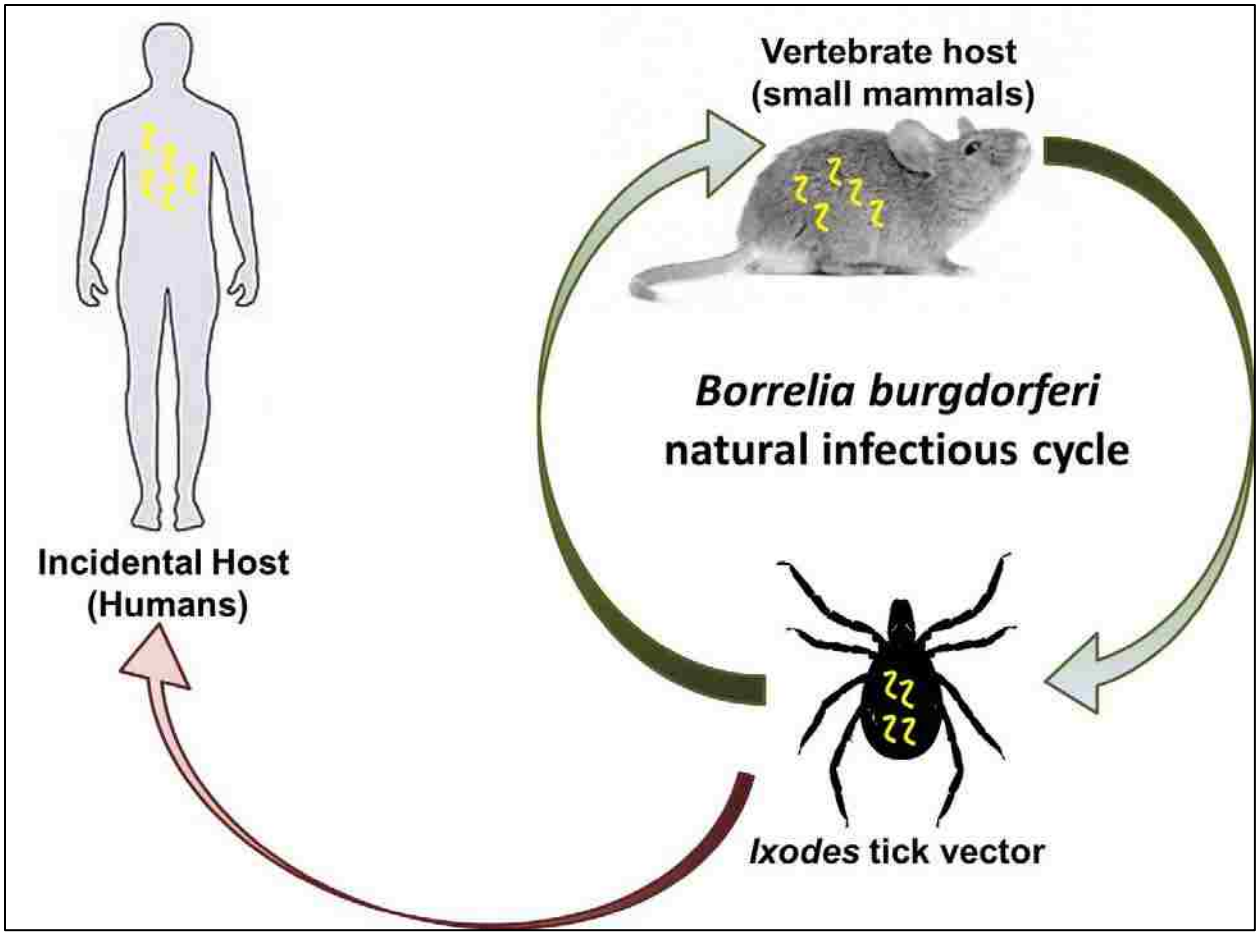

Figure 1: Natural enzootic life cycle of *Borrelia burgdorferi*.

Spirochetes are transferred between the *Ixodes* spp. tick vector and mammalian hosts, which include small rodents and deer. Humans are not part of this natural life cycle and only acquire these spirochetes accidentally when they get bitten by infected ticks.

1.2: The Unique Segmented *B. burgdorferi* Genome.

Borrelia species are unique compared to other bacteria due to the presence of a fragmented genome [7, 8]. The *B. burgdorferi* genome is comprised of a single small linear chromosome that is about 900 kb in size along with 12 linear and 9 circular

plasmids ranging in size from 5 kb to 56 kb (Fig. 2). The genome also has a very low G+C content of approximately 28% [1]. Comparative genomics studies have revealed that the chromosomes of *B. burgdorferi* isolates have relatively constant gene content and organization and carry mostly housekeeping genes. Conversely, the plasmids have been found to be more variable between isolates and carry genes for several surface proteins that interact with the tick or mammalian host and show differential expression in the two environments [1]. The genetic composition of the plasmids is quite intriguing. Even though approximately 40% of the *B. burgdorferi* genome is composed of plasmids, less than 10% of the plasmid-encoded genes have any known function [7-9]. Also, a majority of the annotated pseudogenes in the genome are located on the plasmids and not on the chromosome [9]. Another unusual finding is the presence of multiple prophage plasmids within a single cell, since traditionally, prophages have been known to prevent the integration of additional incoming bacteriophages into the host genome allowing only one to be integrated and expressed at a time [1, 10, 11]. It has been observed that plasmids are lost during serial passage and manipulation *in vitro* and the loss of some of the plasmids, like lp28-1 and lp25, has been shown to be related to the loss of infectivity and persistence in the host [12-15]. Another plasmid that is essential for *B. burgdorferi* mammalian infectivity is linear plasmid 36 (lp36) [16].

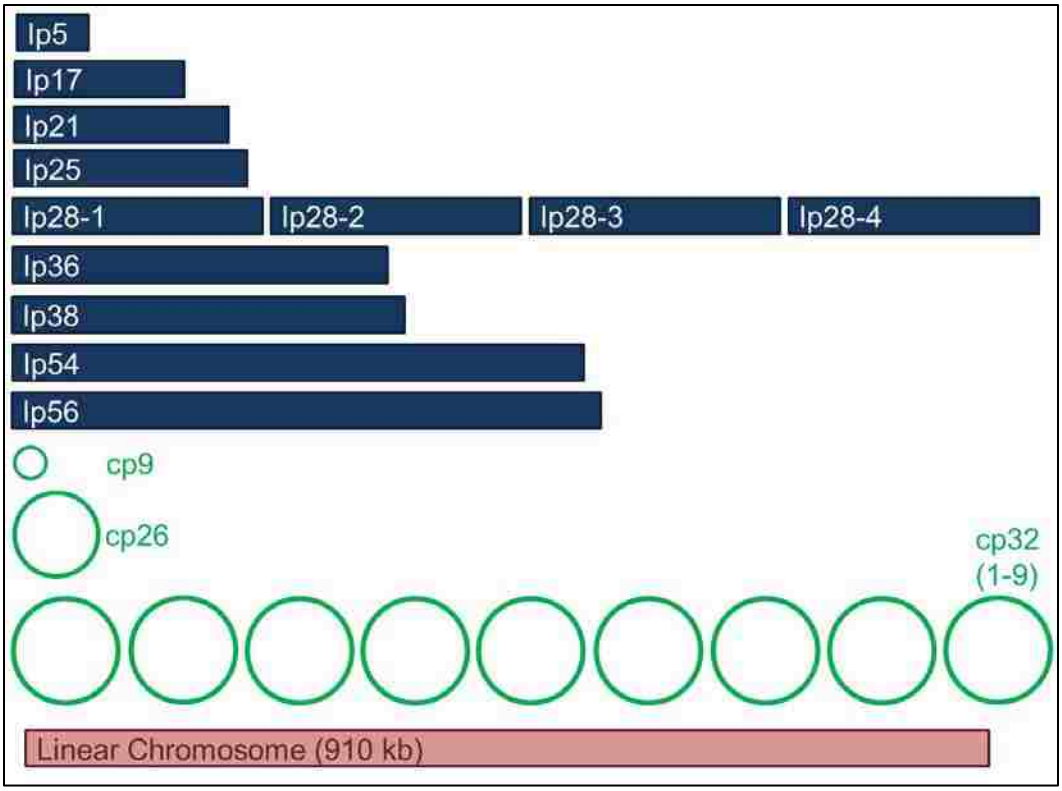

Figure 2: Schematic representation of the segmented genome of *B. burgdorferi.*

The genome of *Borrelia burgdorferi* contains one chromosome, 12 linear and 9 to11 circular plasmids. The chromosome is about 900 kilobases (kb) in size while the plasmids range in size from 5 kb to 56 kb**.**

1.3: Linear Plasmid 36

Lp36 is approximately 36 kb in size and carries 56 putative open reading frames majority of which have no predicted function [7, 8, 17]. It has been observed that this plasmid is rarely lost among natural isolates [18]. Sequence variation has been observed between different strains of *B. burgdorferi* in the region extending from *bbk35* to *bbk50* on lp36 and the presence of this region has been associated with clones

demonstrating more severe disseminated infection [15, 19, 20]. Mutant *B. burgdorferi* lacking lp36 show no deficiency in survival in ticks; however, these spirochetes do not readily survive in the mammalian host [16]. The ID_{50} of a $lp36^{-}$ clone was found to be about 10^8 spirochetes as compared to 10^3 for wild-type spirochetes, which indicates that this plasmid is critical for *B. burgdorferi* mammalian infectivity [21].

Even though it is known that lp36 is required for the spirochetes to survive within the host, there is very little known about the lp36-encoded genes that contribute to this essential function. Apart from a few lipoproteins, *bbk07* and *bbk50* that have putative immunogenic functions [22-25] few other genes had been found to have any known functions. During the course of our research, Lin et al. (2012) published their work analyzing a comprehensive signature tagged mutagenesis (STM) mutant library in *B. burgdorferi* with the aim of identifying gene candidates important for infectivity [26]. This study identified that mutants with disruptions in the genes located in the region from *bbk02.1* to *bbk04* and genes *bbk05, bbk07, bbk13, bbk17, bbk45, bbk46* to *bbk50* on lp36 showed reduced mammalian infectivity. The two lp36-encoded genes that have been studied most extensively for their possible roles in infectivity are *bbk32* and *bbk17*. The *bbk32* gene encodes a fibronectin binding protein that is believed to be involved in motility and dissemination of the spirochetes from the site of the tick bite through the extracellular spaces to different tissues and organs [27-32]. The *bbk17* gene encodes an adenine deaminase, a 548 amino acid enzyme involved in conversion of adenine to hypoxanthine, a critical step in the *B. burgdorferi* purine salvage pathway for the synthesis of DNA and RNA [8, 16]. Both of these genes have been studied separately

and each has been found to have a minor contribution to mammalian infection [21, 26, 31-33]. The increase in the ID_{50} for spirochetes lacking either gene; however, was much lower than that seen due to the loss of the entire linear plasmid 36 [16, 32]. Thus the essential role of lp36 in mammalian infectivity is partially, but not entirely, attributed to each of these genes. Moreover, as suggested by the work of Lin *et al.* (2012), there are likely additional genes on lp36 that contribute to the role of this plasmid in *B. burgdorferi* pathogenesis.

1.4: Gene Regulation And Differential Expression.

Borrelia burgdorferi survive within highly dissimilar environments during their life cycle. They exist in the insect vector, *Ixodes scapularis*, and then through the bite of the tick they enter into the mammalian host. The spirochetes encounter changes in the environmental temperature, pH and availability of nutrients and are required to evade the host immune response. They have developed highly complex regulatory mechanisms by which they manipulate the expression of various metabolic and antigenic factors in order to survive [34-51].

The regulation of gene expression in *Borrelia burgdorferi* is a highly complex process involving various genes (Table 1). The major regulator of expression of virulence genes in *B. burgdorferi* is the alternative sigma factor RpoS which has been shown to be essential for mammalian infectivity [52]. RpoD and RpoS are believed to be involved in regulation of genes during tick colonization [1].

Table 1:Genes invovled in gene regulation in *Borrelia burgdorferi.*

Gene	Description	Reference
rpoS	Sigma S	[51]
rpoN	Sigma 54	[51]
rrp2	Response regulator	[51]
hk	Histidine kinase	[51]
ntrC	Nitrogen regulatory protein C	[51]
pta	Phosphate acetyltransferase	[51]
bosR	Borrelia oxidative stress regulator	[51]
csrA	Carbon storage regulator A	[51]
dsrA	Downstream region A	[51]
hfq	Host factor required for Qβ bacteriophage replication	[51]
rpoD	Sigma-70	[53, 54]
ebfC (ybaB)	DNA-Binding protein	[51, 55]
bpaB (parB)	DNA-binding protein	[51, 55, 56]

The blood meal taken by the tick is believed to trigger a cascade of events that involves the repression of phosphate acetyltransferase (Pta) by the carbon storage regulator A (CsrA) resulting in the generation of acetyl phosphate which is believed to activate the response regulator Rrp2 subsequently resulting in the activation of sigma factor RpoS via activated RpoN [51, 57-59]. The RpoN-RpoS pathway remains activated during the mammalian infection and together these two factors form a regulatory cascade that controls the expression of several genes during the enzootic life cycle. BosR is another gene implicated as a regulator of RpoS and believed to be essential for mammalian infectivity but not for survival in the tick [51, 60, 61]. BosR has also been shown to regulate the expression of some genes in an independent manner exclusive of the RpoN-RpoS pathway [62]. $DsrA_{Bb}$ is a small RNA (sRNA) involved in the temperature mediated regulation of RpoS expression [51]. Ebfc and BpaB are believed to bind to upstream regions of *erp* (*ospE, ospF* and *elp*) genes and regulate

their expression [56, 63-65]. Two of the best studied antigenic factors controlled by the RpoN-RpoS pathway are the outer surface proteins OspC and OspA encoded on the circular plasmid 26 and linear plasmid 54, respectively [66]. OspA is required for survival in the tick and shows increased expression while the spirochetes are in the tick; whereas, OspC plays a critical role in spirochete migration from the tick mid-gut and the initial establishment of a mammalian infection [47, 48, 67, 68] and its expression increases as spirochetes migrate out of the tick into the mammal. Other genes known to be of importance during tick colonization of *B. burgdorferi* are *bbe16, bb0365* and *bb0690* [69-71].

There are several other genes that also show differential expression during the mammalian phase of the spirochete lifecycle whose regulation can be rather complex and whose exact function is not always understood (Table 2) [51]. CRASPs (complement regulator-acquiring surface proteins) are a group of proteins that bind to host extracellular proteins and plasminogen and are believed to play a role dissemination [72]. *bbk32* is a fibronectin binding protein which is believed to be involved in dissemination within the mammalian host [27-32]. RevA is another fibronectin binding protein that was found to have antigenic properties [73]. The purine salvage pathway genes *bbk17, guaA and guaB* have been shown to be critical for survival in the mammalian host [16, 74]. The purine transporters BBB22 and BBB23 play a vital role in mammalian infectivity as well [75].

Table 2: Genes known to be expressed in the mammalian host.

Gene	Predicted/ deduced gene product	Reference
BB0036	DNA topoisomerase 4 (pare)	[76]
BB0056	Phosphoglycerate kinase	[22]
BB0096	V-type ATPase subunit E, putative	[76]
BB0108	Peptidylprolyl isomerase	[22]
BB0134	Hypothetical Protein	[76]
BB0147	Flagellar filament (FlaB)	[22, 77-79]
BB0181	Flagellar hook-associated protein (FlgK)	[22]
BB0215	Phosphate ABC trasporter (PstS)	[22]
BB0238	Hypothetical Protein	[22]
BB0260	Hypothetical Protein	[22]
BB0279	Flagellar protein (FliL)	[22]
BB0283	Flagellar hook protein (FlgE)	[22]
BB0286	Flagellar protein (FlbB)	[22]
BB0323	Hypothetical Protein	[22, 79]
BB0328	Oligopeptide ABC transporter (OppA-1)	[22, 79]
BB0329	Oligopeptide ABC transporter (OppA-2)	[22, 79]
BB)330	Oligopeptide ABC transporter (OppA-3)	[79]
BB0337	Enolase	[22]
BB0348	Pyruvate kinase	[22]
BB0359	Carboxyl-terminal protease	[22]
BB0365	Lipoprotein LA7	[22, 79]
BB0383	Basic membrane protein A (BmpA)	[79]
BB0385	Basic membrane protein D (BmpD)	[22]
BB0408	Phosphotransferase system, fructose-specific IIABC	[22]
BB0476	Translation elongation factor TU (tuf)	[22]
BB0518	Molecular chaperone (DnaK)	[22]
BB0543	Hypothetical Protein	[22]
BB0560	Hsp90	[79]
BB0565	Purine binding chemotaxis protein (CheW-2)	[76]
BB0567	Chemotaxis histidine kinase (CheA-1)	[76]
BB0603	P66 outer membrane protein	[22, 79-81]
BB0649	Chaperonin (GroEL)	[22]
BB0652	Protein export protein (SecD)	[22]
BB0668	Flagellar filament outer layer protein (FlaA)	[22]
BB0669	Chemotaxis histidine kinase (CheA-2)	[76]
BB0681	Methyl-accepting chemotaxis protein (Mcp-5)	[22, 76]
BB0741	Chaperonin (GroES)	[76]
BB0751	Hypothetical Protein	[22]

Gene	Predicted/ deduced gene product	Reference
BB0772	Flagellar P-ring protein (FlgI)	[22]
BB0774	Flagellar basal body cord protein (FlgG)	[22]
BB0805	Polyribonucleotidyltransferase (PnpA)	[22]
BB0811	Hypothetical Protein (COG1413)	[22]
BB0844	Hypothetical Protein	[22, 76]
BBA03	Hypothetical Protein	[22, 79]
BBA04	“S2 antigen”	[22]
BBA07	Hypothetical Protein	[22]
BBA15	Outer surface protein A	[22, 82]
BBA16	Outer surface protein B	[22, 82]
BBA19	Hypothetical Protein	[22]
BBA25	Decorin binding protein B (DbpB)	[22, 76, 83]
BBA34	Oligopeptide ABC transporter (OppA-5)	[22]
BBA36	Lipoprotein	[22, 76]
BBA40	Hypothetical protein	[22]
BBA48	Hypothetical protein	[22]
BBA57	Hypothetical protein	[22]
BBA64	Hypothetical protein (P35)	[22, 79]
BBA66	Hypothetical protein	[22, 79]
BBA72	Hypothetical protein	[76]
BBA73	“P35”	[76]
BBB09	Hypothetical protein	[22]
BBB14	Hypothetical protein	[22]
BBB16	Oligopeptide ABC transporter (OppA-4)	[22]
BBB17	GuaA	[74]
BBB18	GuaB	[74]
BBB19	OspC	[22, 47, 48, 67, 76, 84]
BBB22	Purine transport protein	[75]
BBB23	Purine transport protein	[75]
BBC03	Hypothetical protein	[22]
BBC06	EppA (BapA)	[22]
BBC10	RevA	[22]
BBE09	Hypothetical protein	[22]
BBF03	BdrS (BdrF1)	[22]
BBF33	VlsE	[22, 85, 86]
BBG18	Hypothetical protein	[22]
BBG33	BdrT (BdrF2)	[22]
BBH06	Hypothetical protein	[22]
BBH13	BdrU (BdrF3)	[22]
BBI36/38	Hypothetical Protein	[79]
BBI42	Hypothetical protein	[22]

Gene	Predicted/ deduced gene product	Reference
BBJ23	Hypothetical protein	[76]
BBJ24	Hypothetical protein	[22]
BBJ31	Hypothetical protein	[76]
BBJ51	VlsE1 (authentic frame shift)	[76]
BBK07	Hypothetical protein	[22]
BBK12	Hypothetical protein	[22]
BBK13	Hypothetical protein (COG2859)	[22]
BBK17	Adenine deaminase	[16]
BBK19	Hypothetical protein	[22]
BBK23	Hypothetical protein	[22]
BBK32	Fibronectin-binding protein	[22, 29, 87]
BBK50	Putative P37 antigenfamily lipoprotein	[88]
BBK52	“P23”, putative lipoprotein	[22]
BBK53	Hypothetical protein	[22]
BBL03	Hypothetical protein	[22]
BBL15	Hypothetical protein	[76]
BBL27	BdrO (BdrE1)	[22]
BBL39	ErpN (CRASP-5)	[22, 79]
BBL40	ErpO	[22, 76, 79]
BBM27	RevA	[22, 79]
BBM34	BdrK (BdrD2)	[22]
BBM36	Hypothetical protein	[22]
BBN11	Hypothetical protein	[22]
BBN27	BdrR (BdrE2)	[22]
BBN28	MlpL	[22]
BBN34	BdrQ (BdrD10)	[22]
BBN38	ErpP (CRASP-3)	[22, 79]
BBN39	ErpQ	[22]
BBO34	BdrM (BdrD3)	[22]
BBO39	ErpL	[22]
BBO40	ErpM	[22]
BBP34	BdrA (BdrD4)	[22]
BBP39	ErpB	[22]
BBQ03	Hypothetical protein	[22]
BBQ04	Hypothetical protein	[22]
BBQ13	Hypothetical protein	[22]
BBQ19	Hypothetical protein	[22]
BBQ34	BdrW (BdrE6)	[22]
BBQ35	MlpJ	[22]
BBQ40	Partition protein	[22]
BBQ42	BdrV (BdrD5)	[22]

Gene	Predicted/ deduced gene product	Reference
BBR12	Hypothetical protein	[22]
BBR35	BdrG	[22]
BBR42	ErpY	[22]
BBS30	MlpC	[22]
BBS41	ErpG	[22]

1.5: *B. burgdorferi* Immune Evasion Strategies.

Evasion of the host immune response is one of the major mechanisms of survival within the mammalian host. The Erp lipoproteins are expressed during murine infection and are believed to bind to the host complement inhibitory factor H thereby providing protection from the host immune response [44]. Lmp1 is a surface exposed antigen believed to provide protection against host immunity [89]. The outer surface antigenic protein VlsE undergoes random recombination between its central locus and the adjacent silent cassettes, which gives rise to protein products of varying sequences being presented at the cellular surface. This mechanism of generating antigenic variation is believed to be essential for long term survival of spirochetes within the mammalian host and occurs at different time points during the mammalian infection creating differences in the antigenic profile of the spirochete surface and aiding in host immune evasion [13, 14, 18, 85, 86, 90-93].

1.5: Methods to Identify Genes Expressed *In Vivo.*

The unique nature of the *B. burgdorferi* genome with its segmented structure and high A/T content, its lack of stability and loss of plasmids during *in vitro* passage have hampered the experimental study of this organism. The biggest challenge for the

genetic manipulation of these spirochetes has been its extremely low transformability that make mutagenesis and subsequent complementation difficult [94]. The first genetically defined mutants were generated a number of years after the initial discovery of *B. burgdorferi* as the causative agent of Lyme disease [95-97]. Since those early days, molecular genetic tools have been developed and utilized extensively to better understand the mechanisms underlying the survival and pathogenesis of this organism. *B. burgdorferi* have been grown *in vitro* under varying conditions of temperature and pH, that are believed to duplicate those found in the tick or the mammal and they have been subjected to DNA microarrays and proteomic analyses in order to determine the changes in gene expression that are observed under these different environmental conditions [98-100]. Another method used to study *B. burgdorferi* gene expression in response to the mammalian host environment is the growth of spirochetes in dialysis membrane chambers (DMCs) within the peritoneal cavity of rats [76, 101-103]. *In vivo* expression technology (IVET) has been used to identify novel genes that are expressed within a microbe while it is present in a host or a particular experimental environment [104, 105]. In this system a virulence and/or growth essential gene within a microbe is deleted, thus creating a clone that is incapable of surviving within the environment of interest. A genomic library of DNA fragments is then cloned upstream of the essential gene. The fragments of the genome that carry promoters that are active *in vivo* will drive the expression of the essential gene and will be selected by the host environment because only those clones with transcriptionally active promoters will be able to survive in the host. This method has been used to identify novel virulence genes in several

pathogenic organisms [106-110] but has not yet been used to identify virulence factors in *Borrelia burgdorferi*.

1.6: Hypothesis

We hypothesize that, though there are some known contributors to the effects of lp36 on *B. burgdorferi* pathogenesis in mammalian hosts, there are possibly additional, as of yet unknown, genes on lp36 that also contribute towards the essential role of this plasmid in mammalian infectivity. Using an *in vivo* genetic screen we will generate a list of candidate genes expressed *in vivo* that can be further investigated for virulence functions.

In vivo expression technology identifies a novel virulence factor critical for *Borrelia burgdorferi* persistence in mice.

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CHAPTER TWO: *IN VIVO* EXPRESSION TECHNOLOGY IDENTIFIES A NOVEL VIRULENCE FACTOR CRITICAL FOR *BORRELIA BURGDORFERI* PERSISTENCE IN MICE.

2.1: Introduction

Lyme disease is a multi-stage inflammatory disease caused by the pathogenic spirochete *Borrelia burgdorferi*, which is transmitted by the bite of an infected tick [6]. *B. burgdorferi* has an enzootic life cycle that requires persistence in two disparate environments, the arthropod vector and the mammalian host. *B. burgdorferi* is well adapted to modulate its expression profile in response to the different conditions encountered throughout its infectious cycle [51]. Although the specific environmental signals that induce changes in spirochete gene expression are not fully defined, it has been reported that changes in temperature, pH, the presence or absence of mammalian blood, as well as changes in bacterial growth rate, can affect patterns of gene expression [51, 76, 98-100, 102, 111]. DNA microarray analysis and proteomics have been used to examine changes in the global expression profile of *B. burgdorferi* grown under *in vitro* conditions that partially mimic the tick and mouse environments [98-100]. A rat dialysis membrane chamber (DMC) implant model, together with microarray technology, has been used to help identify *B. burgdorferi* genes expressed in response to mammalian host-specific signals [76, 101-103]. Although the data reported in these studies provide insight into the molecular mechanisms of gene regulation, they may not fully reflect the patterns of *B. burgdorferi* gene expression during an active mammalian infection. Furthermore, transcriptome analysis of *B. burgdorferi* during murine infection

has proven difficult given that spirochete loads in the blood and tissues are too low to recover sufficient spirochete RNA for direct microarray analysis [42].

In vivo expression technology (IVET) is a gene discovery method used to identify transcriptionally active portions of a microbial genome during interaction of the microorganism with a particular environment or host organism [104, 105]. In this system, the environment itself directly selects for upregulated bacterial loci [110]. The IVET selection system functions on the premise that deletion of a biosynthetic gene can lead to attenuation of growth and persistence of a pathogen in the host environment. This attenuation can be complemented by expression of the biosynthetic gene driven by promoters that are transcriptionally active *in vivo*. Thus, in the environment of interest, *in vivo* transcriptionally active promoters can be selected from a genomic library of DNA fragments cloned upstream of the essential biosynthetic gene [104, 105, 107, 110]. IVET is a sensitive and versatile method for identification of *in vivo*-expressed genes that has been used with pathogenic bacteria and fungi in a wide variety of host environments and has identified a number of previously uncharacterized virulence genes [106-110].

Using IVET we have developed and applied a genome-wide genetic screening approach to identify *B. burgdorferi* genes that are expressed during an active murine infection. This is the first time that an IVET strategy has been applied to *B. burgdorferi*. Moreover, we have identified a novel gene on virulence plasmid lp36 that is strongly induced *in vivo* and required for *B. burgdorferi* persistent infection in the mouse.

2.2: Results

2.2.1: Mammalian Host Adapted Spirochetes Demonstrate A 100-Fold Decrease In ID_{50} Relative To *In Vitro* Grown Spirochetes.

It is clear that *B. burgdorferi* modulates its gene expression profile at different stages of the infectious cycle [51]. The infectious dose of wild-type *B. burgdorferi* varies depending on the environment from which the spirochetes are derived. For example, the 50% infectious dose (ID_{50}) of spirochetes derived from partially fed ticks has been found to be two orders of magnitude lower than that of log phase *in vitro* grown *B. burgdorferi* [112]. In order to quantitatively assess the impact that adaptation to the mammalian environment has on *B. burgdorferi* infectivity the 50% infectious dose (ID_{50}) of spirochetes derived directly from the mammalian host was determined and compared to that of log phase *in vitro* grown spirochetes. *B. burgdorferi* are only transiently present in the blood of immunocompetent mice [113], whereas spirochetes persist longer in the blood of immunocompromised mice [114]. Therefore, the blood of severe combined immunodeficiency (*scid*) mice infected with *B. burgdorferi* was used as a source of spirochetes adapted to the mammalian environment. Strikingly, an inoculum containing approximately eight *in vivo*-derived spirochetes was able to infect five out of six mice, whereas, 5,000 *in vitro* grown spirochetes were required to obtain this level of infectivity (Table 3).

Table 3: *In vivo*-adapted *B. burgdorferi* are highly infectious.

***In vivo* grown spirochetes**		***In vitro* grown spirochetes**	
Spirochete dose	Number of mice infected[a]/ number of mice analyzed	Spirochete dose	Number of mice infected[a]/ number of mice analyzed
$8x10^2$	6/6	$5x10^4$	6/6
$8x10^1$	6/6	$5x10^3$	6/6
$8x10^0$	5/6	$5x10^2$	2/6
		$5x10^1$	1/6
		$5x10^0$	0/6
ID_{50}[b]	< 8 spirochetes	ID_{50}[b]	660 spirochetes

[a]Mouse infection was determined 3 weeks post inoculation by serological response to *B. burgdorferi* proteins and reisolation of spirochetes from ear, bladder and joint tissues.
[b]The ID_{50} was calculated according to method of Reed and Muench [115].

The ID_{50} for *in vivo*-derived spirochetes was found to be less than eight organisms. In contrast, the ID_{50} for *in vitro* grown spirochetes was calculated to be 660 organisms. These data indicate that mammalian host-adapted spirochetes are 100-fold more infectious than *in vitro* grown spirochetes, likely due to appropriate coordinate expression of *in vivo*-expressed genes important for murine infectivity.

2.2.2: The *B. burgdorferi* IVET System Is A Robust Method For Selection Of *B. burgdorferi* Sequences That Are Expressed During Murine Infection.

We have developed a genome-wide genetic screening method to identify *B. burgdorferi* genes that are expressed during mouse infection using an *in vivo* expression technology (IVET) approach [104, 105]. The *in vivo* expression technology vector, pBbIVET, carries the *B. burgdorferi bmpB* Rho-independent transcription terminator sequence [116] repeated in triplicate (3XTT), to prevent any read-through promoter activity from the pBSV2* *Borrelia* shuttle vector backbone, followed by the promoter-less *in vivo*-essential *pncA* gene (Figure 3) [12, 117]. Spirochetes lacking linear plasmid (lp) 25 are non-infectious in mice and severely compromised in the tick vector [13, 14, 18, 118-121]. The *pncA* gene, located on lp25, encodes a nicotinamidase that is sufficient to restore murine infectivity to *B. burgdorferi* lacking the entire lp25 plasmid [12]. Genetic transformation of low-passage, infectious *B. burgdorferi* occurs at low frequency and efficiency hampering introduction of a complex DNA library into an infectious background [122, 123]. Because *B. burgdorferi* clones lacking lp25 and lp56 demonstrate increased transformability [124], we isolated a clonal derivative of the low-passage infectious clone A3 that lacks both lp25 and lp56. This clone was designated A3 68-1 [125]. Clones A3 and A3 68-1 were transformed by

electroporation with 20 µg of a *Borrelia* shuttle vector. The transformation frequency and efficiency of A3 68-1 was determined relative to that of the A3 parent. As expected, genetic transformation of A3 68-1 occurred at a high frequency and efficiency. We recovered approximately 2,000 transformants/ml in clone A3 68-1, whereas no transformants were recovered with a parallel transformation of clone A3 (data not shown). In order to test the function of the *B. burgdorferi* IVET system, the promoter for the *in vivo* essential *ospC* gene [126], was cloned in front of the promoter-less *pncA* gene in pBbIVET (Figure 3), creating plasmid pBbIVET-$ospC_p$. This plasmid, along with pBbIVET alone, was transformed into the non-infectious, low-passage, highly transformable *B. burgdorferi* clone A3 68-1. All clones were tested for their abilities to infect groups of 6 C3H/HeN mice at an infectious dose 100 (ID_{100}) of $1x10^4$ spirochetes [16], indicating the presence or absence of an active promoter sufficient to drive expression of *pncA* thereby restoring infectivity. Spirochetes were reisolated from the ear, bladder and joint tissues of 5/6 mice infected with *B. burgdorferi* harboring pBbIVET-$ospC_p$. No spirochetes were reisolated from mice (0/6) infected with *B. burgdorferi* carrying the promoter-less pBbIVET alone. Together these data demonstrated that our promoter trap system functioned with a known *in vivo* active promoter.

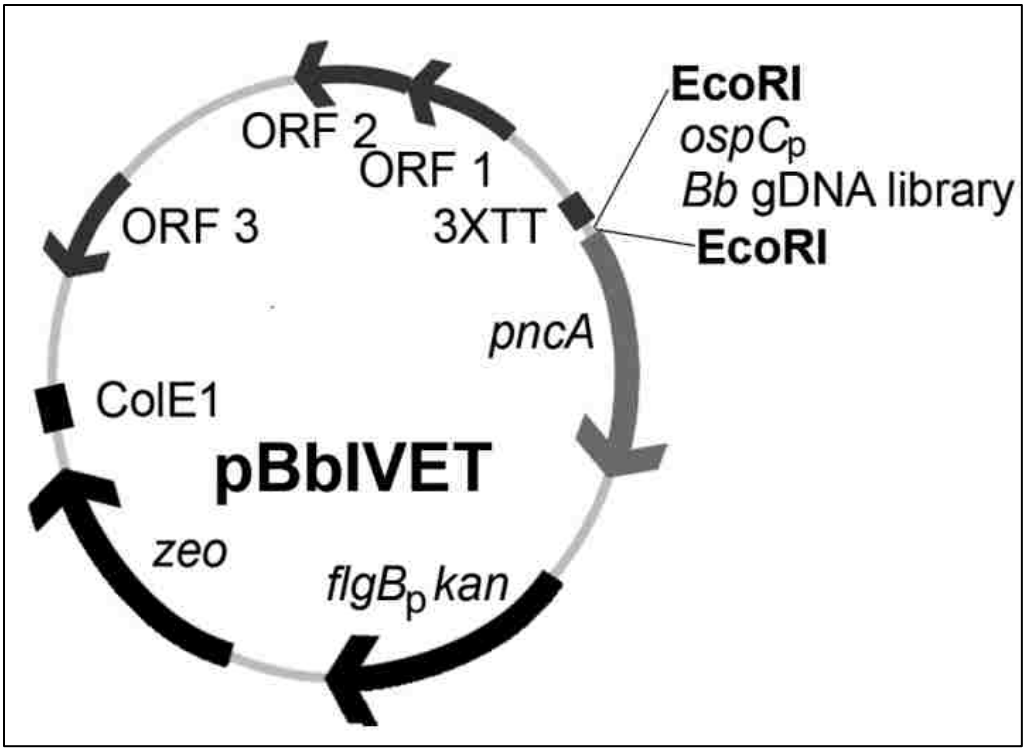

Figure 3: Schematic representation of the pBbIVET vector.

Features of this vector include: 3XTT, the transcriptional terminator sequence for *bmpB* [116] repeated in triplicate; *pncA*, promoterless *pncA* gene; *flgB*$_p$*kan*, kanamycin resistance cassette; *zeo*, zeocin resistance marker; ColE1, *E. coli* origin of replication; ORFs 1, 2, 3, *B. burgdorferi* cp9 replication machinery. The EcoRI restriction site was used to clone the *B. burgdorferi* control *in vivo*-expressed promoter, *ospC*$_p$, as well as the *B. burgdorferi* (*Bb*) gDNA library, in front of the promoterless *pncA* gene. The pBbIVET vector was derived from the *B. burgdorferi* shuttle vector pBSV2* [127].

2.2.3: Screening For *B. burgdorferi* Genes Expressed During Murine Infection.

A *B. burgdorferi* genomic DNA library using an average DNA fragment size of approximately 200 bps was constructed upstream of the promoter-less *pncA* gene (Figure 1) in the pBbIVET vector in *E. coli*, yielding approximately 30,000 independent clones. A small subset of individuals from the 30,000 clone library in *E. coli* was

analyzed by PCR and DNA sequencing and determined to carry non-identical *B. burgdorferi* DNA fragments. The strategy used to construct the pBbIVET library allowed the DNA fragments to be cloned in either the forward or reverse orientation relative to the *pncA* gene. Therefore, a library of 30,000 clones each harboring a unique 200 bp DNA fragment represented approximately 2X coverage of the 1.5 Mb genome of *B. burgdorferi*. Although the initial analysis of the transformation efficiency of *B. burgdorferi* clone A3 68-1 demonstrated that each transformation of 20 µg of a single purified plasmid into this genetic background yielded approximately 10,000 transformants, this transformation efficiency was not achieved when 20 µg of complex library plasmid DNA was transformed into A3 68-1. Forty four transformations of the library plasmid DNA resulted in recovery of approximately 15,000 individual clones in *B. burgdorferi* A3 68-1, representing an IVET library in *B. burgdorferi* with approximately 1X coverage of the spirochete genome. As described for the pBbIVET library in *E. coli*, a subset of individuals from 15,000 clone library in *B. burgdorferi* were analyzed and found to carry non-identical *B. burgdorferi* DNA fragments.

Like the BbIVET system described here, many IVET strategies are based upon complementation of auxotrophy. For microorganisms other than *B. burgdorferi* these strategies have allowed negative selection against “promoter-less” clones in minimal medium in which the auxotroph mutants are unable to grow [128]. *B. burgdorferi* lacking *pncA* are not attenuated for growth in the complex, undefined *B. burgdorferi* medium, BSKII. Moreover, there is currently no minimal medium available that supports the growth of wild-type *B. burgdorferi*. Therefore, the BbIVET system did not include

negative selection against “promoter-less” clones *in vitro*. 179 mice were infected with pools from the *B. burgdorferi* IVET library of approximately 100 clones each, with each clone at a dose of $1x10^4$ spirochetes resulting in a total dose of $1x10^6$ spirochetes per mouse. Three weeks post inoculation, mice were sacrificed and ear, heart, bladder and joint tissues were harvested for reisolation of infectious spirochetes. 175 out of 179 mice became infected with *B. burgdorferi* as determined by reisolation of spirochetes from at least two or more of the tissue sites analyzed (Table 4). However, due to the potentially stochastic nature of the kinetics of infection [129] and/or tissue-specific promoter activity of distinct *B. burgdorferi* genomic fragments not all four tissue sites from all 175 infected mice were found to be positive for spirochete reisolation. Nonetheless, the recovery of live spirochetes from infected mouse tissues suggested that these spirochetes harbored *in vivo* active promoter(s) in the pBbIVET plasmid sufficient to drive expression of the *in vivo*-essential *pncA* gene to restore spirochete mouse infectivity.

Table 4: The *B. burgdorferi* IVET system selects for *in vivo* active promoters.

Number of BbIVET clones screened	Positive reisolation of infectious spirochetes from mouse tissues[a]				Number of unique genomic fragments recovered
	Ear	Heart	Bladder	Joint	
~15,000	175 /179	173/179	172/179	174/179	289

[a]Number of mice positive for spirochete reisolation/ number of mice analyzed. Four mice were reisolate-negative for all tissues analyzed. Three mice were reisolate-negative for the bladder tissue. One mouse was reisolate-negative for the heart and joint tissues. One mouse was reisolate-negative for the heart tissue.

Total genomic DNA was isolated from each pool of reisolated spirochetes from each of the four mouse tissues and the pBbIVET plasmid DNA rescued in *E. coli*. Colony PCR using primers targeting the genomic DNA insert region of the pBbIVET vector was performed on 24 of the resulting *E. coli* colonies from each plasmid rescue transformation. No reisolated spirochetes were found to harbor a pBbIVET plasmid lacking a genomic DNA fragment insert. The amplified inserts were analyzed by restriction digest using a cocktail of the A/T-rich restriction enzymes to identify those DNA fragments with distinct restriction patterns, suggesting that these fragments represent different *in vivo* active promoters. Up to eleven non-identical restriction digest patterns were detected for every subset of 24 *E. coli* transformants carrying pBbIVET DNA that were analyzed (Fig. 4). The DNA fragments corresponding to each distinct restriction digest pattern were further analyzed by DNA sequencing and the identities of the sequences determined by microbial genome BLAST analysis. Screening of approximately 15,000 BbIVET clones through mice resulted in the identification of 289 non-identical *B. burgdorferi in vivo*-expressed (*Bbive*) DNA fragments from across the chromosome and all 21 plasmid replicons of the *B. burgdorferi* B31 segmented genome (Table 4). Although the 1:1 molar ratio of insert to vector used to generate the pBbIVET library did not preclude insertion of more than one fragment into each clone, only 20 out of the 289 clones were found to harbor two distinct DNA fragments. Of these clones the 3' DNA fragment, proximal to the *pncA* ORF, was assumed to be the active promoter and was included in the subsequent analyses.

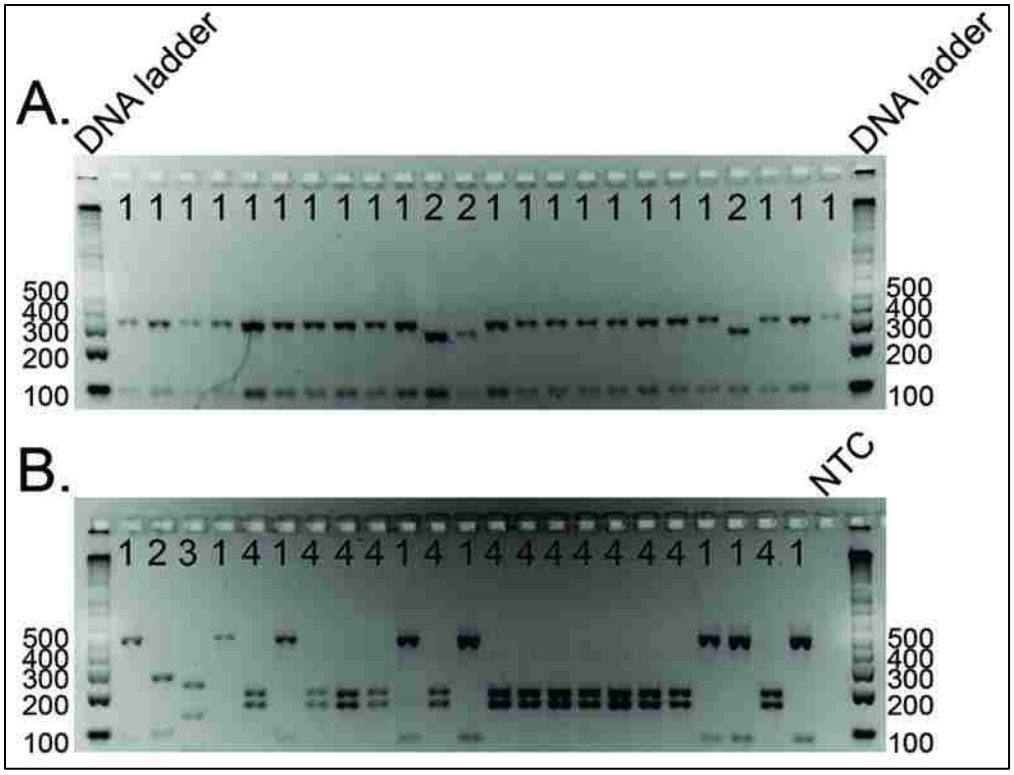

Figure 4: Representative restriction digest analysis of individual pBbIVET plasmids rescued in *E. coli.*

Colony PCR to amplify the *in vivo*-expressed DNA fragment was performed on a random subset of twenty four *E. coli* transformants carrying the rescued pBbIVET plasmids from infected mouse tissues. The PCR products were digested with a cocktail of the restriction enzymes DraI, SspI and AseI and separated on a 1% agarose gel. Numbers across the top of each image identify each non-identical restriction digestion pattern detected for the amplified pBbIVET DNA fragments. Representative data from two mouse tissues (A) and (B) are shown. Migration of the DNA ladders is shown in base pairs on both sides of each image. NTC, PCR no template control.

2.2.4: *B. burgdorferi In Vivo* Expressed Promoters Map To Distinct Classes Of Putative Regulatory Sequences Across The Genome.

Genomic mapping of the 289 unique *Bbive* promoters identified in this genetic screen demonstrated that 67% of the sequences mapped to sense DNA in the same

direction as annotated open reading frames, 27% mapped to antisense DNA in the opposite direction to annotated open reading frames and 6% mapped to intergenic regions lacking annotated open reading frames. Of the large percentage of sense sequences, 41%, which represented 28% of the total *Bbive* sequences, mapped to regions just upstream of and in the same orientation to annotated open reading frames, suggesting that these sequences are promoters for the associated open reading frames and that these open reading frames are candidate *in vivo*-expressed genes. The remaining 59% of the sense sequences, which represented 39% of the total *Bbive* sequences, mapped within annotated open reading frames, suggesting the possibility for promoter elements within *B. burgdorferi* genes. Similar findings of putative transcriptional start sites within genes and operons have been reported for other bacterial pathogens [130, 131]. The sequences that mapped to putative promoter locations in the genome and the genes associated with these promoters were prioritized for further analysis. Among the list of 80 sequences, 9 promoter regions were represented by two overlapping genomic DNA fragments. Five of these overlapping sequence pairs shared the same 3' end, suggesting that the sequences belonging to each pair contained the same promoter. Whereas, the other four overlapping sequence pairs harbored distinct 3' ends, suggesting that each sequence contained a unique promoter. The 71 *in vivo*-expressed candidate genes have been annotated to encode

proteins in various functional categories including: cell division, cell envelope, replication, metabolism, motility, protein synthesis, transport and unknown functions (Table 5).

2.2.5: IVET Identified Candidate Gene *bbk46* On Virulence Plasmid lp36.

Linear plasmid 36 is required for *B. burgdorferi* mouse infection; however, the genetic elements on lp36 that contribute to this phenotype have not been fully defined [16]. IVET identified a candidate *in vivo*-expressed promoter sequence, *Bbive162*, which mapped to lp36. This sequence was found to be 60 bp long, with 48 bp immediately upstream of and in the same direction as the BBK46 open reading frame (Fig. 5), suggesting that the *bbk46* gene may be expressed during mammalian infection and may contribute to the essential role of lp36 in *B. burgdorferi* infectivity. Therefore, the *bbk46* gene was selected for further analysis.

2.2.6: Expression Of The *bbk46* Gene Is Induced During Murine Infection.

Our BbIVET screen identified gene *bbk46* as a putative *in vivo*-expressed gene. The BbIVET screen was designed to identify *B. burgdorferi* DNA fragments that are expressed *in vivo* and did not discriminate between those promoters that are specifically induced *in vivo* and those promoters that are expressed both *in vitro* and *in vivo*. Therefore, quantitative reverse transcription PCR (qRT-PCR) was used to validate the expression of *bbk46 in vivo* and to determine whether *bbk46* expression was upregulated *in vivo* compared to *in vitro*.

1 **Table 5: *B. burgdorferi in vivo* expressed candidate genes organized by functional category.**

***Bbive* clone**[a]	**Replicon**	**ORF**[b]	**Protein designation, Annotated function**[c]
Cell division			
289	chromosome	BB0715	FtsA cell division protein
Cell envelope			
15	chromosome	BB0213	Putative lipoprotein
94	chromosome	BB0760	Gp37 protein
175	lp54	BBA36	Lipoprotein
271	lp54	BBA57	Lipoprotein
297	lp25	BBE16	BptA
151	lp28-2	BBG01	Putative lipoprotein
267	lp38	BBJ34	Putative lipoprotein
269	lp38	BBJ51	VlsE paralog, pseudogene
162	lp36	BBK46	Immunogenic protein P37, authentic frameshift
77	cp32-8, cp32-3, cp32-7, cp32-9, lp56, cp32-4,cp32-6, cp32-1	BBL28, BBS30, BBO28, BBN28, BBQ35, BBR28, BBM28, BBP28	Mlp lipoprotein family
DNA replication			
274	chromosome	BB0111	DnaB replicative helicase
226	chromosome	BB0632	RecD exodeoxyribonuclease V, alpha chain
152	lp28-3	BBH13	RepU replication machinery
Energy metabolism			
62	chromosome	BB0057	Gap glyceraldehyde-3-phosphate dehydrogenase, type 1
34	chromosome	BB0327	Glycerol-3-phosphate O acyltransferase
44	chromosome	BB0368	NAD(P)H-dependent glycerol-3-

***Bbive* clone**[a]	**Replicon**	**ORF**[b]	**Protein designation, Annotated function**[c]
			phosphate dehydrogenase
47	chromosome	BB0381	Trehalase
81	chromosome	BB0676	Phosphoglycolate phosphate
Fatty acid and phospholipid metabolism			
85	chromosome	BB0704	AcpP acyl carrier protein
Motility and chemotaxis			
14	chromosome	BB0181	FlbF putative flagellar protein
29	chromosome	BB0293	FlgB flagellar basal body rod
290	chromosome	BB0755	Flagellar hook-basal body complex protein
65	chromosome	BB0551	CheY-1 chemotaxis response regulator
222	chromosome	BB0568	Chemotaxis response regulator protein-glutamate methylesterase
Prophage function			
295	cp32-8, cp32-7, cp32-1, cp32-3, cp32-6, cp32-4	BBL23, BBO23, BBP23, BBS23, BBM23, BBR23	Holin BlyA family
Protein fate			
193	chromosome	BB0031	LepB signal peptidase I
Protein synthesis			
202	chromosome	BB0113	RpsR ribosomal protein S18
216, 217	chromosome	BB0485	RplP ribosomal protein L16
58	chromosome	BB0495	RpsE 30S ribosomal protein S5
59	chromosome	BB0496	50S ribosomal protein L30
219	chromosome	BB0503	RplQ ribosomal protein L17
232	chromosome	BB0660	GTP-binding Era protein

***Bbive* clone**[a]	**Replicon**	**ORF**[b]	**Protein designation, Annotated function**[c]
288	chromosome	BB0682	TrmU tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase
Regulation			
208	chromosome	BB0379	Protein kinase C1 inhibitor
50	chromosome	BB0420	Hk1 histidine kinase
Nucleoside salvage			
148	lp25	BBE07	Pfs protein, pseudogene
Transcription			
1	chromosome	BB0389	RpoB DNA-directed RNA polymerase, beta subunit
287	Chromosome	BB0607	PcrA ATP-dependent DNA helicase
84	chromosome	BB0697	RimM 16S rRNA processing protein
Transport			
204	chromosome	BB0318	MglA methylgalactoside ABC transporter ATP-binding protein
46	chromosome	BB0380	MgtE Mg^{2+} transport protein
Unknown			
56	chromosome	BB0049	Hypothetical protein
69	chromosome	BB0063	Pasta domain protein
2	chromosome	BB0102	Conserved hypothetical
8	chromosome	BB0138	Conserved hypothetical
13	chromosome	BB0176	ATPase family associated with various cellular activities
23	chromosome	BB0265	Conserved hypothetical
212	chromosome	BB0428	Conserved hypothetical
52, 53	chromosome	BB0429	Conserved hypothetical
220	chromosome	BB0504	Conserved hypothetical
67	chromosome	BB0562	Conserved hypothetical
223	chromosome	BB0577	Conserved hypothetical
71	chromosome	BB0592	Caax amino protease family

Bbive clone[a]	Replicon	ORF[b]	Protein designation, Annotated function[c]
73	chromosome	BB0619	DHH family phosphoesterase function
96	chromosome	BB0799	Conserved hypothetical
240	cp26	BBB27	Unknown essential protein
145, 146	lp25	BBE0036	Hypothetical protein
147	lp25	BBE01	Conserved hypothetical
265, 266	lp38	BBJ30	Conserved hypothetical
171	lp38	BBJ36	Conserved hypothetical
173	lp38	BBJ46	Conserved hypothetical
129, 296	cp32-8, cp32-1, cp32-7, lp56, cp32-9	BBL41, BBP40, BBO42, BBQ48, BBN41	Conserved hypothetical
130, 151	cp32-8, cp32-1, cp32-6	BBL42, BBP41, BBM41	Conserved hypothetical
117	cp32-6, lp56, cp32-9, cp32-8, cp32-3, cp32-1, cp32-4	BBM18, BBQ25, BBN18, BBL18, BBS18,BBP18, BBR18	Conserved hypothetical
182, 183	lp56	BBQ41	PF-49 protein
188	lp56	BBQ84.1	Conserved hypothetical
189	lp56, lp28-3, lp17	BBQ89, BBH01, BBD01	Conserved domain protein
244	cp32-4, cp32-3, cp32-6, lp56, cp32-8, cp32-9	BBR05, BBN05, BBM05, BBQ12, BBL05, BBO05, BBP05	Lyme disease protein of unknown function
157	lp28-4	BBI07	Conserved hypothetical

[a]In some cases two *Bbive* clones shared overlapping, non-identical sequence, as indicated by two *Bbive* clone numbers.
[b]ORF, open reading frame that maps just downstream and in the same orientation to the *Bbive* sequence.
[c]Annotation described by Fraser *et al.* [7].

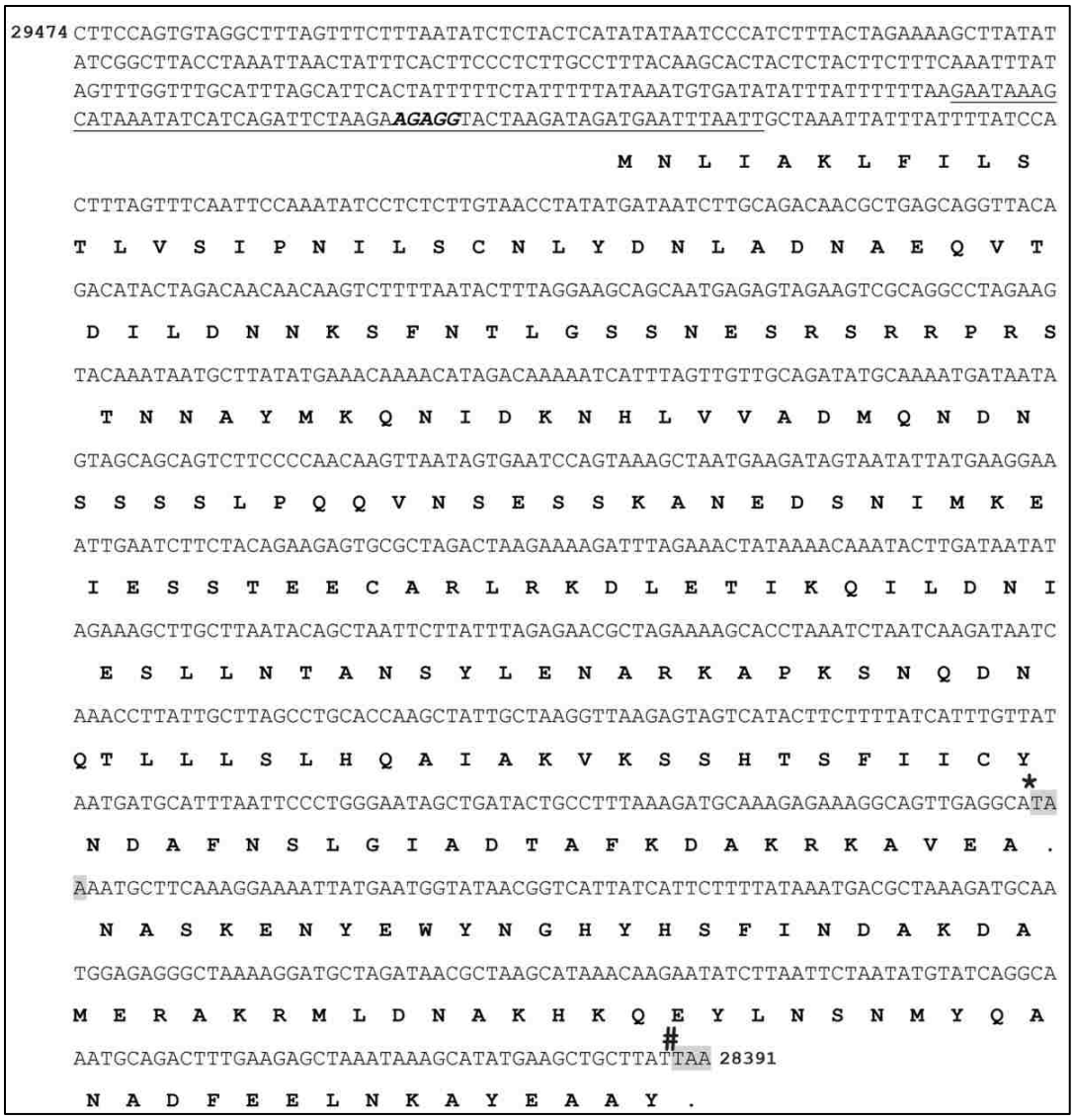

Figure 5: The nucleotide and putative amino acid sequence of the BBK46 open reading frame.

The reverse complement of nucleotides 28391 to 29474 on lp36, encompassing *bbk46* (Genbank GeneID: 1194234) and its putative promoter sequence. The nucleotide sequence of *Bbive162* is underlined. The putative ribosome binding site is shown in bold italics. The putative BBK46 amino acid sequence is shown in bold. The stop codons at nucleotides 625 and 820 are highlighted in gray. The position of the inserted FLAG-epitope tag sequence is indicted with a star (*).The position of the inserted cMyc-epitope tag sequence is indicated with a number sign (#).

Total RNA was isolated from bladder tissue collected from mice infected with $1x10^5$ wild-type *B. burgdorferi* three weeks post-inoculation as well as log phase *in vitro* grown spirochetes. RNA was converted to cDNA using random hexamer primers and the mRNA level of each target gene was measured relative to the constitutive *recA* gene using quantitative PCR. The gene expression levels of *flaB* and *ospC* were also measured as control constitutively-expressed and *in vivo*-induced genes, respectively. These data demonstrated that although *bbk46* was expressed during *in vitro* growth, expression of this gene was increased more than 100-fold during mammalian infection (Fig. 6A). Consistent with their known patterns of gene regulation, *flaB* expression was relatively unchanged *in vivo* compared to *in vitro*; whereas, *ospC* demonstrated a nearly 1000-fold increase in expression *in vivo* compared to *in vitro* (Fig. 6A). Moreover, the relative amount of *bbk46* expression during *in vitro* growth was found to be approximately 10-fold more than that of *ospC*. Whereas, the *in vivo* expression levels of genes *bbk46*, *ospC* and *flaB* were similar.

RpoS is a global regulator that controls expression of genes expressed during mammalian infection, including *ospC* [51]. Because *bbk46* expression was induced *in vivo* in a manner similar to that of *ospC*, we sought to determine whether, like *ospC*, *bbk46* is an RpoS-regulated gene. RNA was isolated from stationary phase temperature-shifted wild-type and Δ*rpoS* mutant spirochetes, a growth condition previously shown to induce expression of *rpoS* and *rpoS*-regulated genes [52]. Quantitative RT-PCR was then performed for genes *bbk46*, *flaB, ospC* and *recA*, as

described above. As expected, *ospC* expression was increased approximately 20 times in the presence compared to the absence of *rpoS* (Fig. 6B). In contrast, *bbk46* expression was RpoS-independent under these growth conditions (Fig. 6B). Likewise, no RpoS-dependent change in gene expression was detected for *flaB*. Interestingly, the amount of *flaB* expression detected in the stationary phase temperature-shifted spirochetes (Fig. 6B) was dramatically decreased compared to the amount of *flaB* expression detected in log phase and *in vivo* grown spirochetes (Fig. 6A), suggesting that *flaB* is not expressed at the same level under all growth conditions. Together these data demonstrated that *bbk46* was highly induced during murine infection and *bbk46* expression was not controlled by RpoS during *in vitro* growth.

2.2.7: The *bbk46* Open Reading Frame Fails To Produce Detectable Amounts Of Protein During *In Vitro* Growth.

The *bbk46* gene is a member of paralogous gene family 75, which also includes lp36-encoded genes *bbk45*, *bbk48* and *bbk50*, all of which are annotated to encode putative P37 immunogenic lipoproteins [7, 8, 132] (Fig. 7). These genes are located on the right arm of lp36 in *B. burgdorferi* clone B31, which is a highly variable region among distinct *B. burgdorferi* isolates [132].

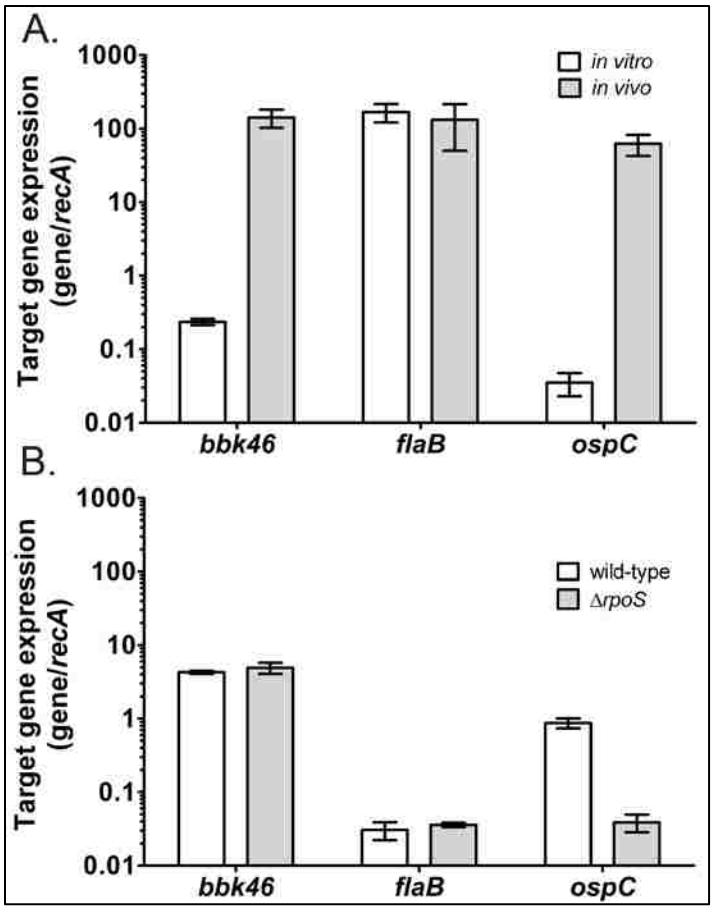

Figure 6: Expression of the *bbk46* gene is upregulated during murine infection and is RpoS-independent.

Total RNA was isolated from bladder tissue collected from (A) mice infected with $1x10^5$ wild-type *B. burgdorferi* three weeks post-inoculation (*in vivo*, gray bars) and from log phase *in vitro* grown spirochetes (*in vitro*, white bars) or (B) stationary phase temperature-shifted stationary phase *in vitro* grown wild-type (white bars) or Δ*rpoS* (gray bars) *B. burgdorferi*. RNA was reverse transcribed to cDNA using random hexamer primers. The expression of *bbk46*, *flaB* and *ospC* were quantified using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and a standard curve analysis method. The mRNA levels of the *bbk46, flaB* and *ospC* gene transcripts were normalized to that of the constitutive *recA* gene. The data are expressed as the gene transcript/*recA* transcript. The data represent the average of triplicate qRT-PCR analyses of 3 biological replicates. Error bars represent the standard deviation from the mean.

The members of paralogous gene family 75 are conserved within *B. burgdorferi* isolates but are not present in the relapsing fever spirochetes. The *B. burgdorferi* clone B31 BBK45, BBK48 and BBK50 proteins are predicted to be 301, 288 and 332 amino acids, respectively. However, *B. burgdorferi* clone B31 *bbk46* is annotated as a pseudogene as a result of an authentic frame shift resulting in a TAA stop codon at nucleotide 625 [7, 8, 132], thereby producing a putative 209 amino acid protein. In contrast, the BBK46 homolog in clone N40, BD04, harbors a CAA codon at nucleotide 625, resulting in a glutamic acid residue at amino acid 209 and producing a putative 273 amino acid protein [66]. Sequence analysis of the cloned *bbk46* open reading frame confirmed the presence of the TAA stop codon at nucleotide 625 (Fig. 5). To experimentally determine the size of the BBK46 protein produced in *B. burgdorferi* B31 the *bbk46* ORF along with a FLAG epitope tag sequence prior to the stop codon at nucleotide 625 and a cMyc epitope tag sequence prior to the stop codon at nucleotide 820 (Fig. 5) was cloned into the *B. burgdorferi* shuttle vector pBSV2G under the control of either the constitutive *flaB* promoter or the putative endogenous *bbk46* promoter.

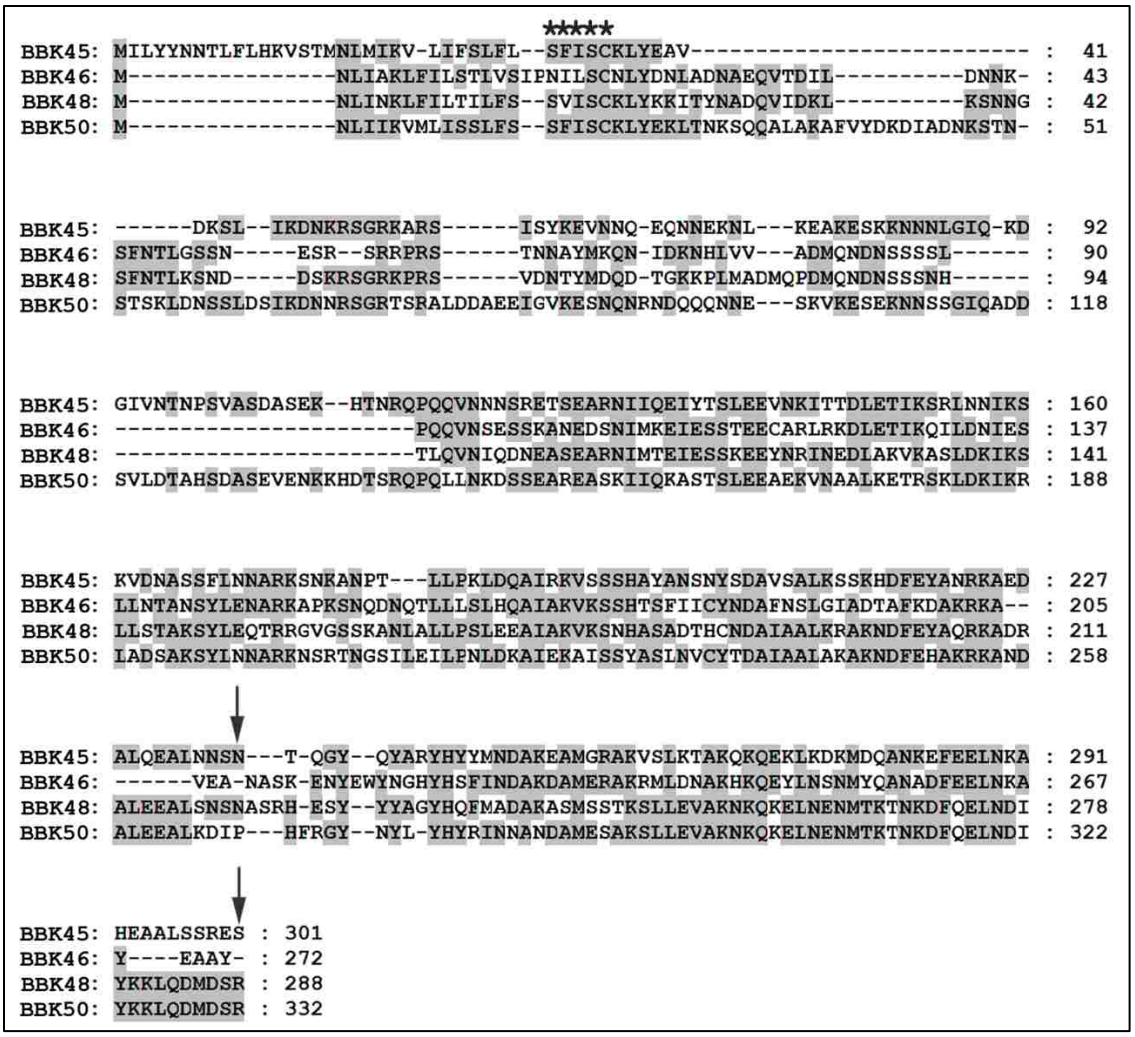

Figure 7: Amino acid alignment of the putative members of the immunogenic protein P37 family encoded on lp36.

Shown is an amino acid alignment of the P37 protein family members BBK45 (GenBank accession no. NP_045617.2), BBK46 (translated *bbk46*, Genbank GeneID: 1194234), BBK48 (GenBank accession no. NP_045619.1) and BBK50 (GenBank accession no. NP_045621.1). Amino acids identical to the consensus sequence are shaded. The predicted SpLip lipobox sequence [133] is indicted with five stars. Dashes represent spaces introduced for optimal sequence alignment. The positions of the two stop codons in the *bbk46* translation are indicated with arrows. Amino acid sequences were aligned using the CLUSTAL W algorithm in the MEGALIGN program from the DNASTAR Lasergene suite.

A mutant clone lacking the entire BBK46 open reading frame was constructed by allelic exchange and verified by PCR analysis (Fig. 8A and 8B). The *bbk46* mutant clone was transformed with the shuttle vectors carrying the epitope tagged *bbk46* constructs. All transformants were verified to contain the plasmid content of the parent clone. BBK46 protein production was assessed in both *E. coli* and *B. burgdorferi*. Immunoblot analyses using αFLAG and αcMyc monoclonal antibodies resulted in detection of a FLAG-epitope tagged protein of an approximate molecular mass of 23 kDa, which is the predicted size of the 209 amino acid BBK46 protein, in the *E. coli* clones carrying both the *flaB*$_p$-driven and the *bbk46*$_p$-driven constructs (Fig. 9). Surprisingly, no FLAG epitope tagged protein was detected in either *B. burgdorferi* clone (Fig. 9), although *bbk46* gene expression was observed in these clones (data not shown), indicating that the lack of detectable BBK46 protein was not likely the result of a transcription defect. Furthermore, no cMyc epitope tagged protein was detected in either *E. coli* or *B. burgdorferi*. Together these data suggested that although the *bbk46* ORF is competent to produce a 23 kDa protein in *E. coli* and the transcript is expressed in *B. burgdorferi* during *in vitro* growth, the protein is either not produced or is rapidly turned over in log phase *in vitro* grown *B. burgdorferi*.

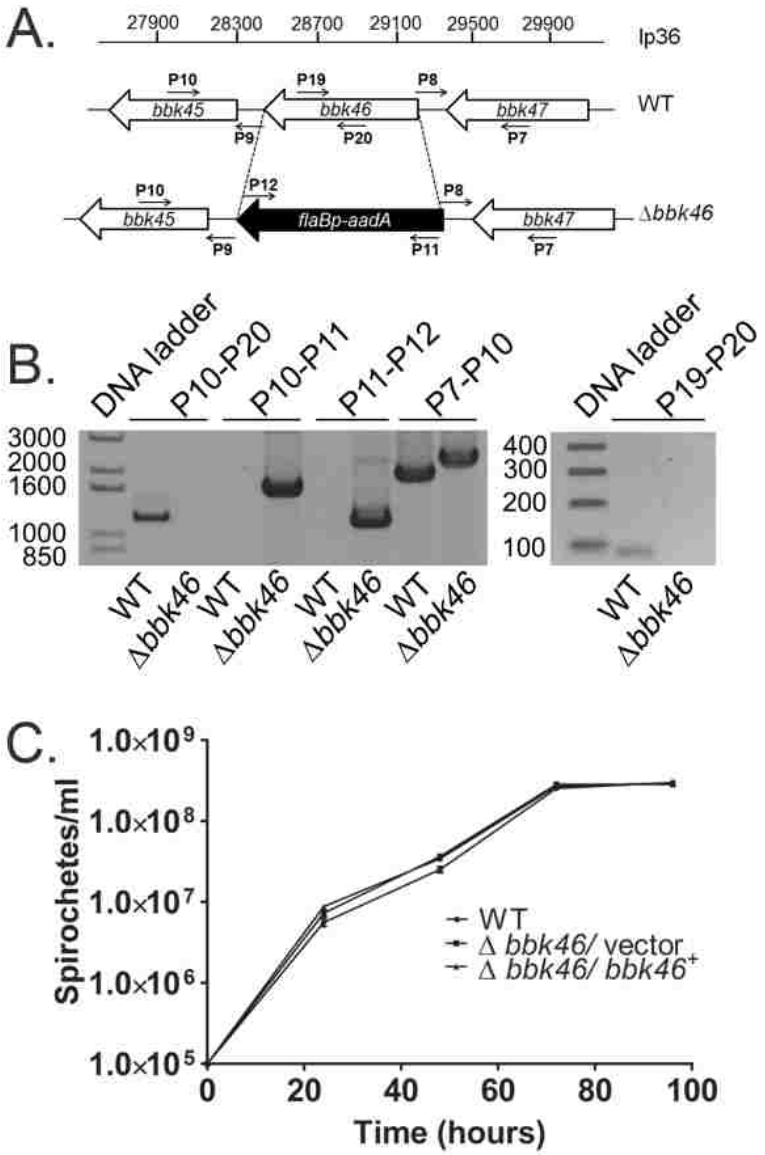

Figure 8: Generation of the Δ*bbk46* mutant and genetic complemented clones of *B. burgdorferi.*

(A) Schematic representation of the wild-type (WT) and Δ*bbk46* loci on lp36. The sequence of the entire *bbk46* open reading frame was replaced with a *flaBp-aadA* antibiotic resistance cassette [16, 134]. Locations of primers for analysis of the mutant clones are indicated with small arrows and labels P7-P12, P19 and P20. Primer sequences are listed in Table 5. (B) PCR analysis of the Δ*bbk46* mutant clone. Genomic DNA isolated from WT and Δ*bbk46*/ vector spirochetes served as the template DNA for

PCR analyses. DNA templates are indicated across the bottom of the gel image. The primer pairs used to amplify specific DNA sequences are indicated at the top of the gel image and correspond to target sequences as shown in (A). Migration of the DNA ladder in base pairs is shown to the left of each image. (C) In vitro growth analysis of mutant clones. A3-68ΔBBE02 (WT), *bbk46::flaBp-aadA*/ pBSV2G (Δ*bbk46*/ vector) and *bbk46::flaBp-aadA*/ pBSV2G-*bbk46* (Δ*bbk46*/ *bbk46*$^{+}$) spirochetes were inoculated in triplicate at a density of 1x105 spirochetes/ml in 5 ml of BSKII medium. Spirochete densities were determined every 24 hours under dark field microscopy using a Petroff-Hausser chamber over the course of 96 hours. The data are represented as the number of spirochetes per ml over time (hours) and is expressed as the average of 3 biological replicates. Error bars indicate the standard deviation from the mean.

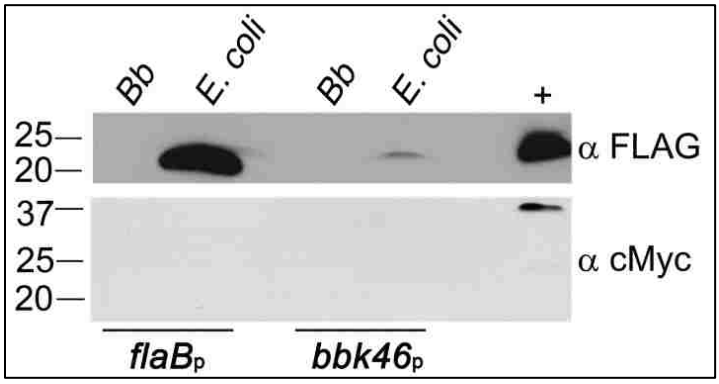

Figure 9: BBK46 protein production is detectable in *E. coli* but not in *B. burgdorferi.*

Immunoblot analysis of total protein lysate prepared from 1.5x10^{8} *B. burgdorferi* Δ*bbk46* (*Bb*) or *E. coli* harboring either pBSV2G *flaB*$_{p}$-*bbk46-FLAG-cMyc* (*flaB*$_{p}$) or pBSV2G *bbk46*$_{p}$-*bbk46-FLAG-cMyc* (*bbk46*$_{p}$). Protein lysates were separated by SDS-PAGE and immunoblots performed using anti-FLAG monoclonal antibodies (α FLAG) and anti-cMyc monoclonal antibodies (α cMyc). 300 ng of purified PncA-FLAG [117] and GST-BmpA-cMyc [135] proteins served as positive controls (+) for each antibody. The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

As a putative member of the P37 immunogenic lipoprotein family, BBK46 is predicted to localize to the spirochete outer surface and to be immunogenic during mammalian infection. Therefore, recombinant BBK46, lacking the first 32 amino acids that are predicted to comprise the signal sequence for the lipoprotein, was produced in *E. coli* as an N-terminal fusion to glutathione *S*-transferase (GST). To assess the immunogenicity of the BBK46 protein, immunoblot analysis was performed using purified rGST-BBK46 probed with mouse immune serum collected 21 days post inoculation with $1x10^4$ wild-type *B. burgdorferi.* The rGST-BBK46 protein was found to be non-immunoreactive with mouse immune serum, in contrast to the control antigen BmpA (Fig. 10). These data suggest that, if produced in *B. burgdorferi*, BBK46 is not an immunoreactive antigen. However, these data do not rule out the possibility that the immunogenic epitope is not present or available in the recombinant protein produced in *E. coli*.

2.2.8: The *bbk46* Gene Is Required For *B. burgdorferi* Persistence In Immunocompetent Mice.

In vitro growth analysis demonstrated that the *bbk46* mutant and complemented clones had no detectable *in vitro* phenotypes (Fig. 8C). Therefore, to examine the role of *bbk46* in mouse infectivity, groups of five C3H/HeN female mice were needle inoculated intradermally under the skin of the upper back with $1x10^4$ wild-type, Δ*bbk46*/vector or Δ*bbk46*/*bbk46*$^+$ spirochetes.

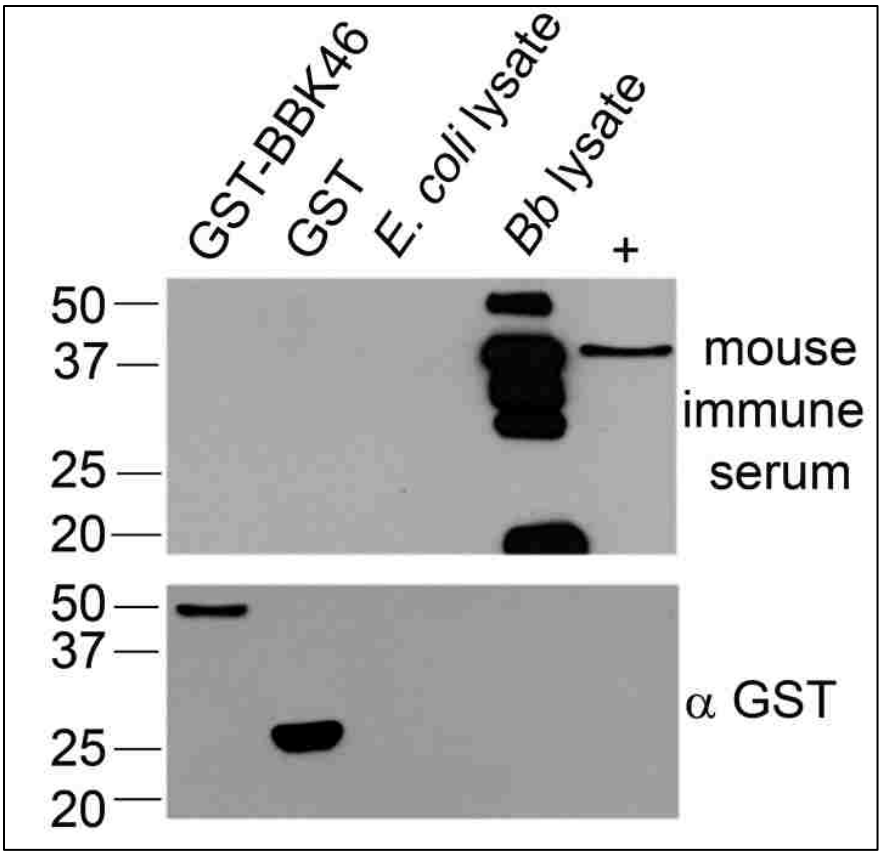

Figure 10: The BBK46 protein is non-immunogenic in mice.

Recombinant GST-BBK46 and GST alone produced in and purified from *E. coli*, along with total protein lysate from *E. coli* and *B. burgdorferi* (*Bb* lysate) and *E. coli* producing the *B. burgdorferi* antigen BmpA (+) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed using immune serum collected from mice infected with wild-type *B. burgdorferi* and anti-GST monoclonal antibodies (α GST). The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

Three weeks post inoculation, mice were assessed for *B. burgdorferi* infection by serology and reisolation of spirochetes from the inoculation site, ear, bladder and joint tissues. All five mice from each infection group were seropositive for anti-*B. burgdorferi* antibodies (Fig. 11, Table 6). Surprisingly however, no spirochetes were reisolated from all tissues examined from the five mice inoculated with the ∆*bbk46*/vector clone (Table 6), whereas, all five mice inoculated with the wild-type or the ∆*bbk46*/*bbk46*$^{+}$ clone resulted in reisolation of spirochetes from all tissues analyzed (Table 6). Together these data demonstrated that spirochetes lacking the *bbk46* gene transiently infected and elicited a humoral response in mice, but were unable to maintain a persistent infection in mouse tissues. To further define the contribution of the host immune response to the inability of spirochetes lacking the *bbk46* genes to cause a persistent infection, groups of five *severe combined immunodeficiency* (*scid*) mice were inoculated with 1x10^{4} wild-type, ∆*bbk46*/vector or ∆*bbk46*/*bbk46*$^{+}$ spirochetes. Three weeks post inoculation the animals were assessed for infection by reisolation of spirochetes from the ear, bladder and joint tissues. Consistent with the hypothesized role of *bbk46* in immune evasion and persistence, four out of five immunodeficient mice inoculated with the ∆*bbk46* mutant were positive for spirochete reisolation from all tissues examined (Table 6). These data demonstrated that a functional host immune response is required for the clearance of spirochetes lacking *bbk46* from mouse tissues 3 weeks post infection, indicating that *bbk46* is essential for the ability of *B. burgdorferi* to avoid killing by the host immune system in order to establish a persistent infection.

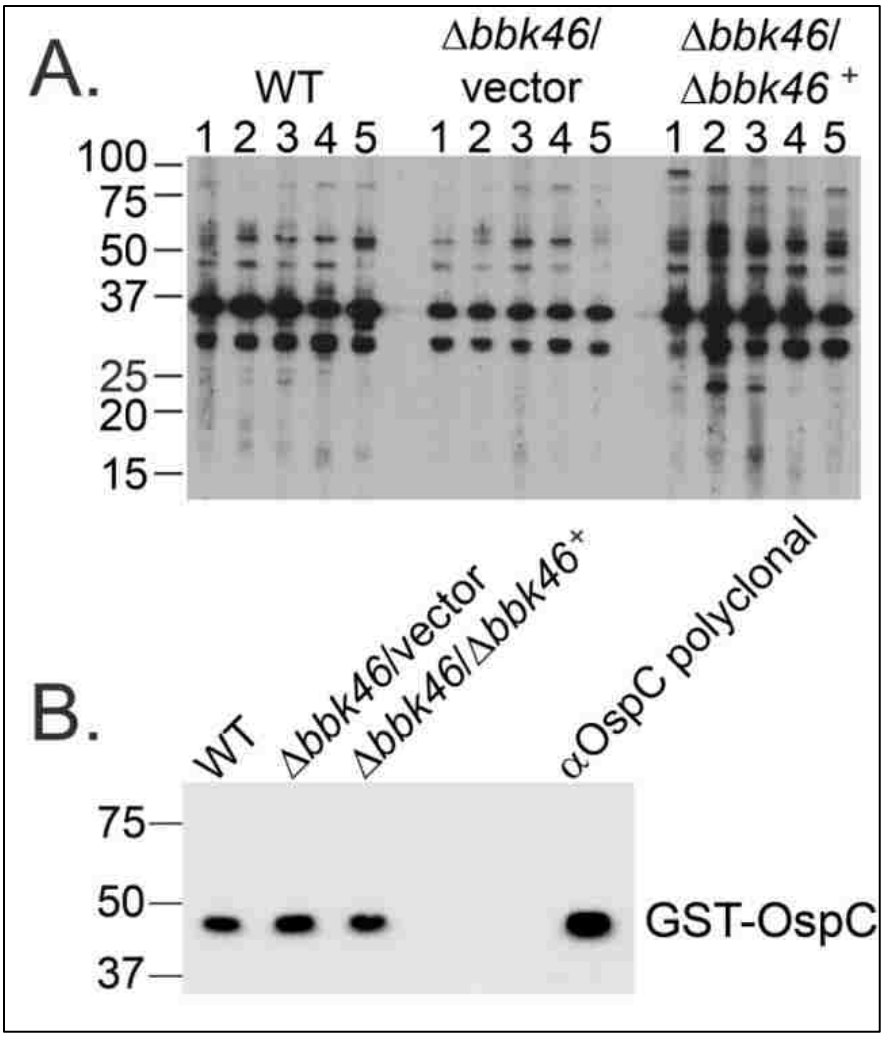

Figure 11: Spirochetes lacking *bbk46* retain seroreactivity in mice.

Immunoblot analysis of sera collected three weeks post inoculation from groups of five C3H/HeN mice inoculated with clone A3-68ΔBBE02 (WT), *bbk46*::*flaB*$_p$-*aadA*/ pBSV2G (Δ*bbk46*/ vector) and *bbk46*::*flaB*$_p$-*aadA*/ pBSV2G-*bbk46* (Δ*bbk46*/ *bbk46*$^+$) at a dose of 1x10^4 spirochetes per mouse. (A) Total protein lysate from *B. burgdorferi* clone B31 A3 was probed with the serum from each individual mouse (1-5). (B) Purified recombinant GST-OspC protein was probed with pooled sera from the five mice in each infection group or αOspC polyclonal antibodies. The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

Table 6: The *bbk46* gene is required for persistent infection of immunocompetent mice.

Clone	Serology[a]	Positive reisolation of spirochetes from mouse tissues[b]			
		Inoculation site	Ear	Bladder	Joint
Immunocompetent mice					
wild-type	5/5	5/5	5/5	5/5	5/5
Δ*bbk46*/vector	5/5	0/5	0/5	0/5	0/5
Δ*bbk46*/*bbk46*$^{+}$	5/5	5/5	5/5	5/5	5/5
Immunodeficient mice					
wild-type	NA	NA	5/5	5/5	5/5
Δ*bbk46*/vector	NA	NA	4/5	4/5	4/5
Δ*bbk46*/*bbk46*$^{+}$	NA	NA	5/5	5/5	5/5

[a]Determined 3 weeks post inoculation by serological response to *B. burgdorferi* total protein lysate and recombinant OspC protein. NA, not applicable.
[b]Number of mice positive for spirochete reisolation/ number of mice analyzed. NA, not applicable.

2.3: Discussion

In this study we have successfully adapted and applied for the first time an IVET-based genetic screen for use in *B. burgdorferi* for the purpose of identifying spirochete genes that are expressed during mammalian infection. Historically, genetic manipulation of low passage, infectious *B. burgdorferi* has been challenged by the low transformation frequencies of these spirochetes, preventing application of classic *in vivo* genetic screening techniques such as *in vivo* expression technology (IVET) and signature-tagged mutagenesis (STM) [136] to identify *B. burgdorferi* genetic elements important for pathogenicity. However, advances in the understanding of the *B. burgdorferi* restriction modification systems that inhibit transformation [124, 137-140] have recently allowed construction and characterization of a comprehensive STM mutant library in infectious *B. burgdorferi* [26]. The foundation for our strategy for development of IVET in *B. burgdorferi* was based upon the spirochete's requirement of lp25 for both restriction modification and virulence functions. Spirochetes lacking lp25 are highly transformable but non-infectious in mice [13, 14, 18, 124]. Restoration of the lp25-encoded *pncA* gene to lp25$^-$ spirochetes restores wild-type infectivity [12] but maintains high transformation frequency. At the time of the development of the pBbIVET system the true start codon of the *pncA* gene was not defined; therefore, the promoter-less *pncA* gene construct in the pBbIVET plasmid used an engineered AUG start codon and was missing the first 24 nucleotides of the now defined *pncA* ORF [117]. Furthermore, this construct was purposefully designed without a ribosome binding site (RBS) and was dependent upon

the cloned *B. burgdorferi* DNA fragments to contain both a promoter and a functional RBS. Although we acknowledge that this requirement may have limited the number of clones identified in our screen, during development of the BbIVET system we found that inclusion of an RBS sequence in the promoterless *pncA* construct resulted in vector-driven PncA production in the absence of a promoter. Thus, in order to reduce the possibility of recovering false positive clones, the pBbIVET system was designed without an RBS. The enzyme Tsp509I was selected to generate the DNA fragments for the pBbIVET library because the AATT restriction site of this enzyme is present approximately every 58 bp in the *B. burgdorferi* B31 genome. However, it is possible that DNA fragments generated with this enzyme will not result in sequences that contain a 3' RBS appropriately distanced from the start codon of the *pncA* ORF, thereby limiting the number of clones identified in the screen.

Screening of a 15,000 clone *B. burgdorferi* genomic library in mice identified 289 DNA sequences from across all 22 *B. burgdorferi* replicons capable of promoting *pncA* expression resulting in an infectious phenotype. It is likely that the BbIVET screen did not achieve saturation because the number of clones analyzed was only estimated to cover the *B. burgdorferi* genome one time, under the assumption that each cloned DNA fragment in the library was unique. Analysis of the pBbIVET library in *B. burgdorferi* suggested that the library was composed of 15,000 unique clones. However, because only a small fraction of the library was examined for the sequences of the DNA fragment inserts, our findings do not rule out the potential that the library was composed of less

than 15,000 non-identical clones and therefore, may represent less than 1X coverage of the genome. Of the 175 mice infected with the pBbIVET library, 10% resulted in reisolation of a single clone, 62% resulted in reisolation of two to five unique clones, and 28% resulted in reisolation of six to eleven unique clones. Furthermore, 57% of the 289 *Bbive* sequences were only recovered once; whereas, 39% of the sequences were recovered two to five times and 4% of the sequences were recovered six to twelve times. These data are indicative of the amount of redundancy in the screen and suggest that although the screen may not have been representative of the entire *B. burgdorferi* genome, a large percentage of mice became infected with multiple clones and many of the *Bbive* sequences were recovered more than once.

We found that 71 of the *Bbive* sequences mapped to canonical promoter positions upstream of annotated open reading frames in the *B. burgdorferi* genome. Unexpectedly, the well characterized *in vivo*-expressed *ospC* promoter was not among these sequences. However, the $ospC_p$ was successfully recovered in our functional validation of the BbIVET system, suggesting that the BbIVET screen had not reached complete saturation of the genome and with further screening of the BbIVET library the $ospC_p$ sequence may be recovered. Alternatively, given that *ospC* expression is known to be down-regulated after the initial stages of infection [42, 43, 141-143] it is possible that in the context of a mixed infection individual pBbIVET clones carrying the $ospC_p$ lack a fitness advantage due to decreased expression three weeks post inoculation and may not be recovered in our screen. This explanation may appear to conflict with the

findings reported herein that *ospC* expression is high at three weeks post inoculation and the *ospC*$_p$ served as a robust positive control promoter for the BbIVET system. However, down-regulation of *ospC* expression at this time point in infection is a stochastic process that occurs at the level of the individual spirochete and does not occur simultaneously across the entire population [142]. Although at the population level the *ospC*$_p$ is expressed at this time point in our studies, in the context of the BbIVET screen individual clones carrying the *ospC*$_p$ may express reduced amount of *pncA* and may be out competed by other BbIVET clones carrying stronger promoters.

A subset of the genes identified in the BbIVET screen included known *in vivo*-expressed genes, which provided validation that our genetic system was working as expected and was sufficiently powerful. The screen recovered the promoter for genes *bba36* (*Bbive175*), *bba57* (*Bbive271*), *bbb27* (*Bbive240*), *bbj34* (*Bbive267*), *bbj36* (*Bbive171*), *bbj51* (*Bbive269*), *bb0213* (*Bbive15*) and *bb0760* (*Bbive94*), all of which have been shown previously to be expressed during mammalian infection [42]. Furthermore, *bba57* was recently reported to be up-regulated *in vivo* and to contribute to pathogenesis in the mouse [144]. The *bptA* gene encodes a function that has been shown to be required for *B. burgdorferi* survival in the tick and to contribute to mouse infectivity [120, 121]. In addition, *Bbive14*, *58*, *232*, *84*, *269*, *295* and *77* are associated with genes that have been shown to be up-regulated in *in vivo*-like conditions and/or gene products that are immunogenic in humans and mice [76, 100, 145, 146]. Notably, few *in vivo*-expressed candidate genes identified using BbIVET were previously

observed to be up-regulated in mammalian host-adapted spirochetes derived from growth within rat dialysis membrane chambers (DMCs). Genes identified in our analyses that have also been detected by microarray analysis of DMC grown spirochetes include *bba36* [76, 103], *bbj51* [76, 102], *bb0551*, *bbm28* [76], *bb0495,* and *bb0660* [102]. The results of the DMC microarray studies are reported as genes that are significantly up-regulated in DMC-derived spirochetes relative to spirochetes grown *in vitro*; whereas, the BbIVET screen does not distinguish between genes that are specifically induced *in vivo* and genes that are expressed both *in vitro* and *in vivo*. Furthermore, the environmental cues within the DMCs may not fully reflect those experienced by *B. burgdorferi* during an active infection. Finally, the BbIVET system specifically selects for promoters that are capable of driving expression of *pncA* allowing the spirochetes to survive throughout a three week mouse infection. Together, these technical and biological differences between the DMC microarray and BbIVET screen likely contributed to the distinct results obtained from the two methods of gene expression analysis. In addition, few genes that have been previously established to be RpoS-regulated *in vitro* and/or within DMCs [58, 103] were identified by the BbIVET screen. RpoS-regulated genes *bba36*, *bba57*, *bb0265* and *bbh01* [58, 103] were among the *in vivo*-expressed *Bbive* candidate genes. Similarly, only one putative BosR-regulated gene, *bb0592* [61], was identified in the BbIVET screen. Although it is unclear why only a small number of know RpoS-regulated promoters were recovered, the recently identified AT-rich BosR binding site [61] contains the restriction site for the

Tsp509I restriction enzyme used to generate the BbIVET library. Therefore, it is possible that the BosR binding sites were subject to cleavage by Tsp509I, perhaps resulting in a limited number of DNA fragments that contained BosR-dependent promoters.

The BbIVET screen was carried out in such a way that both DNA fragments that are expressed *in vitro* and *in vivo*, as well as those fragments that are specifically induced *in vivo*, could be recovered. Therefore, it was not surprising that genes encoding cell division, DNA replication, energy metabolism, protein synthesis and transcription functions were identified, all of which are likely functions essential for spirochete growth under all condition. These findings were consistent with those categories of genes not recovered by genome-wide transposon mutagenesis, suggesting that these genes encode essential functions [26]. The BbIVET screen identified genes that encode proteins in functional categories that may contribute to *B. burgdorferi* infectivity and pathogenesis including, putative lipoproteins, motility and chemotaxis proteins, transport proteins and proteins of unknown function. Similarly, transposon mutagenesis analysis indicated that motility and chemotaxis genes as well as transport genes are important for *B. burgdorferi* survival in the mouse [26].

Linear plasmid 36 is known to be critical for *B. burgdorferi* survival in the mouse; however, the genes on lp36 that contribute to this requirement have not been fully characterized [16]. The recently published comprehensive STM study suggests that many of the genes encoded on lp36 participate in *B. burgdorferi* infectivity [16, 26].

BbIVET identified gene *bbk46* on lp36. We found that *bbk46* was expressed both *in vitro* and *in vivo.* However, *bbk46* expression was dramatically induced in spirochetes isolated from infected mouse tissues as compared to spirochetes grown *in vitro*, suggesting a possible role for this gene in *B. burgdorferi* infectivity. Moreover, consistent with lack of identification of *bbk46* as an RpoS-regulated genes in previous studies of the RpoS regulon [103, 147],control of *bbk46* expression was found to be RpoS-independent under *in vitro* growth conditions that typically induce expression of *rpoS* regulated genes [52, 58, 76, 99]. These findings highlight the power and uniqueness of the IVET-based approach for identification of *B. burgdorferi in vivo*-expressed genes, which might not be discovered using other genome-wide gene expression methods. Surprisingly, BBK46 protein was not detected in spirochetes expressing FLAG epitope tagged *bbk46* under the control of the putative native promoter or the constitutive *flaB* promoter. Moreover, sera from *B. burgdorferi* infected mice were non-immunoreactive against recombinant BBK46 protein. In support of these data, no peptide corresponding to BBK46 has been detected in genome-wide proteome analysis of *B. burgdorferi* under different environmental conditions [148]. Our findings suggest that despite high gene expression, the encoded BBK46 protein is produced at low levels in the spirochete and/or BBK46 is rapidly turned over in the cell. Alternatively, *bbk46* may function as an RNA. The molecular nature of the functional product of *bbk46* is currently under investigation.

Deletion of *bbk46* from low-passage, infectious *B. burgdorferi* resulted in no observable *in vitro* growth defect. Immunocompetent mice needle inoculated with spirochetes lacking *bbk46* were found to be seropositive for *B. burgdorferi* antibodies three weeks post-infection, although the serological responses appeared to be slightly diminished relative to those of mice infected with the wild-type and complemented clones. Surprisingly, however, no live spirochetes were reisolated from all tissues examined from the mutant infected mice at this same time point. Conversely, all mice infected with the wild-type or complemented clone were both seropositive and reisolation positive. Furthermore, *bbk46* was not required for spirochete survival in immunocompromised mice. These data indicate that *bbk46* is dispensable for the initial stages of *B. burgdorferi* murine infection but this gene is essential for *B. burgdorferi* persistence in mouse tissues and may contribute to a mechanism of spirochete evasion of host-acquired immune defenses.

B. burgdorferi survival in the mammalian host requires diverse mechanisms that allow the spirochete to resist and evade the host's immune responses. However, the genetic components of these important properties of the pathogen have yet to be well defined. Here we demonstrate that spirochetes lacking *bbk46* establish an initial infection and are seroreactive but are unable to persist in murine tissues following host antibody production. To our knowledge a similar phenotype has been documented for only two other *B. burgdorferi* genes, the lp28-1 encoded *vls* antigenic variation locus [14, 18, 86, 90, 91] and the chromosomally encoded *lmp*-1(*bb0210*) gene [89].

Moreover, analogous to the *bbk46* mutant, the phenotypes of spirochetes lacking a functional *vls* locus as well as spirochetes lacking *lmp-1* have been shown to be dependent on the host immune response as these mutants demonstrate wild-type survival under immune privileged growth conditions and in immunocompromised mice [14, 18, 89-91]. Although it is clear that the antigenic switching mechanism conferred by the *vls* locus is essential for *B. burgdorferi* persistence in the host [90, 91], the precise mechanism of *vls*-dependent immune evasion remains unknown. Similarly the mechanism of *lmp-1*-dependent protection of *B. burgdorferi* against the host's humoral immune response is unknown [89]. VlsE and Lmp-1 are highly antigenic proteins present on the outer surface of the spirochete [85, 89, 149]. The BBK46 open reading frame appears to encode a lipoprotein with a predicted signal sequence for outer surface localization; however, recombinant BBK46 protein produced in *E. coli* was not found to be seroreactive when analyzed by immunoblot using immune sera collected from mice infected with wild-type *B. burgdorferi*. Future studies are focused on elucidation of the role of *bbk46* in the pathogenesis of *B. burgdorferi*.

In conclusion, we have developed and applied the IVET technology to *B. burgdorferi* to identify spirochete genes expressed during mammalian infection. This represents the first use of this system in *B. burgdorferi*. The power of this system was validated by identification of a subset of genes that have been demonstrated previously to be upregulated *in vivo*. Furthermore, IVET identified *bbk46*, a novel, uncharacterized gene located on essential virulence plasmid lp36. We have presented evidence that

bbk46 is highly upregulated during *B. burgdorferi* murine infection and is critical for the spirochete's ability to persistently infect immunocompetent mouse tissues. Further analysis of the molecular mechanism of *bbk46*-promoted survival, as well as identification and characterization of other putative virulence factors identified by BbIVET, will contribute to advancing understanding of *in vivo* persistence and pathogenicity of *B. burgdorferi*.

2.4: Materials and Methods

2.4.1: Ethics Statement.

The University of Central Florida is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and were reviewed and approved by the University of Central Florida Institutional Animal Care and Use Committee (Protocol numbers 09-38 and 12-42).

2.4.2: Bacteria Clones And Growth Conditions.

All *B. burgdorferi* clones used were derived from clone B31 A3. Clone A3 68-1, which lacks lp25 and lp56 [125] was used for the pBbIVET library. The B31 A3 wild-type and *rpoS*::*kan B. burgdorferi* clones [150] were used for gene expression experiments. All low-passage *B. burgdorferi* mutant and complemented clones generated herein were derived from infectious clone A3-68ΔBBE02, which lacks cp9, lp56 and gene *bbe02* on lp25 [140]. *B. burgdorferi* was grown in liquid Barbour-Stoenner-Kelly (BSK) II medium

supplemented with gelatin and 6% rabbit serum [151] and plated in solid BSK medium as previously described [152, 153]. All spirochete cultures were grown at 35^{o}C supplemented with 2.5% CO2. Kanamycin was used at 200 µg/ml, streptomycin was used at 50 µg/ml and gentamicin was used at 40 µg/ml, when appropriate. All cloning steps were carried out using DH5α *E. coli*, which were grown in LB broth or on LB agar plates containing 50 µg/ml kanamycin, 300 µg/ml spectinomycin or 10 µg/ml gentamicin.

2.4.3: Generation of the pBbIVET Plasmid.

The promoterless *pncA* gene was amplified from *B. burgdorferi* B31 genomic DNA using primers 1 and 2 (Table 7) and Taq DNA polymerase (New England Biolabs). The EcoRI/XbaI-digested *pncA* fragment was cloned into EcoRI/XbaI-linearized plasmid pBSV2*TT [117], creating plasmid pBbIVET. The *in vivo*-expressed *ospC* promoter with EcoRI ends was amplified from *B. burgdorferi* B31 genomic DNA using primers 3 and 4 (Table 7) and cloned into the EcoRI-cut, Antarctic phosphatase-treated (New England Biolabs) pBbIVET plasmid in front of the promoterless *pncA* gene, resulting in plasmid pBbIVET *ospC*$_{p}$. All plasmids were analyzed and verified by restriction digest and sequence analysis. The pBbIVET and pBbIVET *ospC*$_{p}$ plasmids were each transformed by electroporation into A3 68-1 [125] as described [16] and transformants selected in

solid BSK medium containing kanamycin and confirmed by PCR using primers 1 and 2 (Table 7). Total genomic DNA was prepared from PCR-positive clones and screened for the presence of the *B. burgdorferi* plasmid content [150]. The clones that retained the plasmid content of the parent clone were used in further experiments.

2.4.4: Generation Of The pBbivet Library.

Total genomic DNA was isolated from a 250 ml culture of *B. burgdorferi* B31 clone A3 grown to a density $1x10^8$ spirochetes/ml using the Qiagen genomic DNA buffer set and Genomic-tip 500/G, according to the manufacturer's protocol (Qiagen). A3 genomic DNA was partially digested with Tsp509I (New England Biolabs). The partial digests were electrophoretically separated on a 0.8% agarose gel. The 300 to 500 bp range of DNA fragments extracted and ligated in a 1:1 molar ratio with EcoRI-digested and Antarctic phosphatase-treated pBbIVET. Library ligations were electroporated into *E. coli* Top10 cells (Life Technologies) and transformants selected on LB agar containing 50 µg/ml kanamycin, resulting in approximately 30,000 independent clones. Plasmid DNA was isolated from these cells and 20 µg aliquots of the plasmid library were transformed by electroporation into *B. burgdorferi* A3 68-1, as previously described [153]. One fifth of each transformation was plated on solid BSK medium containing kanamycin. *B. burgdorferi* pBbIVET colonies were verified to contain *B. burgdorferi* DNA fragments by PCR using primers 5 and 6 (Table 7) and the number of transformants recovered quantitated. The approximately 15,000 *B. burgdorferi* clones

recovered over 40 transformations were stored in aliquots of pools of approximately 100 BbIVET clones each in 25% glycerol at -80°C.

2.4.5: Selection Of *B. burgdorferi* Clones Having *In Vivo* Expressed DNA Fragments.

Each BbIVET pool (~100 clones) was grown in 10 ml of fresh BSKII medium to a density of $1x10^8$ spirochetes/ml. In groups of approximately 20 animals, 144 6-8 week old C3H/HeN female mice were each inoculated (80% intraperitoneal and 20% subcutaneous) with a dose $1x10^6$ spirochetes of a unique pool of ~100 BbIVET clones, under the assumption that each clone was present at dose $1x10^4$ spirochetes. A fraction of each inoculum was plated on solid BSK medium and colonies screened for the presence of virulence plasmid lp28-1. Three weeks post inoculation, spirochetes were reisolated from ear, heart, bladder and joint tissues in 10 ml BSKII medium containing 20 µg/ml phosphomycin (Sigma), 50 µg/ml rifampicin (Sigma) and 2.5 mg/ml amphotericin B (Sigma) in 0.2% dimethyl sulfoxide (Sigma). Total genomic DNA was isolated from each spirochete cultures using the Wizard genomic DNA purification kit (Promega) and transformed into chemically competent *E. coli* DH5α cells and colonies selected on LB agar containing kanamycin to recover the pBbIVET plasmids. Twenty four transformants were chosen at random from each plasmid rescue and colony PCR performed using primers 5 and 6 (Table 7) to amplify the *in vivo*-expressed DNA fragment. PCR products were subsequently digested with a cocktail of restriction enzymes (DraI, SspI and AseI) and visualized on a 1% agarose gel. Approximately 14,000 *E. coli* clones were analyzed in this manner. All unique BbIVET fragments, as

determined by the restriction digest pattern (Fig. 4), were analyzed by direct sequencing of the PCR product using primer 5 (Table 7). Each individual sequence was identified by blastn analysis and mapped to its location in the *B. burgdorferi* B31 genome.

2.4.6 Deletion of *bbk46.*

We used a PCR-based overlap extension strategy to delete the *bbk46* gene. A spectinomycin/ streptomycin resistance cassette, *flaBp-aadA* [154] with blunt ends, was amplified from genomic DNA isolated from clone Δ*guaAB* [125] using Phusion High-fidelity DNA polymerase (Thermo Scientific) and primers 11 and 12 (Table 7). The 500 bp flanking region upstream of the *bbk46* ORF was amplified from the *B. burgdorferi* B31 clone A3 genomic DNA using the Phusion High-fidelity DNA polymerase and primers 7 and 8 (Table 7). This introduced a 25 bp sequence at the 3' end of this fragment that was complementary to the 5' end of the *flaBp-aadA* cassette. Similarly, the 500 bp flanking region downstream of the *bbk46* ORF was amplified using the primers 9 and 10 (Table 7), which introduced a 5' sequence of 30 bp that was complementary to the 3' end of the resistance cassette. The PCR products from the above 3 reactions were mixed in equal volumes and used as a template for a fourth amplification reaction using Phusion High-fidelity DNA polymerase and primers 7 and 10 (Table 7) in order to generate a product containing the resistance cassette flanked by the 500 bp sequences upstream and downstream of the *bbk46* ORF. This product was ligated with linear pCR-Blunt using a Zero Blunt PCR cloning Kit (Life technologies), yielding the allelic exchange plasmid pCR-Blunt-Δ*bbk46-flaBp-aadA*. *B.*

burgdorferi A3-68ΔBBE02 was transformed with 20 µg of pCR-Blunt-Δ*bbk46-flaBp-aadA* purified from *E. coli* as previously described [16]. Streptomycin-resistant colonies were confirmed to be true transformants by PCR using primer pairs 7 and 10 and 11 and 12 (Table 7). Positive Δ*bbk46-flaBp-aadA* clones were screened with a panel of primers [150] for the presence of all of the *B. burgdorferi* plasmids of the parent A3-68ΔBBE02 clone [140], and a single clone was selected for further experiments.

2.4.7: Complementation Of Δ*bbk46* Mutant.

A PCR-based overlap extension strategy was used to create a DNA fragment encompassing the *bbk46* gene and putative upstream promoter sequence with the introduction of a FLAG epitope tag immediately upstream of the putative premature stop codon and a cMyc epitope tag immediately upstream of the downstream stop codon. This was done by using Phusion High-fidelity DNA polymerase (New England Biolabs) and the primers pairs 13 and 14, 15 and 16, and 17 and 18 (Table 7). A KpnI restriction site was introduced at the 5' end of this fragment and a SalI site at the 3' end. The KpnI+SalI-digested PCR product was ligated into KpnI+ SalI-digested *B. burgdorferi* shuttle vector pBSV2G [155] and cloned in *E. coli*. The pBSV2G *bbk46*$_p$-*bbk46-FLAG-cMyc* plasmid structure and sequence were confirmed by restriction digest and DNA sequence analysis. In addition, a 400 bp DNA fragment encompassing the *flaB* promoter with KpnI and BamHI ends was amplified from B31 A3 genomic DNA using primers 27 and 28 (Table 7). The KpnI+BamHI-digested PCR product was ligated into KpnI+ BamHI-digested *B. burgdorferi* shuttle vector pBSV2G [155]. The *bbk46-FLAG-*

cMyc gene without the putative *bk46* promoter sequence and with BamHI and SalI ends was amplified from pBSV2G *bbk46*$_p$-*bbk46-FLAG-cMyc* plasmid DNA using Phusion High-fidelity DNA polymerase (New England Biolabs) and primers 29 and 18 (Table 7). The BamHI+SalI-digested PCR product was ligated into BamHI+SalI-digested pBSV2G*flaB*$_p$ and cloned in *E. coli*. The pBSV2G *flaB*$_p$-*bbk46-FLAG-cMyc* plasmid structure and sequence were confirmed by restriction digest and DNA sequence analysis. The ∆*bbk46* mutant was transformed with 20 µg of pBSV2G *bbk46*$_p$-*bbk46-FLAG-cMyc,* pBSV2G *flaB*$_p$-*bbk46-FLAG-cMyc* or pBSV2G alone isolated from *E. coli* and positive transformants selected as previously described [16, 74]. The clones that retained the *B. burgdorferi* plasmid content of the parent clone were selected for use in further experiments.

2.4.8: Immunoblot Analysis of BBK46-FLAG-cMyc.

Production of the BBK46-FLAG-cMyc protein was examined in both *E. coli* and *B. burgdorferi* carrying pBSV2G *bbk46*$_p$-*bbk46-FLAG-cMyc* or pBSV2G *flaB*$_p$-*bbk46-FLAG-cMyc*. Total *E. coli* protein lysates were prepared from 2x10^9 cells harvested following overnight growth in LB medium at 37°C with aeration. *E. coli* cells were resuspended and lysed in 200 µl B-PER protein extraction reagent (Pierce), followed by the addition of 200 µl 2x Laemmli sample buffer plus 2-mercaptoethanol (Bio-rad). Total *B. burgdorferi* protein lysates were prepared from 2x10^9 spirochetes harvested at mid-log phase. The spirochetes were washed twice in 1 ml cold HN buffer (50 mM Hepes, 50 mM NaCl, pH 7.4) and lysed in 200 µl B-PER protein extraction reagent (Thermo

Scientific), followed by the addition of 200 µl 2x Laemmli sample buffer plus 2-mercaptoethanol (Bio-rad). 30 ml of each protein lysate (~1.5x10^8 cells) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. 300 ng of PncA-FLAG [117] and GST-BmpA-cMyc [135] proteins served as positive controls. Immunoblot analysis was performed using anti-FLAG monoclonal primary antibody (Genscript) diluted 1:500 in Tris-buffered saline, pH 7.4 and 0.5% Tween20 (TBST) and goat anti-mouse IgG+IgM-HRP secondary antibody (EMD Millipore) diluted 1:10,000 in TBST and the signal detected using SuperSignal West Pico chemluminescent substrate kit (Thermo Scientific). The membrane was then stripped using 0.2M NaOH, reblocked using 5% skim milk in TBST and probed with anti-cMyc primary antibody (Genscript) diluted 1:500 in TBST and goat anti-mouse IgG+IgM-HRP (EMD Millipore) and visualized as described above.

2.4.9: Cloning, Purification and Seroreactivity Analysis of rGST-BBK46.

An in-frame glutathinone *S*-transferase (GST)-BBK46 fusion protein lacking the putative BBK46 signal sequence was generated using primers 30 and 31 (Table 5) and purified, as previously described [135]. Approximately 1 µg of GST-BBK46 was separated by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by immunoblot for seroreactivity using immune serum collected 3 weeks post inoculation from mice infected with wild-type *B. burgdorferi* as previously described [135]. Controls included 1 µg of GST alone and total protein lysates generated from BL21 *E. coli*, *B. burgdorferi* B31 A3 and *E. coli* expressing *B. burgdorferi bmpA* [16] prepared as

described above. The membrane was stripped as described above and reprobed with anti-GST primary monoclonal antibody (EMD Millipore) diluted 1:1000 in TBST and goat anti-mouse IgG+IgM-HRP (EMD Millipore) and visualized as described above.

2.4.10: *In vitro* growth analysis.

Wild-type (A3-68ΔBBE02), Δ*bbk46*/vector and Δ*bbk46/bbk46*$^{+}$ spirochetes were inoculated in triplicate at a density of 1x10^{5} spirochetes/ml in 5 ml of BSK II medium. Spirochete densities were determined every 24 hours under dark field microscopy using a Petroff-Hausser chamber over the course of 96 hours.

2.4.11: RNA Isolation From *In Vitro* Grown Spirochetes.

To obtain *in vitro* grown log phase spirochetes, wild-type (B31 A3) spirochetes were grown in triplicate in 5 ml of BSKII medium pH 7.5 at 35°C to a density of 3x10^{7} spirochetes/ml. To obtain stationary phase, temperature-shifted spirochetes, wild-type (B31 A3) spirochetes were grown in triplicate in 5 ml of BSKII medium pH 7.5 at 35°C to a density of 3x10^{7} spirochetes/ml, transferred to 25°C for 48 hours and then returned to 35°C for an additional 24-36 hours to a density of 2x10^{8} spirochetes/ml. A total of 1x10^{7} spirochetes were harvested from each culture and total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA was resuspended in 100µl DEPC-treated dH_2O. RNA was treated with TURBO DNA-free (Life Technologies) to remove any contaminating genomic DNA. 1 µl of Riboguard

(40U/µl) RNAse inhibitor (Epicentre) was added to all samples and RNA stored at -80°C.

2.4.12: RNA Isolation From Infected Mouse Tissue.

B. burgdorferi-infected mouse bladders (see mouse infection experiments below) were manually macerated on ice using sterile scalpels and transferred to a 2 ml sterile tube containing lysing Matrix D (MP Biomedicals). 1ml of RNA pro solution (FastRNA Pro Green kit, MP Biomedicals) was added to each sample on ice. Tissues were homogenized using a PowerGen High-Throughput Homogenizer (Fisher Scientific) following six cycles of beating for 45 sec and 2 minute incubations on ice. Samples were centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper aqueous phase was transferred to new tubes and incubated for 5 minutes at room temperature. 500 µl of 1-bromo-3-chloropropane (Sigma Aldrich) and 45 µl of 5M sodium acetate were added to each sample and samples were incubated for an additional 5 minutes at room temperature. Samples were centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper aqueous phase was transferred to new tubes and RNA precipitated with the addition of 500 µl of absolute ethanol and 1 µl GlycoBlue (Life technologies). RNA was pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C. RNA was washed with 70% ethanol in DEPC-treated dH_20 and resuspended in 100 µl DEPC-treated dH_20. RNA was treated

with TURBO DNA-free (Life Technologies) to remove any contaminating genomic DNA. 1 µl Riboguard (40U/µl) RNAse inhibitor (Epicentre) was added to all samples and RNA stored at -80°C.

2.4.13: Gene Expression Analysis.

cDNA was synthesized from 1.0 µg of each RNA sample using the iScript cDNA synthesis kit (Bio-Rad) with random primers according to the manufacturer's instructions. Parallel cDNA reactions were carried out in the absence of reverse transcriptase. Real-time quantitative PCR (qPCR) reactions were prepared using 1 µg of each cDNA and iQ SYBR Green Supermix (Bio-Rad). Using an Applied Biosystems 7500 instrument, samples were assayed for the *flaB*, *recA*, *ospC* and *bbk46* transcripts using primers pairs 21 and 22, 23 and 24, 25 and 26, and 19 and 20, respectively (Table 5). Standard curves were generated for each gene target using 100 ng, 10 ng, 1.0 ng, 0.1 ng, and 0.01 ng of B31 A3 *B. burgdorferi* genomic DNA and the amount of each gene transcript calculated. The *recA* transcript was used as the endogenous reference to which the transcripts of the other genes were normalized. The *bbk46* primers were confirmed to be specific for their gene target. Three biological replicate samples were analyzed in triplicate and normalized to *recA* mRNA. The data were reported as the average gene transcript/*recA* transcript for each sample. The

amplification of samples lacking reverse transcriptase was similar to that of the no-template control.

2.4.14: Mouse Infection Experiments.

Unless otherwise noted, groups of 6-8 week old C3H/HeN female mice (Harlan) were used for all experiments.

2.4.14.1: ID_{50} Analysis Of Mammalian-Adapted Spirochetes.

A single C3H/HeN SCID mouse (Harlan) was inoculated with $2x10^6$ *B. burgdorferi* B31 A3. Two weeks post infection the infected blood was harvested and used to inoculate groups of six wild-type C3H/HeN (Harlan) mice with 100 µl of undiluted infected blood or 100 µl of infected blood diluted 1:10 or 1:100 in BSK-H medium. The number of live spirochetes in the infected blood and therefore the actual spirochete dose in the inoculum was determined by plating the blood in solid BSK medium and quantitating the number of colony forming units (Table 3). In addition, groups of six wild-type C3H/HeN mice (Harlan) were inoculated with $5x10^4$, $5x10^3$, $5x10^2$, $5x10^1$ or $5x10^0$, *in vitro* grown spirochetes at mid-log phase. The *in vitro* grown spirochetes were confirmed to harbor all plasmids required for infectivity [150].

2.4.14.2: Functional Validation Of The BbIVET System.

Groups of 6 mice were needle inoculated as described [74] with $1x10^4$ spirochetes of clone A3 68-1 carrying pBbIVET or pBbIVET-$ospC_p$. Mouse infection

was assessed 3 weeks post inoculation by reisolation of spirochetes from ear, bladder and joint tissues as previously described [67, 150].

2.4.14.3: Gene Expression Studies.

Three mice were needle-inoculated intradermally under the skin of the upper back with *B. burgdorferi* clone B31 A3 at a dose of $1x10^5$ spirochetes. Three weeks post inoculation mouse infection was determined by serology [67, 150] and bladders harvested for RNA isolation.

2.4.14.4: bbk46 *Mutant Infectivity Studies.*

Groups of five mice were needle-inoculated, intradermally under the skin of the upper back, with *B. burgdorferi* clones wild-type (A3-68ΔBBE02 [140]), Δ*bbk46*/vector or Δ*bbk46/bbk46*$^+$ at a dose of $1x10^4$ spirochetes. The number of spirochetes inoculated into mice was determined using a Petroff-Hausser counting chamber and verified by colony-forming unit (cfu) counts in solid BSK medium. Twelve colonies per inoculum were verified by PCR for the presence of the virulence plasmids lp25, lp28-1 and lp36 in at least 90% of the individuals in the population. Further, total plasmid content of each inoculum was confirmed to be as expected [16, 74, 150]. Mouse infection was assessed three weeks post inoculation by serology using total *B. burgdorferi* lysate, as previously described [16] and 300 ng recombinant GST-OspC [135], as previously described [156]. Spirochetes were reisolated from the inoculation site, ear, bladder and joint tissues, as previously described [16].

Groups of five immunodeficient C3SnSmn.CB17-*Prkdc*$^{<scid>/}$J (Jackson labs stock 001131) were needle-inoculated with *B. burgdorferi* clones wild-type (A368∆BBE02 [140]), ∆*bbk46*/vector or ∆*bbk46/bbk46*$^{+}$ at a dose of 1x10^{4} spirochetes. 80% of the inoculum was delivered intraperitoneal and 20% of the inoculum was delivered subcutaneous. The inoculum cultures were analyzed as described above. Mouse infection was assessed three weeks post inoculation by reisolation of spirochetes from ear, bladder and joint tissues [16].

Table 7: List of primers used in this study

Primer number	Designation	Sequence (5' – 3')[a]
1	pncA 5' EcoRI A	cggaattcatgGCACTTATTTTAATAGATATAC
2	pncA 3' XbaI	gctctagaTTATATATTAAGCTTACTTTGGCTG
3	ospC prom 5' EcoRI	cggaattcTTCTTTTTCATTAATTTGTGCCTCC
4	ospC prom 3' EcoRI	cggaattcTTAATTTTAGCATATTTGGCTTTGCTTATGTCG
5	pUC18R BSV2	AGCGGATAACAATTTCACACAG
6	pncA prom 3' seq	ACTGTTAGATACTGGCAAAGTGCC
7	bbk46Fup500	GTTCTTTTATGGAGCAAGCAACTAA
8	bbk46Rup500	CGGAAGCCACAAGAGGCGACAGACACTATCTTAGTACCTCTTCTTAGAATCTG
9	bbk46Fdown500	GGCGAGATCACCAAGGTAGTCGGCAAATAAATAATACTAATCTTAGATAGCTCAGCTTT
10	bbk46Rdown500	CTAGCTTCACTAGTTTCCCTAGA
11	flaBpaadA F	TGTCTGTCGCCTCTTGTG
12	flaBpaadA R	TTATTTGCCGACTACCTTGGTG
13	K465'kpn1fwd	cggggtaccCTTCCAGTGTAGGCTTTAGTTT
14	K463'FLAGrev	TTAtttatcatcatcatctttataatcTGCCTCAACTGCCTTTCTC
15	K465'FLAGfwd	gattataaagatgatgatgataaaTAAAATGCTTCAAAGGAAAATTATGAATGG
16	K463'C-mycSalIrev	acgcgtcgacTTAcagatcttcttcagaaataagtttttgttcATAAGCAGCTTCATATGCTTTATTT
17	K465'PCR3fwd	CGGGGTACCCTTCCAGTGTAG
18	K463'PCR3rev	ACGCGTCGACTTACAGATCTTCTTCAGAAATA
19	Lp362908F	AGCATTATTTGTACTTCTAGGC
20	Lp3629013R	ACATACTAGACAACAACAAGTC
21	flaBF3	GCATTAACGCTGCTAATCTTAG
22	flaBR3	GCATTAATCTTACCAGAAACTCC
23	recA F	AATAAGGATGAGGATTGGTG
24	recA R	GAACCTCAAGTCTAAGAGATG
25	ospC1 F	ACGGATTCTAATGCGGTTTTACCT
26	ospC1 R	CAATAGCTTTAGCAGCAATTTCATCT
27	flaBp 5' KpnI	gggggtaccTGTCTGTCGCCTCTTGTGGCT
28	flaBp 3' BamHI	gggggatccGATTGATAATCATATATCATTCCT
29	bbk46+S 5' BamHIF	cgggatccATGAATTTAATTGCTAAATTATTTATTTTATCCAC
30	bbk46-S 5' BamHIF	cgggatcc ATGTGTAACCTATATGATAATCTTGCAGAC
31	bbk46 3' XhoIR	ccgctcgag TTAATAAGCAGCTTCATATGCTTTATTTAG

[a]Lowercase indicates all non-*B. burgdorferi* sequence.

CHAPTER THREE: *B. BURGDORFERI* SURVIVAL IN THE MAMMALIAN HOST IS MODULATED BY *bbk46* VIA A REGULATORY PATHWAY CONTROLLING *vlsE* EXPRESSION.

3.1 Introduction

Borrelia burgdorferi is the etiologic agent of Lyme disease, which is considered to be the most prevalent arthropod-borne bacterial disease in the world [157]. These spirochetes gain entry into the mammalian host via the bite of infected *Ixodes scapularis* ticks [6]. The pathogen survives efficiently in these two disparate conditions in the presence of varying nutritional availabilities, changes in surrounding temperature and chemistry and even hostile immune attacks through coordinated differential gene expression to best suit its current requirements. Variation in the expression of surface proteins is believed to help the spirochetes evade the host immune system and establish a long term infection [34-51]. As a result, the elucidation of the mechanisms involved in the expression of surface antigens will contribute to an increased understanding of the pathogenesis of this disease and may identify novel candidates for drug targets and vaccine development.

In our previous study, *bbk46*, a gene located on *in vivo* essential linear plasmid 36 [16], was identified via an *in vivo* expression technology (IVET)-based screen to be expressed during mouse infection [158]. The deletion of this gene resulted in an intermediate infectious phenotype in immunocompetent mice where the spirochetes lacking the gene were seroreactive but no live spirochetes were recovered from any of

the mouse tissues after three weeks post-inoculation. In immunocompromised mice we found that there was no difference between the survival of the mutant and the wild-type spirochetes. These data indicated that *bbk46* plays an important role in the survival of spirochetes within the murine host resulting in the establishment of a stable infection. Despite the fact that the *B. burgdorferi* clone B31 *bbk46* gene has a premature stop codon in comparison with its orthologs in other *B. burgdorferi* isolates, transcription was detected under *in vitro* and *in vivo* growth conditions [158]. This gene is suggested to be a member of paralogous gene family 75 on lp36, which also includes *bbk45, bbk48* and *bbk50*, all of which are predicted to be surface proteins and some of which have been found to have antigenic properties [7, 8, 132, 158]. However, BBK46 is non-immunogenic and production of BBK46 protein not been detected *in vitro* [158]. Interestingly the phenotype of ∆*bbk46* mutant spirochetes is similar to what has been described for spirochetes lacking lp28-1 and more specifically the lp28-1 encoded gene *vlsE* [13, 14, 18, 85, 86]. The *vlsE* gene is required for antigenic variation due to random recombination between various intragenic cassettes resulting in protein products having varying sequences. This mechanism of recombination has been implicated in the ability of the spirochete to evade the adaptive immune response of the host and has been shown to be essential for long term infection in mice [86, 90, 92, 159, 160]. Like spirochetes lacking *vlsE* [86], the ∆*bbk46* mutants survived until 21 days post inoculation within immunocompromised mice but not immunocompetent mice [158].

These data suggest that this gene may be involved in a mechanism of immune response evasion used by the spirochetes.

We now demonstrate that spirochetes lacking *bbk46* survive up to week 2 post inoculation and are capable of migrating to tissues away from the site of inoculation; however, they are eliminated from the mammalian host by week 3. Our data indicate that the deletion of *bbk46* results in the loss of *vlsE* gene expression and that *bbk46* is involved in this regulatory mechanism via its RNA. We found that even though *vlsE* expression was inhibited in the absence of *bbk46* alone, the loss of the entire lp36 plasmid, which encodes *bbk46*, resulted in no change in *vlsE* expression, suggesting the presence of a gene on lp36 that represses *vlsE* expression in the absence of *bbk46*. Based upon these findings, we have proposed a novel mechanism of regulation of the *vlsE* gene expression by *bbk46* via an lp36 encoded repressor gene.

3.2 Results

3.2.1: Spirochetes Lacking *bbk46* Are Reisolated From Infected Mouse Tissues At 2 Weeks Post-Inoculation But Are Cleared From The Host By Week 3.

Previously we found that the spirochetes lacking *bbk46* are unable to survive within immunocompetent mice three weeks post inoculation, although the mice were found to be seropositive for anti-*B. burgdorferi* antibodies at this same time point [158]. Moreover, the *bbk46* mutant clone demonstrated no survival defect in immunocompromised mice [158]. Together these data suggested that *bbk46* contributes

to *B. burgdorferi's* ability to evade the adaptive immune response of the host. In order to obtain a more detailed timeline of the survival and immune clearance of these spirochetes within their host we infected groups of 5 mice intra-dermally with *B. burgdorferi* clones wild-type (A3- 68ΔBBE02) [140], Δ*bbk46*/vector or Δ*bbk46/bbk46*$^{+}$ [158] at a dose of 1x10^{4} spirochetes. The infection was then assessed at 48 hours, 1 week, 2 weeks and 3 weeks post-inoculation via serology and reisolation of spirochetes from the inoculation site, ear, bladder and joint tissues (Table 8). At 48 hours post inoculation none of the mice were seropositive and live spirochetes were reisolated from inoculation site tissues of 3 out of 5 mice inoculated with wild-type spirochetes and 4 out of 5 mice inoculated with either Δ*bbk46*/vector or Δ*bbk46/bbk46*$^{+}$ clones (Table 6). At 1 week, 2 out of 5 mice inoculated with wild-type spirochetes and 3 out of 5 mice inoculated with Δ*bbk46*/vector or Δ*bbk46/bbk46*$^{+}$ clones were seropositive. At this time point spirochetes were reisolated from almost all inoculation site tissues and very few of the other tissues. At 2 weeks post inoculation 4 out of 5 mice were seropositive in all groups (Table 8). Consistent with previously defined kinetics of spirochete dissemination [113, 161], at the 2 week time point live spirochetes were reisolated from all tissues of 4 out of 5 mice inoculated with clone Δ*bbk46*/vector. This was comparable to the mice inoculated with the wild-type and Δ*bbk46/bbk46*$^{+}$ clones (Table 8). These data suggest that the Δ*bbk46* mutant spirochetes do not display a qualitative dissemination phenotype. As was expected based on our previous findings [158], at 3 weeks post-inoculation all 5 mice inoculated with clone Δ*bbk46*/vector were seropositive

for anti-*B. burgdorferi* antibodies but no live spirochetes were isolated from any of the tissues analyzed (Table 8). However, 4 out of 5 mice inoculated with wild-type and 5 out of 5 mice inoculated with the Δ*bbk46/bbk46*$^{+}$ clone were seropositive and resulted in reisolation of live spirochetes from tissues.

Table 8: Spirochetes lacking *bbk46* are reisolated from infected mouse tissues at 2 weeks post-inoculation but are cleared from the host by week 3.

48 hours post inoculation					
Clone	Serology[a]	Positive reisolation of infectious spirochetes from mouse tissues[b]			
		Inoculation site	Ear	Bladder	Joint
wild-type	0/5	3/5	0/5	0/5	0/5
Δ*bbk46*/ vector	0/5	4/5	0/5	0/5	0/5
Δ*bbk46*/ *bbk46*$^{+}$	0/5	4/5	0/5	0/5	0/5
1 week post inoculation					
Clone	Serology[a]	Positive reisolation of infectious spirochetes from mouse tissues[b]			
		Inoculation site	Ear	Bladder	Joint
wild-type	2/5	4/5	0/5	1/5	1/5
Δ*bbk46*/ vector	3/5	4/5	0/5	0/5	1/5
Δ*bbk46*/ *bbk46*$^{+}$	3/5	5/5	2/5	2/5	3/5
2 weeks post inoculation					
Clone	Serology[a]	Positive reisolation of infectious spirochetes from mouse tissues[b]			
		Inoculation site	Ear	Bladder	Joint
wild-type	4/5	3/5	4/5	5/5	4/5
Δ*bbk46*/ vector	4/5	4/5	4/5	4/5	4/5
Δ*bbk46*/ *bbk46*$^{+}$	4/5	3/5	5/5	4/5	4/5
3 weeks post inoculation					
Clone	Serology[a]	Positive reisolation of infectious spirochetes from mouse tissues[b]			
		Inoculation site	Ear	Bladder	Joint
wild-type	4/5	4/5	4/5	4/5	4/5
Δ*bbk46*/ vector	5/5	0/5	0/5	0/5	0/5
Δ*bbk46*/ *bbk46*$^{+}$	5/5	3/5	4/5	4/5	4/5

[a]Determined at each time point post-inoculation by serological response to *B. burgdorferi* proteins.
[b]Number of mice positive for spirochete reisolation/ number of mice analyzed

These data demonstrated that in the absence of *bbk46* spirochetes are able to survive within the mouse up to 2 weeks post-inoculation. The mutant spirochetes are capable of eliciting an immune response within the host and are capable of migrating to other tissues from the site of inoculation. This suggested that clones lacking *bbk46* have similar kinetics of infection to wild-type spirochetes and that the massive clearance of the mutant spirochetes does not occur until after 2 weeks post-inoculation. The spirochetes loads in the various tissues at 2 weeks post-inoculation were analyzed in order to determine if there was a quantitative difference between the number of disseminated spirochetes with and without *bbk46*. Total DNA was purified from all tissues and was analyzed using quantitative real time PCR (qPCR). The spirochete numbers were expressed as *flaB* spirochete DNA copies per 1000 *nid1* mouse DNA copies (Fig. 12).

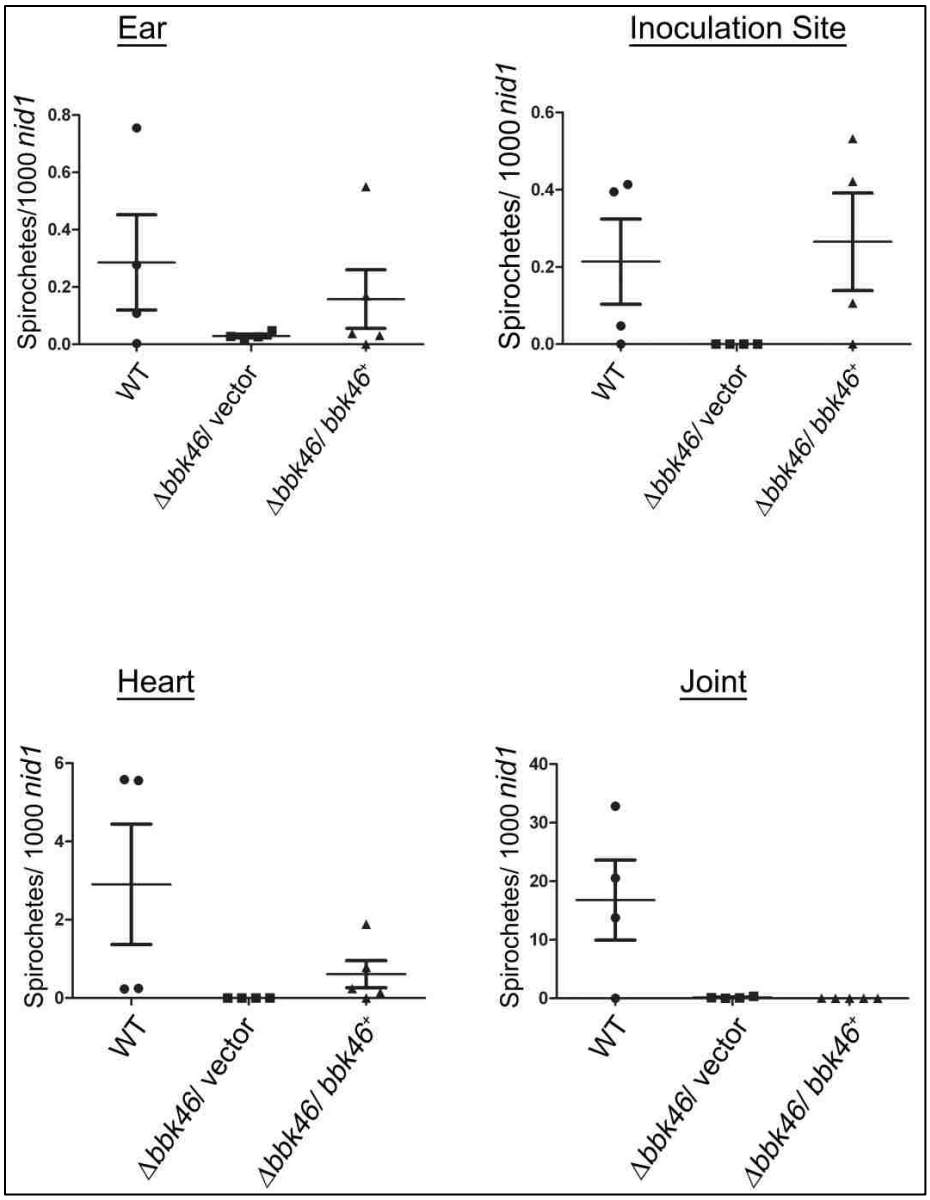

Figure: 12: Spirochete loads in harvested tissues from mice inoculated with wild-type, Δ*bbk46*/vector or Δ*bbk46/bbk46*$^{+}$ clones.

Quantitative PCR analysis of spirochete loads was carried out in mouse tissues collected from groups of 5 mice each at 2 weeks post-inoculation with clones A3-68ΔBBE02 (WT), *bbk46*::*flaB*p-*aadA*/ pBSV2G (Δ*bbk46*/ vector) and *bbk46*::*flaB*p-*aadA*/ pBSV2G-*bbk46* (Δ*bbk46*/ *bbk46*$^{+}$) at a dose of 1x10^{4} spirochetes per mouse. Spirochete numbers are expressed as *flaB* spirochete DNA copies per 1000 *nid1* mouse DNA copies (spirochetes/ 1000 *nid1*).

The qPCR data suggested that the number of Δ*bbk46*/vector spirochetes in the ear, heart and inoculation site tissues was lower than the number of wild-type and complemented spirochetes. A reduced number of the mutant and complemented clones compared to wild-type spirochetes were detected in the infected joint tissues. One caveat to interpretation of these data is that the number of spirochetes at the two week time point may be below the level of detection. Nonetheless, these data suggest that at this time point there are fewer Δ*bbk46* mutant spirochetes in infected tissues, including the site of inoculation, compared to the wild-type and complemented clones. This trend may be a result of impaired ability of the mutant to disseminate and/or to evade the host immune response.

3.2.2: Spirochetes Lacking *bbk46* Do Not Show Seroreactivity For The C6 Peptide Of VlsE In Mice.

Our mouse infectivity experiment data indicated that the loss of *bbk46* results in a defect in a mechanism of evasion of the host immune response. This led us to explore any possible changes in the antigenicity of known surface antigens of the *bbk46* mutant spirochetes. The *bbk46* gene is annotated as a member of the gene family 75 along with lp36 encoded genes *bbk45, bbk48* and *bbk50*. Although BBK46 protein is undetectable in *B. burgdorferi* [158], members of this family encode putative P37 lipoproteins [88]. The BBK50 protein has been shown previously to be antigenic [88] and BBK48 is a putative surface lipoprotein found to be present in the invasive strains of *B. burgdorferi* [162]. Therefore these putative antigens along with the C6 peptide of

VlsE, RevA, DbpA, BBK19, CRASP-2, OspA and OspC were analyzed by immunoblot using immune sera obtained from mice prior to inoculation with spirochetes and at 3 weeks post-inoculation with clones wild-type (A3-68ΔBBE02) [140], Δ*bbk46*/vector or Δ*bbk46/bbk46*$^{+}$ [158]. Strikingly, unlike mice infected with wild-type or *bbk46* complemented spirochetes, mice infected with spirochetes lacking *bbk46* did not develop antibodies against the C6 peptide of the surface antigen VlsE (Fig. 13). No immunogenic differences were detected for any of the other protein antigens.

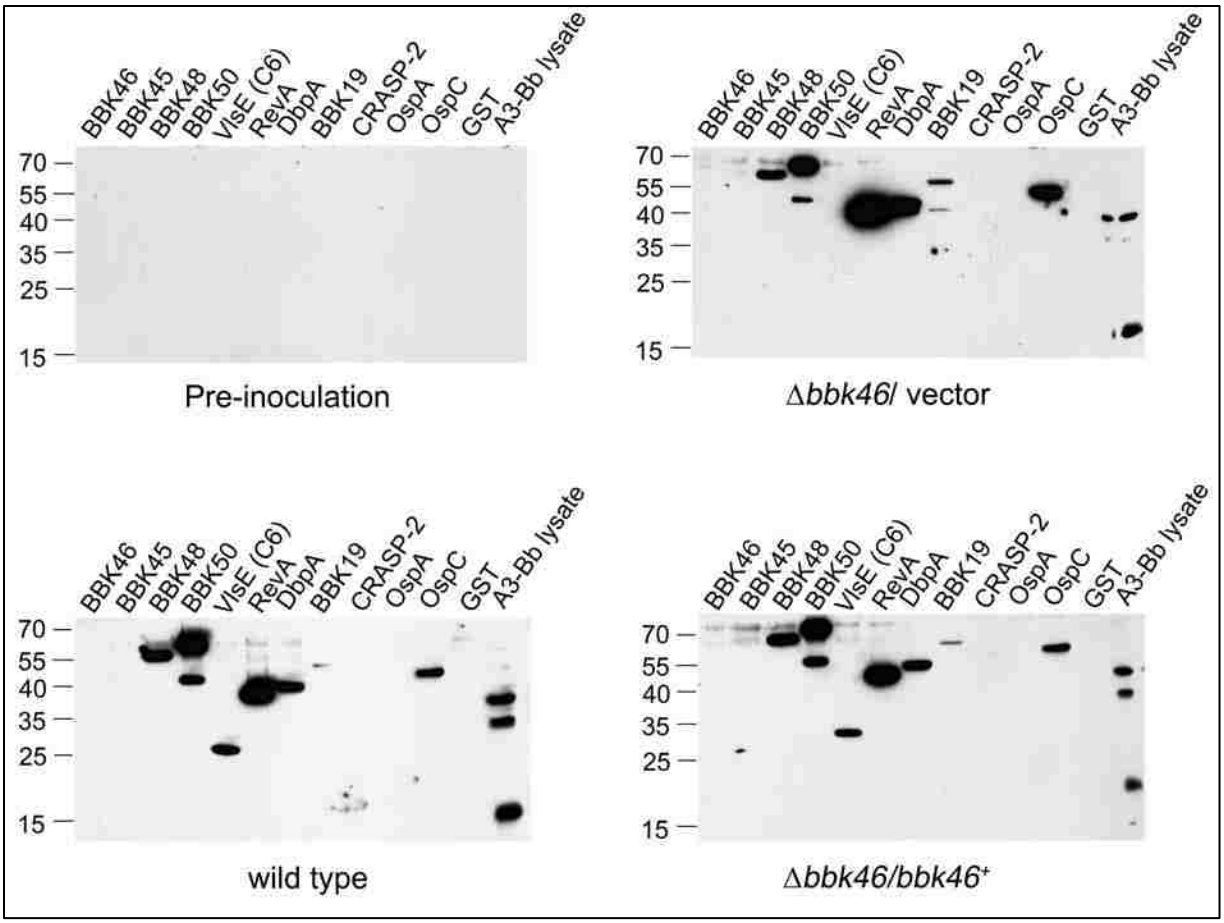

Figure 13: Spirochetes lacking *bbk46* are non-seroreactive for the C6 VlsE peptide in mice.

Immunoblot analysis of sera collected pre-inoculation and 3 weeks post-inoculation from groups of five C3H/HeN mice inoculated with clone A3-68ΔBBE02 (wild-type), *bbk46*::*flaB*p-*aadA*/ pBSV2G (Δ*bbk46*/ vector) and *bbk46*::*flaB*p-*aadA*/ pBSV2G-*bbk46* (Δ*bbk46*/ *bbk46*$^{+}$) at a dose of 1x10^{4} spirochetes per mouse. A panel of recombinant N-terminal GST fusion proteins consisting of known as well as predicted antigenic lipoproteins, was used for testing seroreactivity. The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

All sera were non-reactive to rGST-BBK46, consistent with previous findings [158], rGST-BBK45, rGST-CRASP-2 and rGST-OspA, which was comparable to the reactivity with the negative control, GST alone and the reactivity of the pre-immune sera. All post-inoculation sera were found to be reactive with rGST-BBK48, rGST-BBK50, rGST-RevA, rGST-DbpA, and rGST-OspC. All immune sera demonstrated similar levels of reactivity to the positive control *B. burgdorferi* total protein lysate.

To further evaluate the change in the seroreactivity profile of spirochetes lacking *bbk46*, we analyzed purified recombinant GST-C6 peptide via immunoblot with mouse sera obtained at weeks 1, 2 and 3 post-inoculation with clones wild-type (A3-68ΔBBE02) [140], Δ*bbk46*/vector or Δ*bbk46/bbk46*$^{+}$ [158] (Fig. 14). No reactivity to the C6 antigen was detected at all weeks in mice inoculated with the Δ*bbk46*/vector clone. Mice inoculated with the wild-type and Δ*bbk46/bbk46*$^{+}$ clones showed antibody reactions to rGST-C6 at week 3 but not at weeks 1 or 2.

Previous studies have shown that lp36 is essential for mammalian infectivity and that re-introduction of *bbk17* alone into spirochetes lacking the entire lp36 plasmid restores infectivity to that of wild-type [16]. These data appeared to be in contrast to our findings herein, that spirochetes lacking only *bbk46* are non-infectious 3 weeks post-inoculation. In an attempt to more clearly understand the mechanism of *bbk46*-dependent expression of *vlsE* and the ability of lp36^{-}/*bbk17*$^{+}$ spirochetes to infect mice despite lacking *bbk46,* we tested sera from mice inoculated with spirochetes lacking the entire lp36, except for the infectivity essential gene *bbk1*7, with purified rGST-C6

peptide. We found that these sera were positive for antibodies specific for rGST-C6 peptide (Fig 15). These data suggested that in the absence of the entire lp36 plasmid the C6 peptide of VlsE is immunoreactive on the surface of these spirochetes. Moreover, these findings indicated that spirochetes lacking *bbk46* do not display VlsE on their outer membrane surface or they display it in a manner that is not antigenic like the VlsE of wild-type or *bbk46* complemented spirochetes. We confirmed that the spirochetes reisolated from the mice infected with spirochetes lacking *bbk46* were lp28-1 and/or *vlsE* positive by PCR amplification (data not shown).

VlsE is a surface antigen implicated in protection of *B. burgdorferi* from the host adaptive immune response via a mechanism of antigenic variation [86, 90, 92, 159, 160]. Previous studies have demonstrated that loss of the linear plasmid 28-1, which contains *vlsE*, results in an infectious phenotype similar to the one we have observed in spirochetes lacking *bbk46*. The loss of seroreactivity to VlsE in the absence of *bbk46* suggested that the VlsE protein may not present on the surface of the spirochetes lacking *bbk46* and that the expression of the *vlsE* gene might be lost in the absence of *bbk46.* In addition, the uninhibited immunogenicity of VlsE seen in spirochetes lacking the entire lp36 suggested the possibility of a repressor gene on this plasmid that may downregulate the expression of *vlsE* in the absence of *bbk46* and the effect of which is not seen when the entire plasmid is absent.

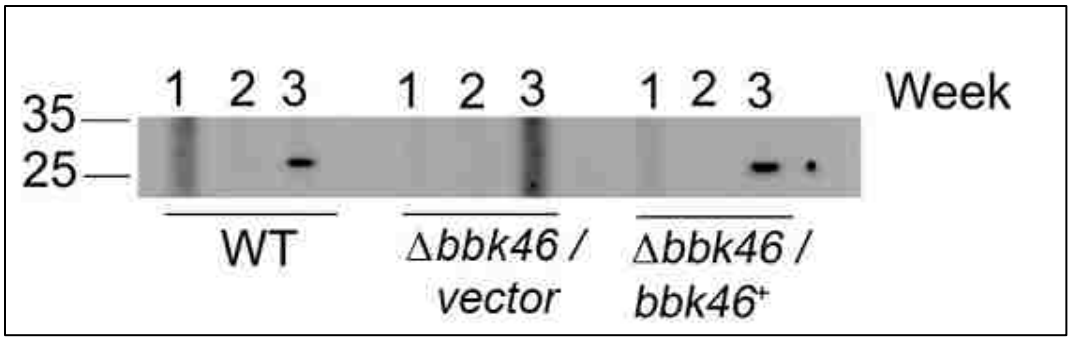

Figure 14: Spirochetes lacking *bbk46* are not seroreactive for VlsE (C6 peptide) in mice up to 3 weeks post inoculation.

Immunoblot analysis of sera collected at 1 week, 2 weeks and 3 weeks post-inoculation from groups of five C3H/HeN mice inoculated with clone A3-68ΔBBE02 (WT), *bbk46*::*flaB*p-*aadA*/ pBSV2G (Δ*bbk46*/ vector) and *bbk46*::*flaB*p-*aadA*/ pBSV2G-*bbk46* (Δ*bbk46*/ *bbk46*$^{+}$) at a dose of 1x10^{4} spirochetes per mouse. Purified, recombinant GST-C6 was used for testing seroreactivity. The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

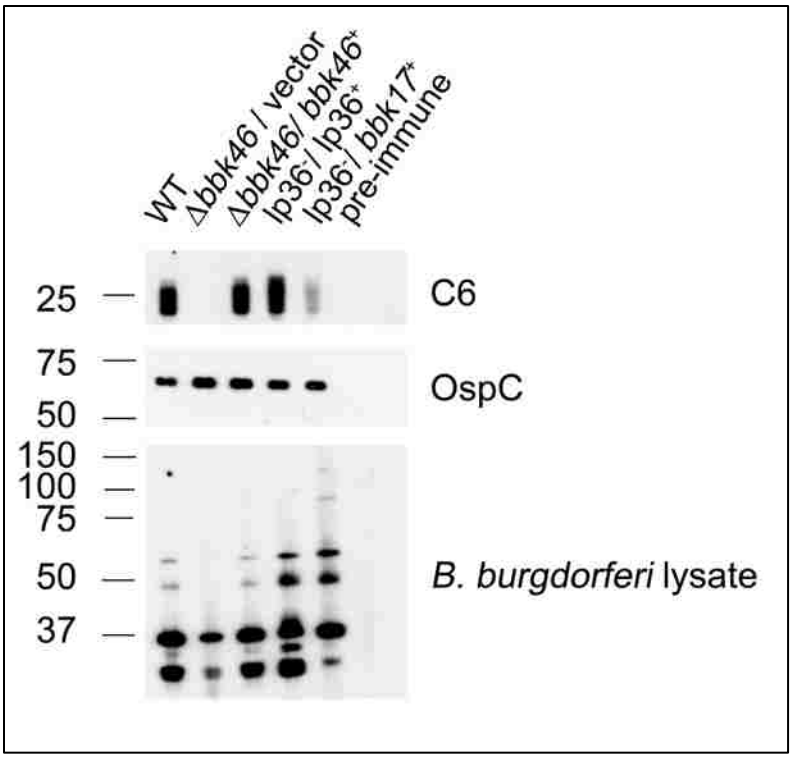

Figure 15: Spirochetes lacking the entire lp36 plasmid are seroreactive for C6 peptide of VlsE.

Immunoblot analysis of sera collected at 3 weeks post inoculation from C3H/HeN mice inoculated with clone A3-68ΔBBE02 (WT), *bbk46*::*flaB*p-*aadA*/ pBSV2G (Δ*bbk46*/ vector) and *bbk46*::*flaB*p-*aadA*/ pBSV2G-*bbk46* (Δ*bbk46*/ *bbk46*$^+$) at a dose of 1x10^4 spirochetes per mouse and A3-M9-lp36$^-$/lp36$^+$(lp36$^-$/lp36$^+$) and A3-M9- lp36$^-$/*bbk17*$^+$ (lp36$^-$/*bbk17*$^+$) at a dose of 1x10^5 per mouse. Purified, recombinant GST-C6 was used for testing seroreactivity (C6). Purified recombinant GST-OspC (OspC) and *B. burgdorferi* total protein lysate (*B. burgdorferi* lysate) were positive controls. Pre-immune serum (pre-immune) collected prior to mouse inoculation was used as the negative control. The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

3.2.3: *vlsE* Expression Is Lost In Spirochetes Lacking *bbk46* Only But Not The Entire lp36 Plasmid.

In order to test the hypothesis that *bbk46* is involved in the regulation of *vlsE* expression, we analyzed the expression of the *vlsE* gene in the Δ*bbk46* mutants via

endpoint reverse transcription (RT)-PCR and by quantitative RT-PCR. In the absence of the *bbk46* gene the *vlsE* transcript was below the level of detection by end-point RT-PCR; whereas, expression of the *vlsE* gene was restored with the complementation of the *bbk46* gene (Fig. 16A). Similarly, qRT-PCR analysis demonstrated that the level of *vlsE* expression was reduced more than 10,000 fold in the absence compared to the presence of *bbk46* (Fig. 16B). These data suggest that *vlsE* expression is regulated either directly or indirectly by *bbk46* and that the inability of the spirochetes lacking *bbk46* to mount a long-term infection in the mouse may be due to the loss of *vlsE* expression.

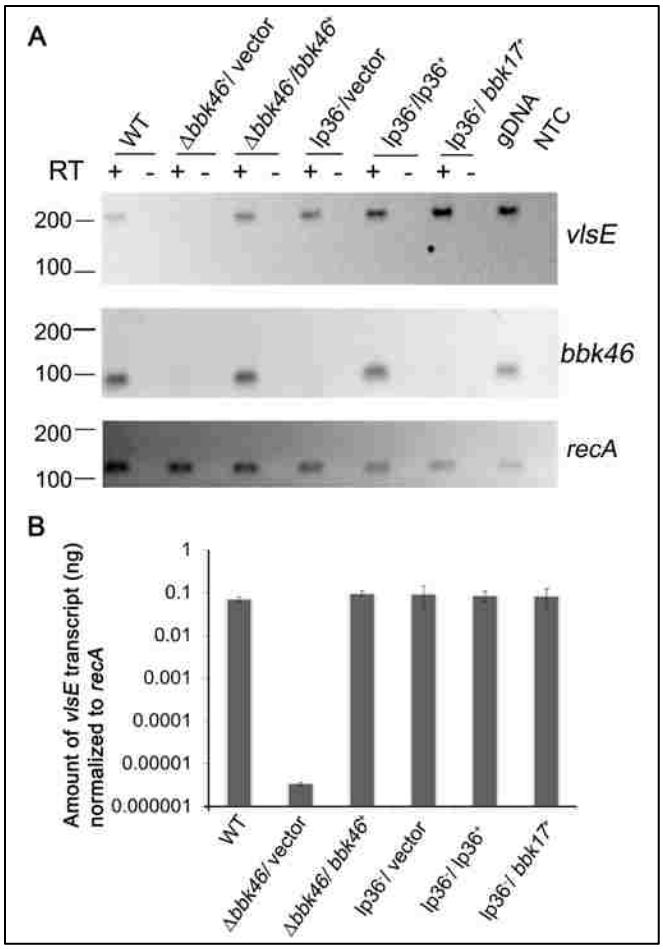

Figure 16: *vlsE* expression is significantly downregulated in spirochetes lacking *bbk46* alone.

Total RNA was isolated from log phase *in vitro* grown A3-68ΔBBE02 (WT), *bbk46*::*flaB*p-*aadA*/ pBSV2G (Δ*bbk46*/ vector) and *bbk46*::*flaB*p-*aadA*/ pBSV2G-*bbk46* (Δ*bbk46*/ *bbk46*$^{+}$), A3-M9-lp36^{-}/lp36^{+} (lp36^{-}/lp36^{+}), A3-M9-lp36^{-}/pBSV2Gv(lp36^{-}/vector) and A3-M9-lp36^{-}/*bbk17*$^{+}$ (lp36^{-}/*bbk17*$^{+}$) *B. burgdorferi*. RNA was reverse transcribed to cDNA using random hexamer primers. The expression of *vlsE*, *bbk46*, and *recA* was obtained using endpoint RT-PCR (A). *B. burgdorferi* genomic DNA was used as positive control (gDNA) and the negative control was the reaction mix with no template DNA (NTC). The positions of markers to the left of the panel depict DNA standard molecular sizes in basepairs. The expression was also quantified using quantitative reverse transcription polymerase chain reaction (qRT-PCR) (B). The mRNA levels of the *vlsE* gene transcripts were normalized to that of the constitutive *recA* gene. The data represent the average of triplicate qRT-PCR analyses. Error bars represent the standard deviation from the mean.

We also analyzed the expression of *vlsE* via end-point RT-PCR, in lp36^{-} and lp36^{-}/*bbk17*$^{+}$ clones that were grown *in vitro* (Fig. 16A). Consistent with our observation that lp36^{-}/*bbk17*$^{+}$ spirochetes produce immunoreactive VlsE protein, we detected *vlsE* transcription in spirochetes lacking lp36 as well as in the lp36^{-}/*bbk17*$^{+}$ clone. As expected, the expression of *bbk46* was not detected in the lp36^{-} and the lp36^{-}/*bbk17*$^{+}$ clones. Similar results were obtained using qRT-PCR analysis (Fig. 16B).

These data demonstrated that in the absence of the entire lp36 plasmid, *vlsE* expression is comparable to that detected in wild-type spirochetes. This expression pattern was also observed when only the *bbk17* gene on lp36 was present. In contrast, *vlsE* expression was severely down regulated in spirochetes lacking the lp36-encoded *bbk46* gene alone (Fig. 16). These findings suggested the possibility of the presence of an additional regulatory gene on lp36 with repressor function that downregulates the expression of *vlsE* in the absence of *bbk46* and that, in turn, itself maybe repressed by *bbk46*. Hence, *vlsE* expression is unhindered in wild-type as well as lp36^{-} spirochetes but is repressed in the absence of *bbk46* alone.

3.2.4: *bbk46* appears to function as an RNA.

We found that *vlsE* expression was lost in the absence of *bbk46*. We have also been unable to detect a protein product for the *bbk46* gene, although *bbk46* gene expression has been detected [158]. These findings suggested the possibility of *bbk46* functioning as an RNA molecule. In order to better understand if *bbk46* was functioning as a regulatory RNA or a regulatory protein to control *vlsE* expression we introduced a

mutation at the putative start codon of the BBK46 open reading frame in order to inhibit *bbk46* translation. The ATG coding for methionine was replaced by TTT that codes for phenylalanine to create the *bbk46*$_{ATG\text{-}TTT}$ mutant (Fig.17A). It was expected that this would result in transcription and production of mRNA from the *bbk46* ORF; however, due to the mutation at the start codon, translation would be obstructed and there would be no protein production. Since BBK46 protein production in *B. burgdorferi* has not been detected, production of the BBK46$_{ATG\text{-}TTT}$ mutant was examined in *E.coli* in order to determine if this mutation abolished translation of *bbk46.* Total protein was extracted from *E.coli* and the expression of the recombinant protein was tested via immunoblot (Fig. 17B) using anti-FLAG antibodies. In contrast to wild-type BBK46-FLAG, which was produced in *E. coli*, no BBK46$_{ATG\text{-}TTT}$-FLAG protein was detected in *E.coli* expressing the *bbk46*$_{ATG\text{-}TTT}$ mutant construct (Fig. 17A). Based on these results it appeared that the *bbk46*$_{ATG\text{-}TTT}$ mutant was unable to produce a detectable protein.

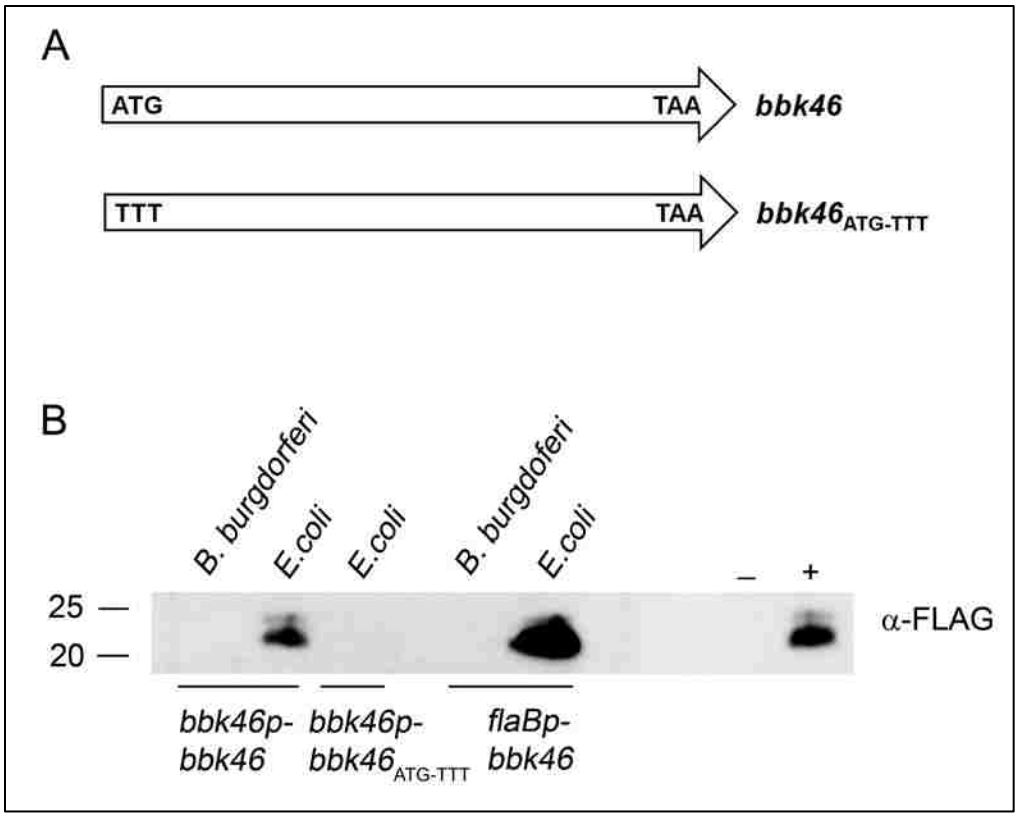

Figure 17: BBK46 protein is not detectable in *E. coli* after mutation of the start codon.

Schematic representation of the *bbk46*$_{ATG-TTT}$ mutant (A). The ATG coding for methionine was replaced by TTT that codes for phenylalanine to create the *bbk46*$_{ATG-TTT}$ mutant. Protein lysates were prepared from *B. burgdorferi* Δ*bbk46* or *E.coli* harboring either pBSV2G *bbk46p-bbk46*-FLAG-*cMyc* (*bbk46p-bbk46*) or pBSV2G *flaBp-bbk46*-FLAG-*cMyc* (*flaBp-bbk46*) and *E.coli* harboring pBSV2G *bbk46p- bbk46*$_{ATG-TTT}$-FLAG-*cMyc (bbk46p-bbk46*$_{ATG-TTT}$) and analyzed by immunoblot (B). The blot was probed with αFLAG antibodies. The positions of markers to the left of the panel depict protein standard molecular masses in kilodaltons.

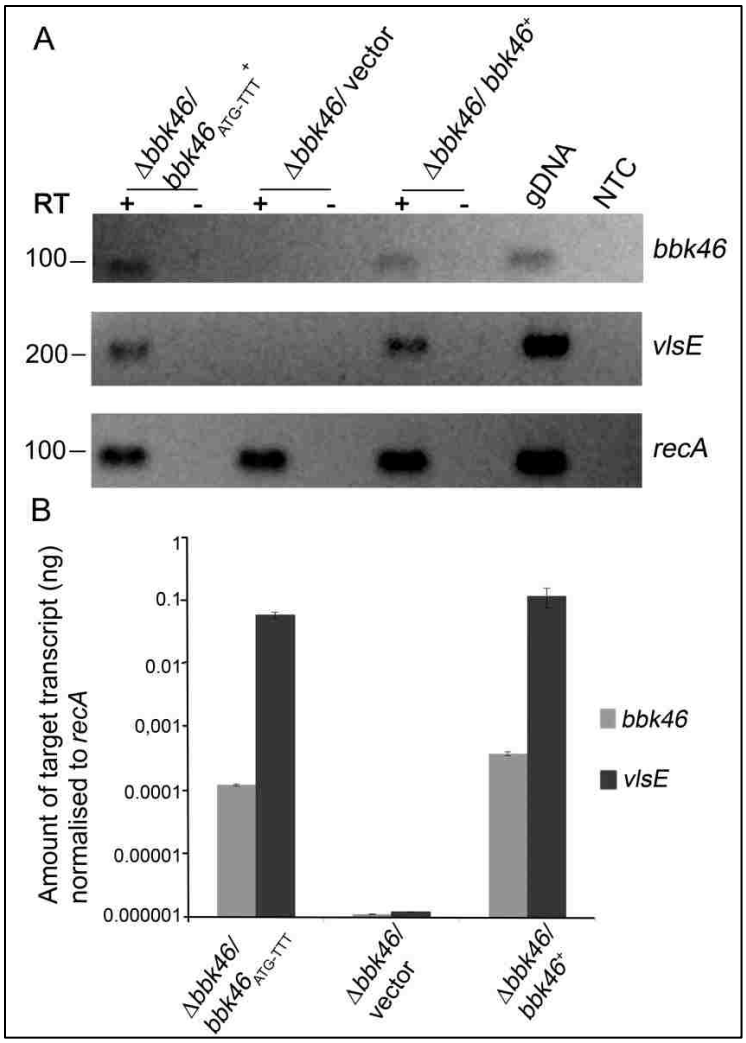

Figure 18: *vlsE* transcription is detected in *bbk46*$_{ATG\text{-}TTT}$ mutant spirochetes.

Total RNA was isolated from log phase *in vitro* grown *bbk46*::*flaB*p-*aadA*/ pBSV2G-*bbk46*$_{ATG\text{-}TTT}^{+}$ (Δ*bbk46/bbk46*$_{ATG\text{-}TTT}^{+}$), *bbk46*::*flaB*p-*aadA*/ pBSV2G (Δ*bbk46*/ vector) and *bbk46*::*flaB*p-*aadA*/ pBSV2G-*bbk46* (Δ*bbk46*/ *bbk46*$^{+}$) *B. burgdorferi*. RNA was reverse transcribed to cDNA using random hexamer primers. The expression of *bbk46*, *vlsE* and *recA* were obtained using endpoint RT-PCR (A). The positions of markers to the left of the panel depict DNA standard molecular sizes in basepairs. Quantitative analysis of *bbk46*, *vlsE* and *recA* expression was carried out using quantitative reverse transcription polymerase chain reaction (qRT-PCR) (B). The mRNA levels of the *vlsE*, and *bbk46* gene transcripts were normalized to that of the constitutive *recA* gene. The data represent the average of triplicate qRT-PCR analyses. Error bars represent the standard deviation from the mean.

To confirm that the *bbk46*$_{ATG\text{-}TTT}$ gene was expressed in *B. burgdorferi* , total RNA was extracted from *in vitro* grown Δ*bbk46*/*bbk46*$_{ATG\text{-}TTT}$ spirochetes and analyzed for the expression of *vlsE* using end point RT-PCR (Fig. 18). Expression of the *bbk46*$_{ATG\text{-}TTT}$ mutant gene in spirochetes lacking *bbk46* resulted in restoration of *vlsE* expression. These data suggested that *bbk46* appears to function as an RNA and not as a protein, to control the expression of *vlsE* (Fig. 18).

3.2.5: Reintroduction Of 12 lp36-encoded Genes To Spirochetes Lacking The Entire lp36 Plasmid Does Not Result in Down Regulation Of The *vlsE* Transcript.

We have demonstrated that *vlsE* expression is repressed in the absence of *bbk46*. We have also shown that in the absence of the entire lp36 plasmid, which contains the *bbk46* locus, there is no detectable loss of *vlsE* expression. These data suggest the possibility of an, as of yet unknown, gene located on lp36 that acts as a repressor of *vlsE* transcription in the absence of *bbk46*. Our next strategy was to try and identify this repressor gene. Our approach has been to add back different fragments of lp36 plasmid to a clone lacking the entire lp36 plasmid in order to create a panel of clones that contain different regions of lp36 in the absence of *bbk46*. We expected that if we are able to introduce the repressor gene into a clone lacking all other lp36 genes including *bbk46*, this gene would be active and *vlsE* expression would be lost. To date, 5 different clones have been created spanning 11 different genes on lp36, as well as the *bbk17* gene (Fig.19).

The genes inserted into the 5 clones were *bbk05* in clone B*, bbk09, bbk08* and putative lipoprotein *bbk07* in clone C*, bbk12, bbk13* (SIMPL family protein), *bbk14* and *bbk15* in clone I, *bbk50* (immunogenic P37 family protein) and *bbk49* (putative outer membrane protein) in clone G and *bbk53* and *bbk52* (putative lipoprotein) in clone H [7]*.* These clones were grown *in vitro* to log phase and total RNA was extracted. The expression of *vlsE* was analyzed using endpoint RT-PCR. We observed no loss or reduction of *vlsE* expression in any of the 5 clones (Fig. 20). These data suggested that the repressor was not present in these regions of linear plasmid 36.

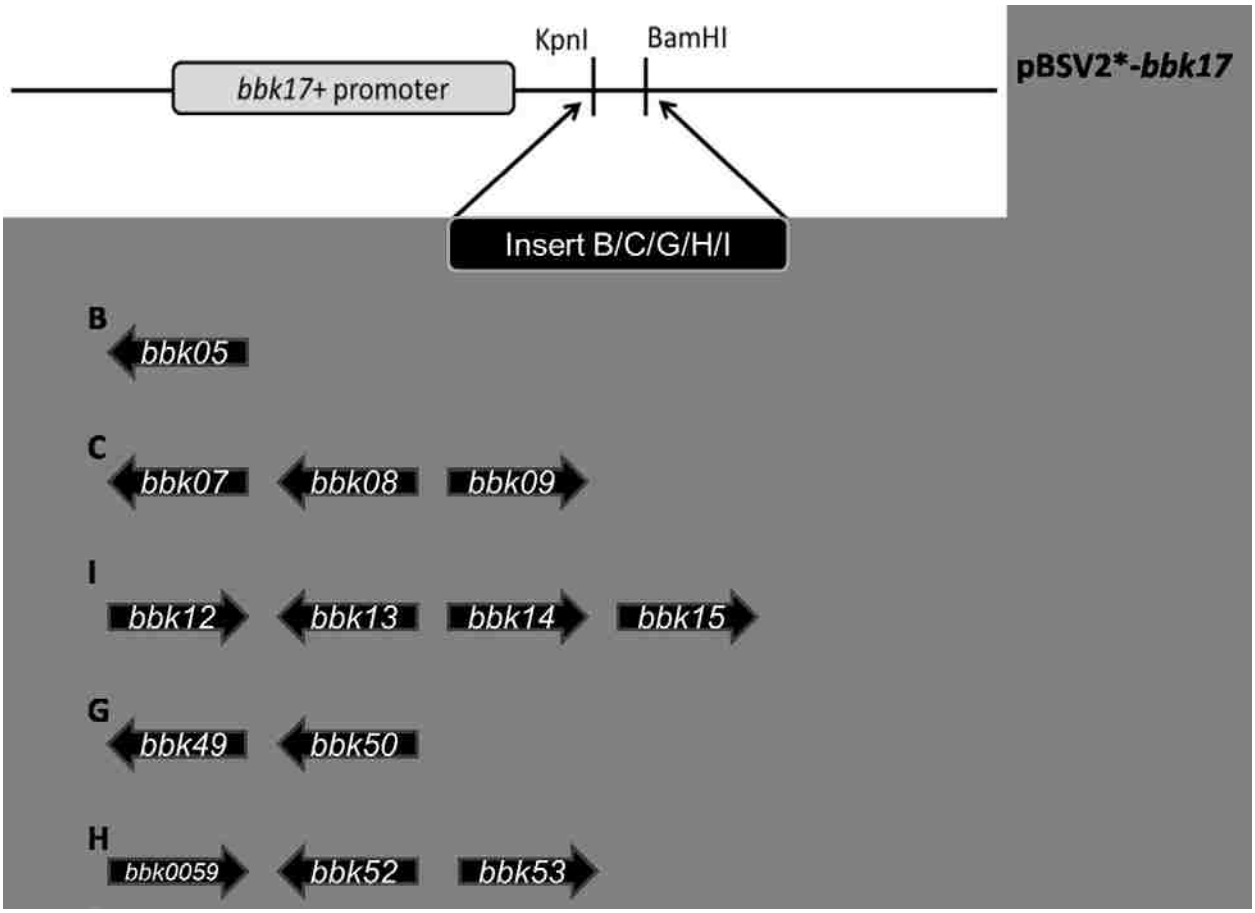

Figure 19: Schematic representation of the shuttle vectors, each containing different fragments of linear plasmid 36 in addition to *bbk17.*

Different fragments B, C, G, H and I containing genes *bbk05, bbk07-bbk08-bbk09, bbk49-bbk50, bbk0059-bbk52-bbk53* and *bbk12-bbk13-bbk15* respectively, of linear plasmid 36 were introduced into a shuttle vector pBSV2* [127] containing the *bbk17* gene.

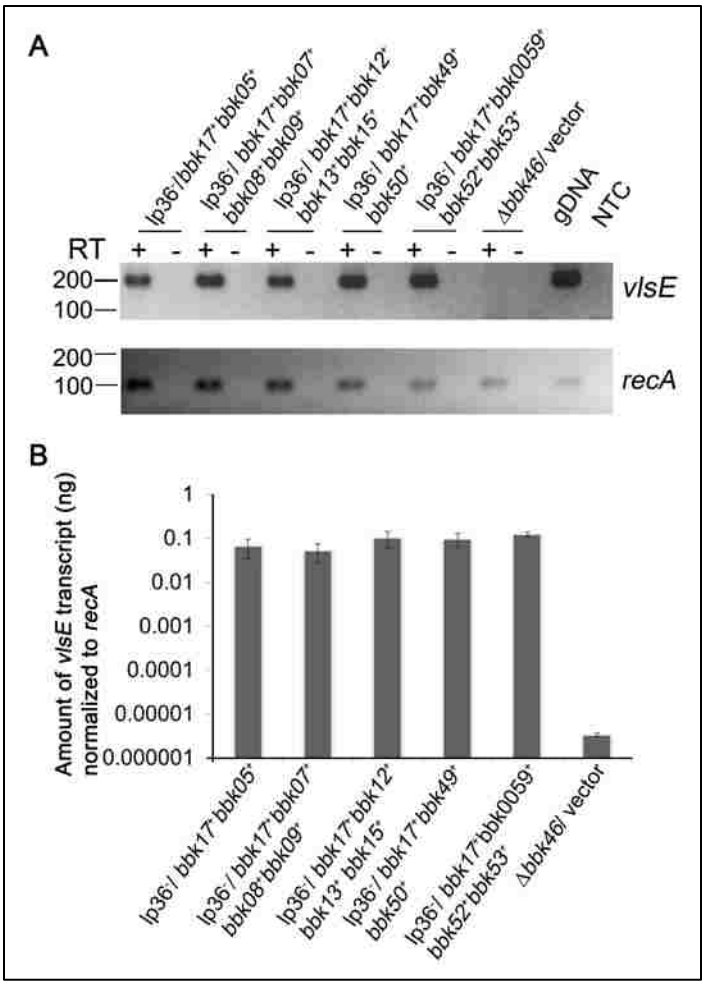

Figure 20: Reintroduction of 12 different genes into spirochetes lacking the entire lp36 plasmid does not result in down regulation of the *vlsE* transcript.

Total RNA was isolated from log phase *in vitro* grown *B. burgdorferi* clones lacking lp36 with distince lp36-encoded genes added back. RNA was reverse transcribed to cDNA using random hexamer primers and in the presence (+) or absence (-) of reverse transcriptase (RT). The expression of *vlsE* and *recA* were obtained using endpoint PCR (A). The positions of markers to the left of the panel depict DNA standard molecular sizes in basepairs. *B. burgdorferi* genomic DNA was used as positive control (gDNA) and the negative control was the reaction mix with no template DNA (NTC). The expression was also quantified using quantitative reverse transcription polymerase chain reaction (qRT-PCR) (B). The mRNA levels of the *vlsE* gene transcripts were normalized to that of the constitutive *recA* gene. These data represent the average of triplicate qRT-PCR analyses. Error bars represent the standard deviation from the mean**.**

3.3 Discussion

The persistence o*f Borrelia burgdorferi* throughout its infectious cycle is attributed to its ability to vary gene expression in response to environmental conditions [51]. Evasion of the host adaptive immune response is one of the survival strategies of these spirochetes and it is believed that one of the mechanisms involved in immune evasion is variation in the patterns of surface antigenic proteins [6].

We previously demonstrated that *bbk46*, a gene located on linear plasmid 36, was required for long term infectivity in mice and suggested that this gene contributes towards *B. burgdorferi's* ability to evade the host adaptive immune response [158]. We now demonstrate that the spirochetes lacking *bbk46* survive up to two weeks post-inoculation in mice (Table 8). Consistent with previous findings [158] the mutant spirochetes were undetectable in immunocompetent mice at three weeks post-inoculation. These findings demonstrate that *bbk46* is not required for the initial stages of *B. burgdorferi* infection. This gene is, however, essential for persistent survival of the spirochetes in infected tissues, likely through a mechanism that contributes to the ability of *B. burgdorferi* to evade the host humoral immune response. The mutant spirochetes are able to migrate to and survive within tissues that are distant from the inoculation site. Although our data suggest that the numbers of spirochetes lacking *bbk46* in infected mouse tissues are lower than that of wild-type spirochetes at the two week time point of infection. Subsequently, between weeks 2 and 3 post-inoculation, the host adaptive immune response targets the spirochetes and those that lack *bbk46* are

unable to evade this immune attack and can no longer survive. Immunoblot and gene expression analyses showed that the spirochetes lacking *bbk46* demonstrated significant downregulation of the critical surface exposed antigen, *vlsE*. Together these data suggest that *bbk46* may be involved in *B. burgdorferi's* ability to evade the host immune response through the regulation of the surface exposed antigen *vlsE*.

vlsE is an outer surface protein that demonstrates antigenic variation of its protein product and has been implicated in the process of evasion of the host adaptive immune response by *B. burgdorferi.* [86, 90, 91]. It is a gene located on lp28-1 and contains a central expression locus that randomly recombines with adjacent silent cassettes causing the production of proteins that have varying sequences resulting in different antigenic properties [92, 159, 160]. This mechanism of antigenic variation has been shown to only occur in the mammalian host and to be essential for evading the host immune response and the long term survival of spirochetes within the host [13, 14, 18, 85, 86, 90-93]. Surprisingly, the only essential protein factors for antigenic switching identified so far have been the Holiday Junction Helicase peptides RuvA and RuvB [90, 91]. A recent study identified pentanucleotide G- runs within the direct repeat regions of the *vls* locus [163] suggesting the formation of G4 DNA structures, which may be involved in the regulation of the antigenic switching between the central expression locus and the silent cassettes. Antigenic variation has been extensively studied in other organisms, particularly in *Neisseria gonorrhea* [164-168] . Several different factors involved in homologous recombination, DNA repair and factors involved in Holiday

junction processing pathways have been found to be essential for its antigenic switching mechanism of the *N. gonorrhea* pilin gene [164-168].

There are a few studies that have investigated *vlsE* gene expression. One such study found that pH and temperature changes influence expression of *vlsE in vitro* and they identified DNA-protein interactions with the promoter region of *vlsE*, suggesting that, yet to be identified, regulatory proteins may control *vlsE* expression [169]. This study identified 37 bp region upstream of the *vlsE* transcription start site to be a minimal promoter important for pH dependent regulation of transcription and additional 5' DNA regions were found to be required for temperature effects [169] . It was found that at 23^{o}C, bacteria expressed higher levels of VlsE than those cultured at 34^{o}C, irrespective of whether the pH was 6.4 or 7. This work also demonstrated the highest expression of VlsE in bacteria cultured at pH 8.0 at either 23^{o}C or 34^{o}C. A recent study demonstrated that the DNA binding protein SpoVG binds within the *vlsE* open reading frame; however, its role in *vlsE* regulation is still unknown [170]. It has also been shown that *vlsE* expression was increased in *in vitro* cultured *B. burgdorferi* that were grown in the presence of human endothelial or neuronal cells suggesting the influence of host factors on regulating *vlsE* expression [171]. Moreover, a unique inverted repeat sequence has been identified in the promoter region of the *vlsE* locus that is predicted to form a stable stem loop structure [171]. Such structures have also been identified in eukaryotic or viral genomes and are believed to be involved in various processes like promoter recognition, plasmid segregation and replication or regulation of gene expression [172-

174]. It is still unclear whether the expression of the *vlsE* gene itself and the recombination events leading to antigenic switching are mutually exclusive processes or might be related and as a result regulated under the same overall mechanism. This is significant since previous studies have mainly targeted the regulation of the process of recombination and not the expression of the gene itself. Our findings, on the other hand, appear to be the first instance of identification of a genetic factor influencing the expression of the *vlsE* gene. We find that, in the absence of *bbk46, vlsE* expression is significantly downregulated and accompanied by loss of long term infection in mice suggesting that *bbk46* might indeed be involved in an, as of yet unknown, mechanism of regulation of *vlsE* expression. Bankhead *et al.,* (2007) [86] found that a non-switching *vlsE* mutant was cleared from infected mice between weeks 2 and 3 post inoculation. In addition they point out that this non-switching mutant was cleared from infected mice at a faster rate than the mutants that lacked the *vlsE* gene and they suggest that this indicates a role of *vlsE* antigenic switching in adaptive immune response evasion. It would seem that a non-switching mutant would be able to present the one non-variant isoform which would be identified by the host thus furthering the idea that non-switching mutants would show seroreactivity for the C6 peptide of VlsE. We observed no seroreacivity for the C6 peptide in mice infected with the ∆*bbk46* mutant, suggesting again, that this mutant does not present any form of VlsE on its cell surface and that *bbk46* deletion downregulates the expression of the *vlsE* gene.

Linear plasmid 36 is essential for spirochete infectivity [16]. Introduction of *bbk17,* an adenine deaminase encoded on lp36, into spirochetes lacking lp36 led to the restoration of infectivity in these spirochetes [16]. There appears to be a dichotomy between these findings and our current data, wherein spirochetes lacking *bbk46* are cleared from the murine hosts by week three post inoculation, whereas spirochetes expressing only *bbk17* and lacking all other lp36-encoded genes including *bbk46* demonstrated a wild-type infectious dose [16]. Our subsequent experiments aimed at reconciling these differences showed us that despite the fact that the spirochetes lacking the entire lp36 plasmid did not show expression of *bbk46*, they showed no inhibition of *vlsE* expression. We also found that serum from mice infected with lp36^{-} /*bbk17*$^{+}$ mutant spirochetes contained antibodies against VlsE. Together these data suggest the presence of another factor on lp36 that appears to repress *vlsE* expression in the absence of *bbk46* alone. However, when the entire lp36 is absent, so is the putative repressor and as a result *vlsE* expression is uninhibited. In the light of these findings, we propose a mechanism of regulation of *vlsE* expression that involves *bbk46* as a repressor of another unknown gene '*x*' located on linear plasmid 36 that, in turn, acts as a repressor of *vlsE* (Fig. 21). We propose that *bbk46*, directly or indirectly, acts as a repressor of gene '*x*' and inactivates it. When repressor gene '*x*' is inactive *vlsE* is expressed. However, if *bbk46* is absent, then gene '*x*' is activated and downregulates the expression of *vlsE* as seen in the mutants that do not contain *bbk46*. If the entire

lp36 is absent then neither the repressor gene'*x*' is present nor is *bbk46* and so *vlsE* expression remains unrestricted as seen in the spirochetes lacking the entire lp36.

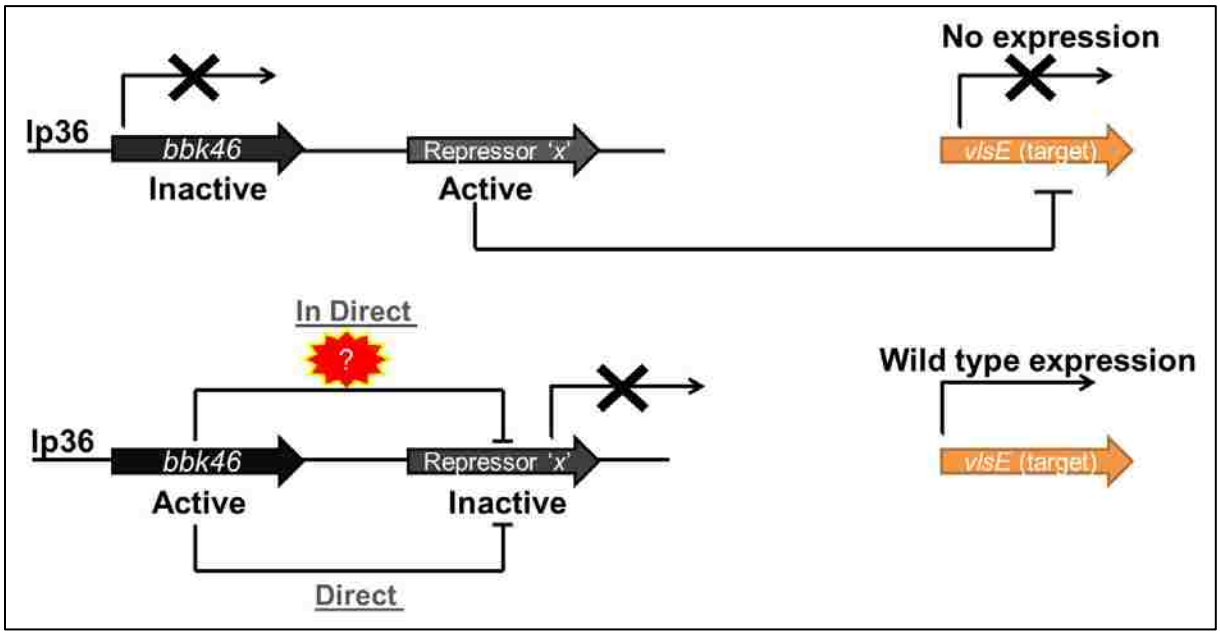

Figure 21: Putative model of regulation of *vlsE* expression via *bbk46* and unknown gene '*x*'.

A schematic representation of a proposed mechanism of regulation of the *vlsE* gene where *bbk46* acts as a repressor of an unknown gene '*x*' located on lp36. In the absence of *bbk46* activity gene '*x*' acts as a repressor of *vlsE* downregulating its expression. When *bbk46* is active it represses gene '*x*' in a direct or indirect manner, allowing wild-type expression of *vlsE*.

Our findings suggest that the regulatory function of *bbk46* is carried out via its RNA product. A protein product for this gene has yet to be detected [158]. Also, the translation mutant, $bbk46_{ATG\text{-}TTT}$ showed no detectable BBK46 protein product but produced transcript comparable to wild-type levels and displayed uninhibited expression of *vlsE*. There is very limited information about the mode of function or even the existence of small regulatory RNAs (sRNAs) within *B. burgdorferi*. [7, 175-177] sRNAs have been studied in *E.coli* [178] and are known to function by binding their target mRNA to either induce or inhibit translation and might also be involved in binding and sequestration of proteins. They are known to interact with the sm-like protein Hfq, which helps stabilize the binding of the sRNAs to their target mRNA and acts like a chaperone protein [179, 180]. Recently an Hfq homolog was found in *B. burgdorferi* and it was shown to be similar in its tertiary structure but varying in sequence similarity to its homologs in other species [181]. It was shown to influence the expression of the regulatory gene *rpoS* in a temperature dependent manner via the small regulatory RNA $DsrA_{Bb}$ [181] . Further studies are needed in order to identify the mechanism of function of the *bbk46* transcript. Our findings suggest that this gene might have a role as a regulatory RNA and further analysis of this possibility is essential for a better understanding of not only *bbk46* function but also understanding of regulatory RNAs in *Borrelia burgdorferi*.

We have proposed a possible mechanism of regulation *bbk46*-dependent *vlsE* regulation; however, we can merely speculate about the factors controlling expression

of *bbk46* itself. We demonstrated previously that *bbk46* expression is dramatically increased in spirochetes isolated from *in vivo* samples as compared to *in vitro* grown spirochetes [158]. We also found that *bbk46* expression was independent of RpoS regulation [158]. Perhaps the induction of this gene is influenced by the environment itself. Various host factors such as different cellular and tissue components that interact with the invading spirochetes and perhaps even immune response factors might act as triggers inducing the increased expression of this gene so that it can play its part within the mechanism of immune protection. Currently, based on our proposed mechanism of regulation, we are in the process of identifying the gene on lp36 that acts as a repressor of *vlsE* expression. Our initial findings have been restricted to a few genes on lp36 and we have determined that *B. burgdorferi* clones lacking lp36 and containing *bbk17* along with either genes *bbk05, bbk07,bbk08, bbk09, bbk12, bbk13, bbk15, bbk49, bbk50, bbk0059, bbk52 or bbk53* do not show a downregulation of *vlsE* expression (Fig. 17), which suggests that none of these genes is the putative repressor. Identification of the putative lp36-encoded repressor of *vlsE* is an area of ongoing research.

In conclusion, we have demonstrated that spirochetes lacking *bbk46* can survive within the mammalian host up to 2 weeks and are eliminated from the host by week 3 post-inoculation. We have also shown that *bbk46* produces an RNA, which appears to be involved in regulation of *vlsE* expression and we have proposed a regulatory mechanism involving a putative repressor gene located on lp36. These findings have broadened our understanding of the role of lp36 in mammalian infectivity. Our current

and future work is directed at identifying the putative repressor of *vlsE* expression, which would be invaluable for our understanding of the mechanisms involved in the expression of this gene.

3.4 Materials And Methods

3.4.1: Ethics Statement.

The University of Central Florida is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and approved by the UCF's Institutional Animal Care and Use Committee.

3.4.2: Bacterial Clones And Growth Conditions.

All *B. burgdorferi* clones used were derived from clone B31. Low passage *B. burgdorferi* complemented clones were generated from the mutant clone Δ*bbk46*::*flaBp-aadA* [158], that lacks the *bbk46* gene. *B. burgdorferi* clones containing various lp36 fragments were generated, using non-infectious clone B31-A [182] and A3-M9 lp36$^-$ that lacks lp36, cp9 and lp21 [16]. *B. burgdorferi* was grown in liquid Barbour-Stoenner-Kelly (BSK) II medium supplemented with gelatin and 6% rabbit serum [151] and plated in solid BSK medium as previously described [152, 153]. All spirochete cultures were grown at 35^{o}C supplemented with 2.5% CO_2. Kanamycin was used at 200 μg/ml, streptomycin was used at 50 μg/ml and gentamicin was used at 40 μg/ml, when

appropriate. All cloning steps were carried out using DH5α *E. coli*, which were grown in LB broth or on LB agar plates containing 50 μg/ml kanamycin or 10 μg/ml gentamycin.

3.4.3: Mouse Infection Experiments.

3.4.3.1: Time course of infectivity of the bbk46 *mutant.*

6-8 week old C3H/HeN female mice (Harlan) were used for all experiments. Groups of five mice were needle-inoculated, intra-dermally under the skin of the upper back [14], with *B. burgdorferi* clones wild-type (A3-68ΔBBE02) [140], Δ*bbk46*/vector or Δ*bbk46/bbk46*$^{+}$ [158] at a dose of 1x10^{4} spirochetes. The number of spirochetes inoculated into mice was determined using a Petroff-Hausser counting chamber and verified by colony-forming unit (cfu) counts in solid BSK medium. Twelve colonies per inoculum were verified by PCR for the presence of the virulence plasmids lp25, lp28-1 and lp36 in at least 90% of the individuals in the population. Further, total plasmid content of each inoculum was confirmed to be as expected [16, 74, 150]. Mouse infection was assessed at 48 hours, 1 week, 2 weeks and 3 weeks post inoculation by serology and re-isolation of spirochetes from inoculation site, ear, bladder and/or joint tissues, as previously described [16].

3.4.3.2: Seroreactivity towards VlsE-C6 in lp36^{-} *mutant spirochetes.*

Groups of 3 mice were needle inoculated as described in [74] with *B. burgdorferi* clones lp36^{-}/lp36^{+} and lp36^{-}/*bbk17*$^{+}$ [16] at a dose of 5x10^{3} spirochetes per mouse. The number of spirochetes inoculated into mice was determined using a Petroff-Hausser counting chamber and verified by colony-forming unit (cfu) counts in solid BSK medium.

Twelve colonies per inoculum were verified by PCR for the presence of the virulence plasmids lp25, lp28-1 and lp36 in at least 90% of the individuals in the population. Further, total plasmid content of each inoculum was confirmed to be as expected [16, 74, 150]. Sera was collected at 3 weeks post inoculation, as previously described [74].

3.4.4: Genomic DNA Extraction From Tissues.

Spirochete DNA was isolated from infected mouse tissues, as previously described. [154, 183] Briefly, *B. burgdorferi*-infected mouse hearts, ears, skin from inoculation site, and joint tissues were collected 2 weeks post-inoculation (see above). The hearts were manually macerated with sterile scalpels and then placed in Collagenase A (1 mg/ml). Ears, skin from inoculation site and joints were directly placed in Collagenase A (1 mg/ml) and all organs were incubated at 37^{o}C for 4 hours. Proteinase K (0.2 mg/ml) was added to all tubes and they were then placed at 55^{o}C for overnight incubation. 2.5 ml each of phenol and chloroform were added to each tube followed by vigorous shaking. The samples were centrifuged at 3600 rpm at 25^{o}C for 20 minutes. From each tube, the clear top layer of liquid (approximately 4.5 ml) containing the DNA was transferred to a new tube. 5 ml of 200 proof ethanol was added to each tube followed by vigorous shaking. Tubes were incubated at -20^{o}C for 2 hours followed by centrifugation at 3600 rpm at 4^{o}C for 20 minutes. The supernatant was discarded and the pellet was allowed to dry at room temperature. The pellet was resuspended in 2 ml of sterile water. 20μl of 1 mg/ml of DNase free RNase was added to each tube and then incubated at 37^{o}C for 1 hour. 1 ml each of phenol and chloroform were added to

each tube and shaken vigorously. The tubes were centrifuged at 3600 rpm at 25^{o}C for 20 minutes. Again, the top layer of liquid, containing the DNA, was transferred to new tubes. 2ml of chloroform was added to each new tube followed by vigorous shaking and then centrifugation at 3600 rpm at 25^{o}C for 20 minutes. The top layer of liquid was transferred to new tubes. 1M NaCl was added to each tube to get final concentration of 0.2M. 5 ml of 200 proof ethanol was added to each tube followed by vigorous shaking. Tubes were incubated at -20^{o}C overnight. Samples were then centrifuged at 3600 rpm at 4^{o}C for 20 minutes. The supernatant was discarded and the pellets were allowed to dry at room temperature. Pellets were resuspended in 100 μl of Tris-EDTA buffer (pH 8.0). The samples were then purified using the Qiaquick PCR purification kit (Qiagen) and the DNA was eluted in 50 μl of Qiagen Elution buffer buffer. All samples were stored at -20^{o}C.

3.4.5: Quantitation Of Spirochete Loads In Mouse Tissues.

Total DNA isolated from infected mouse tissues was used to quantify *B. burgdorferi* loads within the tissue extracts. Real-time quantitative PCR reactions were carried out using 50 μg of DNA per reaction, iTaq Supermix (Bio-rad) and with IDT Primetime Standard qPCR Assay Primers/Probe mix for the *flaB* gene (*B. burgdorferi* chromosome) (primers 1137 and 1138, probe 1139) (Table 9) and the mouse *nidogen1* gene [184] (primers 1140 and 1141, probe 1142) (Table 9) using an Applied Biosystems 7500 instrument. Samples were analyzed in triplicate and the spirochete burden was expressed as *flaB* spirochete DNA copies per 1000 *nid1* mouse DNA copies. The *flaB*

primers were optimized to maximum efficiency using a standard curve with B31 A3 *B. burgdorferi* genomic DNA at concentrations of 10^6 spirochetes/ 5 μl, 10^5 spirochetes/ 5 μl, 10^4 spirochetes/5 μl, 10^3 spirochetes/ 5 μl, 10^2 spirochetes/ 5 μl and 10^1 spirochetes/ 5 μl. The *nid1* primers were optimized using plasmid DNA containing a cloned copy of the *nid1* gene (REFENCE the lp36 paper) at concentrations 5.5 x10^6 copies/ 5 μl, 5.5 x10^5 copies/ 5 μl, 5.5 x10^4 copies/ 5 μl, 5.5 x10^3 copies/ 5 μl and 5.5 x10^2 copies/ 5 μl.

3.4.6: Cloning, Purification And Seroreactivity Analysis Of Recombinant GST-Tagged Proteins.

In-frame glutathione S-transferase (GST) fusion proteins lacking their putative signal sequences were generated for BBK45, BBK48, BBK50, VlsE (C6), RevA, DbpA, BBK19, CRASP-2 and OspA. The above targets were PCR amplified using primer pairs, 1053 and 1054, 1045 and 1046, 1043 and 1044, 1022 and 1023,1143 and 1144, 1145 and 1146, 1147 and 1148, 1149 and 1150, and 1151 and 1152, respectively (Table 9), Phusion polymerase (New England Biolabs) and *B. burgdorferi* genomic DNA. The clones were generated and proteins purified as described previously [135]. Approximately 1 μg of rGST-BBK46 [158], rGST-BBK45, rGST-BBK48 and rGST-BBK50, and 150 ng of the remaining recombinant proteins along with rGST-OspC [135] were separated by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by immunoblot for seroreactivity using immune sera as previously described [135]. The sera were collected, pre-inoculation, and at 1 week, 2 weeks and 3 weeks post inoculation, from mice infected with 1x10^4 wild-type Δ*bbk46*/vector and

Δ*bbk46/bbk46*$^{+}$ *B. burgdorferi,* clones. The sera were also collected, pre-inoculation, and at 3 weeks post inoculation, from mice infected with lp36^{-}/ lp36^{+} and lp36^{-}/ *bbk17*$^{+}$ *B. burgdorferi* clones. Controls included 1 μg of GST alone and total protein lysates generated from *B. burgdorferi* B31 A3.

3.4.7: RNA Isolation From *In Vitro* Grown Spirochetes.

To obtain *in vitro* log phase spirochetes, all clones were grown in triplicate in 5 ml of BSKII medium pH 7.5 at 35°C to a density of 3x10^{7} spirochetes/ml. A total of 1x10^{7} spirochetes were harvested from each culture and total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA was resuspended in 100 μl DEPC treated dH_2O. RNA was treated with TURBO DNA-free (Life Technologies) to remove any contaminating genomic DNA. 1 μl of Riboguard (40U/μl) RNAse inhibitor (Epicentre) was added to all samples and RNA stored at -80°C.

3.4.8: Gene Expression Analysis.

cDNA was synthesized from all RNA samples using the iScript cDNA synthesis kit (Bio- Rad) with random primers according to the manufacturer's instructions. Parallel cDNA reactions were carried out in the absence of reverse transcriptase. The cDNA was used for end-point PCR amplification using *Taq* DNA Polymerase with ThermoPol® Buffer and 1 μg of cDNA per reaction. Simultaneously quantitative reverse transcription PCR (qRT-PCR) reactions were prepared using 1 μg cDNA and iQ SYBR Green Supermix (Bio-Rad). Using an Applied Biosystems 7500 instrument, samples

were assayed for the *vlsE*, *recA*, and *bbk46* transcripts using primers pairs 1063 and 1064, 1123 and 1124, and 1121 and 1122, respectively (Table 9). The *recA* transcript was used as the endogenous reference to which the transcripts of the other genes were normalized. The *bbk46 and vlsE* primers were confirmed to be specific for their gene targets. All primers were optimized to maximum efficiency using a standard curve with *B. burgdorferi* genomic DNA at concentrations of 50 ng/μl, 5 ng/μl, 0.5 ng/μl, 0.05 ng/μl, and 0.005 ng/μl. The amount of gene specific transcripts were quantitated based upon the standard curves generated for each gene and were normalized to *rec*A. Three biological replicate samples were analyzed in triplicate and normalized to *recA* mRNA. The amplification of samples lacking reverse transcriptase was similar to that of the no-template control.

3.4.9: Complementation Of the Δ*bbk46* Mutant With pBSV2G-*bbk46*$_{ATG\text{-}TTT}$.

A PCR-based overlap extension strategy was used to introduce a mutation at the start codon of the *bbk46* gene. The ATG start codon coding for methionine was replaced by TTT that codes for phenylalanine. The generated DNA fragment contained the rest of *bbk46* gene and putative upstream promoter sequence with the introduction of a FLAG epitope tag immediately upstream of the putative premature stop codon and a C-myc epitope tag immediately upstream of the downstream stop codon.

The insert with the mutant start codon was generated using pBSV2G-*bbk46-FLAG-C-myc* DNA as template, primers 1117 and 1062, 1118 and 1053, and 1119 and 1120 (Table 9) and Phusion polymerase (New England Biolabs). The PCR products

were purified and used to generate the mutant clone in *E.coli* as described previously [158]. The Δ*bbk46-flaBp-aadA* clone was transformed with 20 μg of pBSV2G-*bbk46*$_{ATG-TTT}$-*FLAG-C-myc* isolated from *E. coli* and positive transformants selected as previously described [16, 74]. The clones that retained the *B. burgdorferi* plasmid content of the parent clone were selected for use in further experiments.

3.4.10: Immunoblot Analysis of BBK46$_{ATG-TTT}$.

Production of the BBK46$_{ATG-TTT}$-FLAG-cMyc protein was examined in *E. coli* and compared to the production of BBK46-FLAG-cMyc in *E. coli* and *B. burgdorferi* carrying pBSV2G *bbk46*$_p$-*bbk46-FLAG-cMyc* or pBSV2G *flaB*$_p$-*bbk46-FLAG-cMyc*. *E. coli* protein lysates from *E.coli* carrying plasmids pBSV2G *bbk46*$_p$-*bbk46*$_{ATG-TTT}$-*FLAG-cMyc,* pBSV2G *bbk46*$_p$-*bbk46-FLAG-cMyc* and pBSV2G *flaB*$_p$-*bbk46-FLAG-cMyc* were prepared from 2x10^9 cells harvested following overnight growth in LB medium at 37°C with aeration. *E. coli* cells were resuspended and lysed in 200 μl B-PER protein extraction reagent (Thermo Scientific) followed by the addition of 200 μl 2x Laemmli sample buffer plus 2-mercaptoethanol (Bio-rad). Total *B. burgdorferi* protein lysates from clones *bbk46*$_p$-*bbk46-FLAG-cMyc* and *flaB*$_p$-*bbk46-FLAG-cMyc* were prepared from 2x10^9 spirochetes harvested at mid-log phase. The spirochetes were washed twice in 1 ml cold HN buffer (50 mM Hepes, 50 mM NaCl, pH 7.4) and lysed in 200 μl B-PER protein extraction reagent (Thermo Scientific), followed by the addition of 200 μl 2x Laemmli sample buffer plus 2-mercaptoethanol (Bio-rad). 30 μl of each protein lysate (~1.5x10^8 cells) were separated by SDS-PAGE and transferred to a nitrocellulose

membrane. 300 ng of PncA-FLAG [117] protein served as the positive control. Immunoblot analysis was performed using anti-FLAG monoclonal primary antibody (Genescript) diluted 1:500 in Tris buffered saline, pH 7.4 and 0.5% Tween20 (TBST) and goat anti-mouse IgG+IgM-HRP secondary antibody (EMD Milipore) diluted 1:10,000 in TBST and signal was detected using SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific).

3.4.1:1 Generation Of Plasmids Containing lp36 Fragments.

In order to generate the pBSV2*-*bbk17* plasmid the *bbk17* gene was amplified from *B. burgdorferi* genomic DNA using Phusion High-fidelity DNA polymerase and the primers 1125 and 1126 (Table 9). EcoRI restriction sites were introduced at the 5' and 3'ends. The EcoRII-digested PCR product was ligated into the EcoRI-digested *B. burgdorferi* shuttle vector pBSV2* [127]. The plasmid structure and sequence were confirmed by restriction digest and DNA sequence analysis. Different fragments B, C, G, H and I containing genes *bbk05, bbk07-bbk08-bbk09, bbk49-bbk50, bbk0059-bbk52-bbk53* and *bbk12-bbk13-bbk15* respectively, of linear plasmid 36 were introduced into a shuttle vector pBSV2* [127] containing the *bbk17* gene (Fig. 19). The fragments B, C, G, H and I were amplified from *B. burgdorferi* genomic DNA using Phusion High-fidelity DNA polymerase and primer pairs 1127 and 1128, 1129 and 1130, 1131 and 1132, 1133 and 1134, and 1135 and 1136 (Table 9).The fragment B had KpnI restriction sites generated at the 5' and 3' end and the fragment C had HindIII sites. The fragments G, H and K each had sites KpnI at 5' end and BamHI at the 3' end. Each of these fragments

was digested with the respective restriction enzymes and was then ligated with an appropriately digested pBSV2*-*bbk17* plasmid.

B. burgdorferi lacking the entire lp36 plasmid [16] was transformed with 20 μg of pBSV2*-*bbk17* plasmid isolated from *E. coli* and positive transformants selected as previously described [16, 74]. To facilitate transformation of the shuttle vector plasmids into the low-passage lp36$^-$ clone background, first high-passage clone B31-A [182] was transformed with pBSV2*-*bbk17*-B ,pBSV2*-*bbk17*-C,pBSV2*-*bbk17*-G,pBSV2*-*bbk17*-H or pBSV2*-*bbk17*-I isolated from *E.coli*. The presence of the shuttle vector plasmids was determined by PCR amplification using the primer pairs used to generate the respective inserts. The positive transformants were saved at -20^{o}C. Total genomic DNA was purified from each of the B31-A clones carrying the shuttle vector plasmids using Wizard genomic DNA purification kit (Promega). 1 μg of this DNA was transformed into *B. burgdorferi* lacking the lp36 [16] and positive transformants selected as previously described [16, 74]. The clones that retained the *B. burgdorferi* plasmid content of the parent clone were selected for use in further experiments.

Table 9: List of primers and probes used in this study

Primer number	Designation	Sequence (5' – 3')[a]
1117	K465'kpn1fwd	cggggtaccCTTCCAGTGTAGGCTTTAGTTT
1118	K463'C-mycSalIrev	acgcgtcgacTTAcagatcttcttcagaaataagtttttgttcATAAGCAGCTTCATATGCTTTATTT
1119	K465'PCR3fwd	CGGGGTACCCTTCCAGTGTAG
1120	K463'PCR3rev	ACGCGTCGACTTACAGATCTTCTTCAGAAATA
1121	Lp3629018F	AGCATTATTTGTACTTCTAGGC
1122	Lp3629013R	ACATACTAGACAACAACAAGTC
1123	recA F	AATAAGGATGAGGATTGGTG
1124	recA R	GAACCTCAAGTCTAAGAGATG
1063	vlsE Express F	CTTATACTTTTCATTATAAGGAGACGATG
1064	vlsE Express R	GCCTCTGCTACTAACCCAC
1061	K46-mut-F 5'	ATAAAGCATAAATATCATCAGATTCTAAGAAGAGGTACTAAGATAGTTTAATTTAATTGC
1062	K46-mut-R 3'	GCAATTAAATTAAACTATCTTAGTACCTCTTCTTAGAATCTGATGATATTTATGCTTTAT
1053	bbk45-S 5'BamH1F	cgggatccATGTGCAAGCTATATGAAGCTGTAG
1054	bbk45 3' Xho1R	ccgctcgagCTAAGATTCTCTACTACTTAAAGCAGCTTC
1045	bbk48-S 5' BamH1F	cgggatccATGTGTAAATTATACAAGAAGATTACATACAACG
1046	bbk48 3' Xho1R	ccgctcgagTTATCTAGAGTCCATATCTTGCAATTT
1043	bbk50-S 5' BamH1F	cgggatccATGTGTAAATTATATGAAAAGCTTACAAATAAATCGC
1044	bbk50 3' Xho1R	ccgctcgagTTATCTAGAGTCCATATCTTGCAATTT
1125	bbk17 EcoRI 5'	cggaattcCTTTGCGCTATAATTTAAGT
1126	bbk17 EcoRI 3'	cggaattcAAAAACTTTTGAGTTCCTTC
1127	B05fwdKpnI	ggggtaccAACTGGGCATTTTGGTTTACAATTCTAA
1128	B05revKpnI	ggggtaccGCTCATCAAGAATGAAATTCACTATGTTATC
1129	C07fwdHindIII	cccaagcttGGACTGCCAAATTCTCTTAAATTTGAAATT
1130	C07revHindIII	cccaagcttTTAGAATTGTAAACCAAAATGCCCAGTT
1131	GfwdKpnI	ggggtaccGTTGTGTGACATATTCGGTATCTCAAC
1132	GrevBamHI	cgggatccCTAAAGCCTACACTGGAAGTTTTAAAGC
1133	HfwdKpnI	ggggtaccGGTTATTTAGGAGTTCACAGATTTTATGTAGG
1134	HrevBamHI	cgggatccGTTGAGATACCGAATATGTCACACAAC
1135	K121315fwdKpnI	ggggtaccCAAACTGTTTCTGCTGGGAATAAC
1136	K121315revBamHI	cgggatccGATACCAGCAAAGATTATGTGGTAAAG
1137	flaB-taqman-FWD	TCTTTTCTCTGGTGAGGGAGCT

Primer number	Designation	Sequence (5’ – 3’)[a]
1138	flaB-taqman-REV	TCCTTCCTGTTGAACACCCTCT
1140	nid1-taqman-FWD	CACCCAGCTTCGGCTCAGTA
1141	nid1-taqman-REV	TCCCCAGGCCATCGGT
1022	C6 Bb F	cgggatccCATATGAAGAAGGATGATCAGATTG
1023	C6 Bb R	acgcgtcgacTTACTTCACAGCAAACTTTCCATC
1143	REVA F	cgggatccAAAGCATATGTAGAAGAAAAGAAAG
1144	REVA R	acgcctcgagTTAATTAGTGCCCTCTTCG
1145	DBPA F	cgggatccGGACTAACAGGAGCAACAA
1146	DBPA R	acgcctcgagTTAGTTATTTTTGCATTTTTCATCAG
1147	BBK19 F	cgggatccTTTTCAAAAGATTCTCGATCACG
1148	BBK19 R	acgcctcgagTCAATTGTTAGGTTTTTCTTTTCC
1149	CRASP2 F	cgggatccGATGTTAGTAGATTAAATCAGAGAAATATT
1150	CRASP2 R	cgggatccGATGTTAGTAGATTAAATCAGAGAAATATT
1151	OSPA F	cgggatccAAGCAAAATGTTAGCAGCC
1152	OSPA R	acgcctcgagTTATTTTAAAGCGTTTTTAATTTCATCAAG
Probe number	**Designation**	**Sequence (5’ – 3’)**
1139	flaB-taqman-Probe	6-FAM-AAACTGCTCAGGCTGCACCGGTTC-TAMRA
1142	nid1-taqman-Probe	6-FAMCGCCTTTCCTGGCTGACTTGGACA-TAMRA

[a]Lowercase indicates all non-*B. burgdorferi* sequence.

CHAPTER FOUR: CONCLUSIONS AND FUTURE DIRECTIONS

Bacterial pathogenesis is the cause of severe, physical, mental and economic distress faced by the world today. The effects of bacterial infections range from mild fevers, to more debilitating symptoms like tissue necrosis, organ failure or even death. *Borrelia burgdorferi* is an extracellular pathogen that is distinct from many bacterial pathogens in the fact that it does not appear to release any known toxins within its host to cause disease or tissue damage [1]. *B. burgdorferi* is known to activate the host immune response which, if left activated over long periods of time due to untreated infection, can lead to the clinical symptoms of lyme disease [1]. These spirochetes gain entry into the mammalian host via the bite of a tick and survive by evading the host immune system and scavenging nutrients from the host environment. In order to thrive in these hostile conditions, *B. burgdorferi* has developed strategies of antigenic variation to evade the host immune attack and of varying expression of different metabolic genes to compensate for changing nutritional availability [1]. Identification of antigenic as well as metabolic factors essential for virulence has been a major goal of researchers with the aim of gaining better understanding of *B burgdorferi* pathogenesis. Characteristic surface antigens are used for identification of these spirochetes as part of diagnostic kits and can also serve as candidates for vaccine development [185]. However, since *B. burgdorferi* vary their surface complement of proteins throughout the infection cycle there is still a pressing need to identify novel makers of infectivity. The studies described in the preceding chapters were aimed at investigating the virulence essential

linear plasmid 36 and the genes encoded by it so as to identify the factors that contribute toward the critical role of this plasmid in *B. burgdorferi* mammalian infectivity.

We successfully applied a novel *In vivo* expression technology (IVET) screen to identify *B. burgdorferi* genes expressed *in vivo* during murine infection. These results represent the first application of IVET to the *B. burgdorferi* system. We have generated a set of candidate promoters that are active *in vivo* and the associated genes which are putative candidates for virulence factors. From the list of *in vivo* expressed candidate genes we proceeded with further investigation of *Bbive162* since it was located on the virulence essential linear plasmid 36. The data presented herein demonstrate the identified *in vivo*-expressed candidate gene *bbk46* to be essential for persistent infection within the murine host. These data demonstrate the effectiveness of the IVET screen in identifying novel virulence factors and underscore the importance of further investigating the other candidates identified by this screen for discovery of new genes essential for infectivity.

Linear plasmid 36 was previously found to be essential for mammalian infectivity and the genes *bbk17* and *bbk32* have been independently investigated and found to contribute, in part, towards successful infection [16, 28, 32]. The identification of *bbk46* as an additional gene that contributes to mammalian infectivity has added another piece to this body of knowledge regarding virulence factors on lp36. In the *B. burgdorferi* clone B31, *bbk46* gene is annotated as a putative pseudogene due to a premature stop codon relative to the sequence of the open reading frame in other *B. burgdorferi* isolates. We

identified that *bbk46* did in fact, produce a transcript, both *in vivo* and *in vitro*, but no protein product was detected in our studies. We also found that, in the absence of *bbk46, vlsE* expression is significantly downregulated and accompanied by loss of long term infection in mice, indicating that *bbk46* is involved in an, as of yet unknown, mechanism of regulation of *vlsE* expression. Moreover, mutation of the *bbk46* start codon resulted in no detectable BBK46 protein product but its transcript levels were comparable to those of wild-type clones. Surprisingly, the *bbk46* mutant allele restored *vlsE* expression to spirochetes lacking the endogenous *bbk46* gene. Together these data suggest a putative role of *bbk46* as a regulatory RNA. This is one of the few instances of identification of a putative regulatory RNA molecule in *B. burgdorferi* and lays a foundation for future studies to understand the mechanisms underlying the control of genes via such regulatory RNAs. Future studies are aimed at identification of the region of the *bbk46* allele that is required for regulation of the *vlsE* expression. At present the *bbk46* ORF is being analyzed via a truncation approach. A set of shuttle vectors containing fragments of the open reading frame are being generated, all having the same 5' end sequence but each fragment of increasing size and sequence at it 3' end so as to incrementally cover the entire ORF. These plasmids will be transformed in to *B. burgdorferi* clones lacking *bbk46*. The objective is to determine the sequence of the *bbk46* open reading frame that is necessary and sufficient for the expression of *vlsE* in these clones. We expect that the clone containing the entire ORF will express *vlsE* but as the size of the ORF fragments gets shorter, the sequences will no longer include

the region required for expression of the functional *bbk46* transcript and the *vlsE* expression will be repressed. Once identified, such a region can be analyzed for sequence similarities to other known motifs and secondary structures associated with RNA regulation in other organisms and can provide an indication of the function of the *bbk46* RNA product which can then be further investigated.

The mechanism of regulation of expression of *bbk46* also remains to be determined. The expression of the *bbk46* gene was found to be increased in spirochetes isolated from *in vivo* samples as compared to *in vitro* grown spirochetes. We also found that *bbk46* expression was not RpoS-dependent. Perhaps the induction of this gene is influenced by the environment itself. Various host factors such as changes in temperature and pH, different cellular and tissue components that interact with the invading spirochetes and perhaps even immune response factors might act as triggers inducing the increased expression of this critical gene. Microarray data from previous studies looking at the effect of pH and temperature on gene expression have not identified *bbk46* as being influenced by these factors. *In vitro* culture of *B. burgdorferi* in the presence of blood or mammalian neuronal or endothelial cells or growth in rat dialysis membrane chambers could be carried out to identify host factors that might influence the expression of this gene.

VlsE is a surface antigen that shows antigenic variation and is believed to play an essential role in host immune response evasion [13, 14, 18, 85, 86, 90-93]. Identifying the involvement of *bbk46* in the expression of *vlsE* introduces the possibility of a novel

regulatory mechanism for the expression of this critical antigen and is a highly significant contribution from this body of work. We found that spirochetes that lack *bbk46* also lack detectable *vlsE* transcripts and are unable to trigger the generation of antibodies against the C6 peptide of VlsE within infected mice. One of the most surprising findings in this study was that spirochetes lacking the entire lp36 plasmid showed no inhibition of *vlsE* expression even though *bbk46* was absent in these clones. This suggested the presence of another factor on lp36 that was repressing the expression of *vlsE* in the absence of *bbk46* but when the entire lp36 is absent, so is the repressor and as a result *vlsE* expression was uninhibited. In light of these findings, we propose a mechanism of regulation of *vlsE* expression that involves *bbk46* as repressor of another unknown gene '*x*' located on linear plasmid 36, that in turn, acts as a repressor of *vlsE* (Fig. 21). This putative model of *vlsE* regulation forms the basis of an ongoing investigation aimed at identifying the, as of yet, unknown repressor gene. The methodology being used is the construction of a panel of clones lacking the entire lp36 plasmid and containing *bbk17* along with fragments of lp36 that encode its different genes (Fig. 19). We expect that the clone containing the repressor gene in the absence of the rest of lp36 will cause downregulation of *vlsE*. Given the fact that *vlsE* expression is crucial for persistent infection in the mammalian host the repressor might be a gene showing low expression during mammalian infection as compared to tick colonization or it could be a gene that is not differentially expressed throughout the infectious cycle. It is possible that *bbk46* expression is what influences the activity of the repressor. We have

found that *bbk46* expression is greatly upregulated *in vivo* as compared to *in vitro*. Perhaps *bbk46* upregulation *in vivo* causes the repressor to be downregulated thus allowing the expression of *vlsE. bbk45* is a gene located adjacent to *bbk46* on lp36. It is a gene of unknown function with some sequence homology to *bbk46* and is part of the same P37 family of lipoproteins. These features make it a good candidate as a gene that might be regulated by *bbk46,* since it has been observed that non-protein coding genes from gene families can regulate the expression of their family members through their transcripts [186]. Preliminary studies have identified a few other lp36 encoded genes that do not repress *vlsE* expression. As of now, the putative *vlsE* repressor remains unidentified and is the subject of ongoing investigation.

The identification of a novel functional gene on the linear plasmid 36 provides some more information about the role of this plasmid in mammalian infectivity. However, the phenotype of the *bbk46* mutant that involves another repressor gene on the same plasmid suggests that *bbk46* is not the only other factor apart from the previously known *bbk17* and *bbk32* that might be contributing to the importance of lp36. It is also unclear whether *bbk46* regulation of the repressor gene is direct or there are other intermediate factors involved that may or may not be present on lp36. These findings suggest that lp36 might have multiple infectivity phenotypes involving different sets of genes. It has been observed that addition of *bbk17* to an lp36^{-} mutant restored infectivity to the mutant in the absence of *bbk46* which we have found to be involved in a completely different regulation mechanism critical for persistence within the mammalian host. The

recent STM study by Lin *et al*. [26] identified that mutants with disruptions in the genes located in the region from *bbk02.1* to *bbk04* and genes *bbk05, bbk07, bbk13, bbk17, bbk45, bbk46* to *bbk50* on lp36 showed lowered mammalian infectivity. It was interesting to note that the P37 family of genes including *bbk46* was identified by this study to influence infectivity and that they also found *bbk17* to influence infectivity as had been seen previously [16]. The genes in this list provide a set of valuable gene candidates to further investigate for putative roles in *B. burgdorferi* survival and further support the hypothesis that there are additional genes on lp36 that contribute towards its infectious phenotype.

Collectively this body of work introduces IVET as an efficient whole genome screening process in *B. burgdorferi* and identifies a novel virulence factor *bbk46* that regulates the expression of a critical surface antigen. We have been able to identify a novel regulatory mechanism on the essential linear plasmid 36 thereby broadening our understanding of the role of this plasmid in mammalian infection. Based on our data we propose a putative model of regulation that will guide future investigations aimed at improving our understanding of gene regulation in *Borrelia burgdorferi*.

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