DEVELOPMENT OF A NOVEL, PEPTIDE-BASED VACCINE FOR LYME DISEASE

A Dissertation

by

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ABSTRACT

Lyme disease (LD) is the most prevalent arthropod borne illness in the US. Currently, there is no vaccine to prevent infection with LD in humans, rather, prevention of this disease relies on avoiding exposure to the tick vector or treating for LD retroactively. Present research towards a new LD vaccine has focused on the idea of using multimeric, chimeric, and multivalent molecules. The antigens targeted in this approach are highly heterologous between strains and species of *Borrelia burgdorferi*, and as such, this vaccine may require reformulation of antigens to remain relevant. As such, this dissertation explored the idea of using a novel, highly conserved peptide antigen derived from *B. burgdorferi* to prevent infection with LD. This approach utilized reverse vaccinology, *in silico* and *in vitro* analysis of potential protein candidates, and *in vivo* vaccination studies using selected proteins and peptides to evaluate the feasibility of a novel peptide vaccine with potential to be broadly protective against LD. Using this methodology, a previously uncharacterized vonWillebrand factor A domain containing protein, BB0173, was characterized and found to localized to the inner membrane with the VWFA domain exposed to the periplasmic space. Further, a novel, highly-conserved peptide antigen of *B. burgdorferi* (PepB) was identified the extracellularly exposed VWFA domain containing protein BB0172 that demonstrated the ability to generate a protective immune response against *B. burgdorferi* challenge both using the needle and tick based methods of infection.

DEDICATION

To my family, for always lifting me up and encouraging me to succeed.

To my parents, who have always believed in me and encouraged me to achieve my dreams.

Aunt Cheryl, who has always gone above and beyond to support me.

My family by marriage, who have all welcomed me as one of their own.

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NOMENCLATURE

BAT	*Bacteroides* Aerotolerance
BSK-II	Barbour-Stoenner-Kelley II
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CFT	Cattle fever tick
DIVA	Differentiating Infected and Vaccinated Animals
DNA	Deoxyribonucleic Acid
DPP	Days post priming
DUF	Domain of unknown function
ELISA	Enzyme-Linked Immunosorbent Assay
HBSS	Hank's Balanced Salt Solution
HRP	Horseradish Peroxidase
ID_{50}	50% Infectious Dose
Ig	Immunoglobulin
KLH	Keyhole Limpet Hemocyanin
LD	Lyme disease
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with 0.1% Tween-20
PCR	Polymerase Chain Reaction
PK	Proteinase K

qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
TBD	Tick-borne disease
TBS	Tris Buffered Saline
TX-114	Triton X-114
VWFA	vonWillebrand Factor A
WPP	Weeks post priming

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Lyme disease agents

Lyme disease (LD) is the most prevalent arthropod borne illness in the United States, with more than 30,000 cases reported annually according to the Centers for Disease Control and Prevention (CDC). Recent studies, however, suggest the true number of cases is actually 300,000 cases per year in the United States alone [1, 2]. LD is a zoonotic disease caused by wave-like spirochetes in the genus *Borrelia*. Lyme disease is found throughout the temperate regions of the world where competent *Ixodes* vectors are present. In the United States, the majority of cases are located in the Northeast, although the Pacific coast is also a focal point for *B. burgdorferi* infection. It is worth noting that in 2015, a new pathogenic strain of *Borrelia* was detected in the northern regions of the Midwestern US - *Borrelia mayonii*. Unlike *B. burgdorferi*, *B. mayonii* is associated with nausea and vomiting and a higher number of spirochetes in the blood. Infection also presents with a diffuse rash that differs from the characteristic bulls-eye rash. Although symptoms resemble Relapsing Fever, presently, *B. mayonii* is currently genetically classified as LD infection [3].

In Europe and Asia, *B. afzelii* and *B. garinii* in addition to *B. burgdorferi* s.s. are the major etiological agents of Lyme disease. Notably, *B. garinii* is associated with neuroborreliosis more often than the other species of *Borrelia* [4], while *B. afzelii* has

been found more often to cause skin related symptoms, notably acrodermatitis chronica atrophicans [5]. There are several other Borrelial pathogens that are minor contributors to human disease, such as *B. speilmanii* and *B. lusitaniae* [6]. On a more general scale, each species and serotype of *Borrelia* carries minor genetic and protein differences that make vaccinating against these pathogens using one antigen difficult.

Ixodes life cycle and pathogen transmission

The Ixodid tick vector and *B. burgdorferi* are found in temperate regions around the world. *Ixodes scapularis, Ixodes pacificus* (USA), *Ixodes ricinus,* and *Ixodes persculatus* (Eurasia) are the primary vectors of LD transmission that are known to bite humans. *Ixodes affinis* and *Ixodes minor* are also present in the USA and vector *Borrelia* species. These ticks, however, are maintained on wildlife and rarely bite humans [7-10]. The ticks that vector LD are three host ticks with four life stages: egg, larvae, nymph, and adult. The pathogen *B. burgdorferi* is thought to be transmitted transtadially, and as such, eggs hatch into uninfected larvae. Acquisition of *B. burgdorferi* infection occurs after feeding on an infected host, such as the reservoir host, *Peromyscus leucopus (P. leucopus)* [11]. After acquisition, *Borrelia* is transtadially transmitted between tick life stages, and thus the tick can transmit LD to naïve hosts as a nymph or adult, if the early host was infected with *B. burgdorferi*. Ticks attach, take a blood meal, and drop off the host to molt to the next life cycle stage. Adult females feed to repletion and release the host before oviposition. New research suggests that *Borrelia* may be transmitted transovarially in rare instances, however, so the acquisition and transmission of *Borrelia* by its tick vectors may not be as clear-cut as previously thought [12].

The complete life cycle of Ixodid ticks takes approximately 2 years, and human risk for infection with LD correlates with the tick life stages. Due to the activity of nymphal *I. scapularis* peaking in late spring and summer, the majority of human cases are acquired during this high-risk time. Hosts for *I. scapularis* range from small mammals like mice and rabbits to larger mammals such as raccoons and deer. Humans, dogs, and other domestic animals are incidental hosts for the ticks, and may show signs of *B. burgdorferi* infection [13, 14]. The nymphal blood meal can last up to 8 days, while adult *Ixodes* ticks may feed up to 12 days. Once an infected tick has attached to a human, transmission of LD is typically regarded to occur after 36 to 48 hours of attachment, although there are reports of transmission in animal models taking just over 16 hours [15]. Transmission to humans occurs predominately by nymphs because their small size makes them difficult to notice before the blood meal and pathogen transmission is completed [13].

In the tick vector, spirochetes are acquired during a blood meal on an infected host and are maintained in the tick midgut after acquisition. *B. burgdorferi* persists in the lumen until after molting and are transmitted to a naïve host during the following blood meal. As such, the midgut plays a key role in transmission of many tick-borne pathogens. Interestingly, *Ixodes* ticks express a ligand known as the tick receptor for OspA (TROSPA), that was discovered to bind OspA on the *B. burgdorferi* surface. The interaction between the ligand and receptor are thought to be instrumental in maintaining spirochete density in the tick. As such, the midgut plays an even more important role in *B. burgdorferi* transmission [16].

Once the infected tick has attached and the blood meal has started, *B. burgdorferi* begins to alter gene expression. The ability to rapidly alter gene expression is key to the survival of *B. burgdorferi* in the tick vector and mammalian host, due to the need to adapt to changes in environmental pH, temperature, oxygen, and nutrient availability [17, 18]. As a result, surface expression of OspA decreases, thereby decreasing interaction with the TROSPA receptor. OspC expression increases, and the bacteria begin to rapidly multiply in the midgut. During this time, the spirochete begins migrating to the tick salivary glands in preparation for transmission to the mammalian host. After localizing to the salivary glands, *B. burgdorferi* is introduced into the host along with the tick salivary secretions. During the blood meal, the ticks take up blood, nutrients are removed from the blood while saliva with excess water and ions that are unnecessary for the tick are returned back to the host, creating a cycle of feeding that allows for the transmission of pathogens [14, 19].

Tick saliva is key to the tick blood meal as well as to transmission of the spirochete. The functions of the saliva include the release of compounds to reduce pain sensation and inflammation at the bite site, as well as increase blood flow and inhibit clotting. The tick must also compete with the host's innate and adaptive immunity during this time to complete the blood meal. In the presence of salivary proteins, blood continues flowing into the pool the tick feeds from under the host's skin, and the tick can feed unnoticed by the host [20-23]. With the contribution of the tick saliva, it has been shown that *B. burgdorferi* is capable of enhanced transmission and infection versus studies where the spirochetes are administered without the salivary pharmacy [24-26].

For example, the salivary protein Salp15 has been shown to bind the surface of the spirochete through interaction with OspC. Further, Salp15 inhibited complement mediated killing of spirochetes by preventing formation of the membrane attack complex, and also suppresses CD4(+) T cell responses [26, 27]. Bearing in mind these characteristics, Salp15 has been shown to promote transmission of *B. burgdorferi* into mice [28]. Another salivary protein, the tick salivary lectin pathway inhibitor (TSLPI) also functions in inhibition of *B. burgdorferi* lysis in the host by also inhibiting complement mediated lysis. TSLPI also has been shown to decrease neutrophil chemotaxis and phagocytosis, leading to enhanced *Borrelia* transmission [24]. Tick histamine release factor (tHRF) has been shown to stimulate histamine release in the host, and increase blood flow and vascular permeability. The presence of tHRF has been shown to play a role in the transmission and colonization in mice [29]. Thus, there is a notable difference in vaccine studies when infection is performed artificially using a needle compared to infections performed via the tick vector [30].

Differential gene expression of Lyme disease spirochetes

The differential gene regulation of *B. burgdorferi* contributes to its ability to live in a range of disparate environments. The spirochete exists in the unfed tick midgut, migrates to the salivary glands during the blood meal, is transmitted to the mammalian host during feeding, and adapts to the mammalian host after transmission during the blood meal. In order to study the differential gene expression, conditions in the laboratory have been standardized to mimic these events. The tick condition is represented by growth of the spirochetes in BSK-II at a temperature of 23ºC and a pH of

7.6. The mammalian condition is represented by growth at 37ºC and a pH of 6.8. To look at the transition between these phases, spirochetes are grown under the tick condition until adapted and grown to late log phase, then subcultured into the mammalian conditions and analyzed before adaptation. Utilization of these conditions allows for the evaluation of changes in gene expression of the spirochetes [18, 31-36]. Examples of differentially regulated genes expressed in *Borrelia* are described in Table 1-1.

The outer membrane of *B. burgdorferi* has been shown to contain a relatively high density of lipoproteins compared to other bacteria, and a number of these outer proteins have been shown to be differentially regulated [33, 37, 38]. Differentially regulated, surface exposed proteins found on the spirochete's surface change during the course of the enzootic cycle. These proteins function in a multitude of roles, ranging from immune response, to metabolism, to adhesion within the vector or host environment [39]. Due to the inherent immunogenic nature of lipoproteins and the role of these proteins in disease pathogenesis, lipoproteins serve as prime targets for anti-Lyme vaccines [40]. Although expressed in the unfed tick, OspA was targeted in the only vaccine licensed for human use against Lyme disease, LYMErix™. Once the mechanism of action behind the LYMErix™ vaccine was discovered, it was also shown to be the first transmission blocking vaccine [41]. OspC is expressed during the bloodmeal and in the mammalian host, and has been used as a vaccine target in several canine formulations [42-47].

Other differentially regulated proteins of *B. burgdorferi* have been studied as potential vaccine candidates, including decorin binding protein A (*dbpA*), *bbk32*, and

bb0172. These upregulated proteins correspond to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) protein family that function through binding to mammalian extracellular matrix proteins. These proteins have been determined to play a role in the colonization and dissemination processes of the spirochete in the host. Elimination of these proteins may attenuate the pathogen's virulence, highlighting the importance of these genes in pathogenesis [48, 49].

Table 1-1: Differentially regulated genes of *B. burgdorferi*

Gene	Location	Expression	Function	Citation
ospA (outer surface protein A)	lp54	UT	Lipoprotein; binds Tick Receptor for OpsA (TROSPA)	[18, 34, 50]
bptA (*borrelia persistence in ticks A)*	lp25	FT/M	Lipoprotein; necessary for tick persistence	[33, 51]
bicA (borrelia iron- and copper- binding protein A) formerly *napA*	Chrom	FT	Necessary for persistence in ticks	[31, 52]
bba52	lp54	FT	Outer membrane protein; functions in transmission	[53]
bb0365	Chrom	FT	Lipoprotein; necessary for persistence in tick	[54]
ospC (outer surface protein C)	cp26	FT	Required for infectivity	[18, 34, 50]
vlsE (variable major protein-like sequence, expressed)	lp28-1	M	Immune evasion; required for infectivity, expressed after OspC	[34, 55]
dpbA/B (decorin binding protein A/B)	lp54	M	Adhesin; binds decorin/glycosaminoglycans	[56]
ospE (outer surface protein E)	cp32	FT/M	Adhesin/Immune evasion; Binds Factor H	[57]
ospF (outer surface protein F)	cp32	FT/M	Adhesin; binds heparan sulfate/glycosaminoglycans	[58]
bb0172	Chrom	FT/M	Adhesin; binds integrin α3β1	[59]
bbk32	lp56	FT/M	Adhesin/immune evasion; fibronectin binding protein	[60, 61]
cspA (complement regulator-acquiring surface protein A)	lp54	UT	Immune evasion; Binds Factor H	[35, 62]
p66	Chrom	FT/M	Adhesin/Porin; Binds β3 chain integrins	[63, 64]
pncA (pyrazinamidase/ nicotinamidase)	lp25	FT/M	Metabolism; Nicotinamidase activity	[65, 66]
bba34 (oligopeptide permease A5)	lp25	FT/M	Transporter; Putatively binds Sodium Acetate or Sodium Bicarbonate	[67]

Chrom = Chromosomally encoded, UT = Unfed tick, FT = Fed Tick, M = Mammal

One of the most notable differentially regulated genes of *B. burgdorferi* is the variable major protein-like sequence, expressed (VlsE) is a surface exposed, immunodominant protein of *B. burgdorferi.* VlsE is upregulated in the mammalian host, and functions in immune evasion. VlsE consists of two invariable regions flanking the cassette region, that is made up of an alternation of 6 variable and 6 invariable regions, as denoted in Figure 1-1. VlsE is capable of recombination with upstream, silent cassette regions, generating proteins with a range of variable regions exposed on the protein's surface [68, 69].

Invariable region 6 (IR6) is the most conserved invariable region, and surprisingly, is the most immunodominant epitope of the cassette region [70]. It is predicted that IR6 serves as a decoy epitope, and may function through diversion of the immune system away from potentially protective epitopes of VlsE [70]. The C6 ELISA was developed as a diagnostic assay to detect infection of individuals that has shown high specificity and precision based on this conserved IR6 sequence of VlsE [71-74]. Although immunodominant, IR6 is thought to have limited surface exposure on the parent protein, as determined by X-ray crystallography and immunoprecipitation. Further, VlsE C6 antibodies cannot interact with the molecule on the surface of the spirochete, likely due to the confirmation of the protein in the native form on the spirochete, further supporting the hypothesis that IR6 is a decoy epitope [70, 75, 76].

Figure 1-1: Arrangement of the *vlsE* gene. The lipoprotein leader sequence, which directs surface localization is shown in gray, the unique conserved regions flanking the protein are shown in white. The direct repeats flank the cassette region and are shown in red. Additionally, the cassette region is shown between the direct repeats, with the invariable (light green) and variable regions (dark green) described for each of the 6 sections of the cassette region. The dark green region noted with white stripes is invariable region 6 (IR6).

Pathogenesis, detection, and treatment

Symptoms of LD are classified as local and disseminated. Infected patients develop symptoms from few days up to approximately a month after infection. *Erythema migrans* (EM, the bulls-eye rash) presents early during infection in approximately 70% of patients. The presence of this distinctive rash can streamline the diagnosis of LD. Additionally, early infection may present with nonspecific flu-like symptoms such as headache, malaise, and fever. Multiple EM lesions are possible during the early-disseminated phase of LD, and late-disseminated LD may present with neurologic, cardiologic, and rheumatologic complications. Arthritis in the joints is a common symptom of late LD, especially in the US [77]. The discovery of LD by Dr. Alan Steere occurred while investigating the prevalence of arthritis in patients in Connecticut in 1977 [78]. In terms of vaccine production, long term inflammation is troublesome when seen in any vaccine candidate, but especially so for LD vaccines as it may be interpreted as a symptom of the disease rather than a vaccine side effect [79, 80].

Once risk of infection is established through either tick bite, location in an endemic area, or presence of EM, patients can be evaluated for LD infection using laboratory diagnostics. The CDC recommends a two-tiered protocol for testing for Lyme disease that limits the amounts of false positives conveyed by the testing. Initially, the patient will undergo the first tier of the test, an enzyme immunoassay (EIA). The EIA consists of identifying host antibodies generated against either the previously described VlsE C6 antigen or against whole-cell *Borrelia* lysate. A positive or indeterminate test then progresses to the second tier, an immunoblot against either purified, diagnostically significant antigens or *Borrelia* whole cell lysate. These antigens were detected in infected patients and used as diagnostic markers of *Borrelia* infection [81]. Depending on the length of time a patient has been symptomatic, and thereby likelihood of seroconversion, serum will be evaluated for either IgM or IgG antibodies. Samples will be tested for IgM if symptoms have occurred for less than or equal to 30 days, and for IgG if symptoms have occurred for longer than 30 days, due to seroconversion. Samples tested for IgM will be considered positive upon reaction with 2 of 3 bands (OspC, BmpA, Fla), and samples tested for IgG will be considered positive upon reaction with 5 of 10 bands (OspC, BmpA, Fla, and antigens designated by their molecular weights: 18, 28, 30, 45, 58, 66, and 93 kDa proteins) [81-83].

Treatment for LD after exposure to the tick vector consists of antibiotics such as doxycycline, amoxicillin, and cefuroxime axetil. These treatment options are also effective after development of the EM [84]. However, avoiding exposure to the tick vector is the most effective method of avoiding LD infection. According both to the

CDC and the American Veterinary Medicine Association, avoidance of ticks can be accomplished through a range of methods, including: wearing long sleeved and light colored clothing to prevent skin contact with ticks and make visualizing ticks easier, using repellents, and doing tick checks on yourself and your pets after activity in areas that may contain ticks [85, 86]. These recommendations are particularly important for pets that move between the home and outdoors. Pets can carry ticks into proximity of humans, particularly in endemic areas, and as such should be treated with tick preventatives or with the available canine Lyme disease vaccines [86]. Currently there are no available LD-specific methods to prevent infection in humans in the event of a failure of the previously described methods of vector avoidance.

Early Lyme disease vaccine history

After the discovery of *B. burgdorferi* in 1977, much research was done to understand the infectious process of the organism, as well as vaccination and challenge protocols to identify a means of preventing infection with the spirochete. One of the earliest studies performed by Johnson et al. [87] evaluated the contributions of antibodies to preventing infection with spirochetes upon challenge using passive transfer methodology. To this end, immune rabbit serum was transferred to naïve hamsters prior to challenging with spirochetes using a dose of 1000 × ID_{50}. From this study, it was determined that hamsters were completely protected from challenge after transfer of immune sera, and as such, vaccines capable of generating a similar antibody based immune response would also be capable of preventing infection with *B. burgdorferi* [87].

The natural next step, completed later the same year by Johnson et al. [88], was to evaluate active immunization of hamsters. During this study, hamsters were immunized with a single dose of inactivated *B. burgdorferi* and challenged at a dose of 1000 × ID_{50} *B. burgdorferi* cells. When animals were immunized with a dose corresponding to 50 µg of *B. burgdorferi* cells, nearly complete protection (86-100%) was seen. Less protection was seen with lower amounts of vaccine, and similar protection was seen when the amount was doubled [88].

For further vaccine experiments, the mouse model was determined to be an effective model system due to the ability to generate a protective immune response against *B. burgdorferi* in mice with functional immune systems. Using this ideology, the first bacterin vaccine was designed and approved for use in dogs, and a range of these whole cell based vaccines are still employed today in veterinary medicine [42]. A working mouse model for LD was identified in 1990 that showed some similarity to the human course of infection. Although not all murine strains are susceptible to LD symptoms, susceptible strains such as C3H/He develop carditis and arthritis upon infection. Further, these susceptible mice generate an enhanced antibody immune response when compared to resistant strains (C57Bl/6) [89]. Later that same year, the first manuscript documenting the utilization of OspA as a vaccine antigen was published using the described C3H/HeJ mouse model. From this work, Fikrig and colleagues determined that by both passive and active immunization of mice with OspA, immunized mice were protected during challenge with *B. burgdorferi*. Further, these

animals developed a strong antibody response against OspA, and demonstrated no clinical manifestations of LD [90].

Although antibodies were thought to be involved in the protection mediated by OspA due to successful passive transfer experiments, the mode of protection for OspA based vaccines was unknown. Interestingly, after the licensing of the human OspA based LD vaccine LYMErix™, the mode of action was identified. LYMErix™ was the first vaccine licensed for use in humans that functioned to protect individuals by blocking the transmission of the pathogen to the host from the vector. Due to differential gene regulation, OspA is not found on the spirochetal surface in the gut of fed ticks, but is present prior to the start of the blood meal. The transmission blocking aspect of LYMErix™ was discovered through passively transferring antibodies against OspA to naïve mice at various times during the blood meal of infected *I. scapularis*. The spirochete presence and location within the vector as well as the infection status of the mice was then evaluated, and revealed that only when antibodies against OspA were administered prior to the blood meal there was protection against LD seen. Further, in ticks feeding on mice receiving antibodies before the blood meal, spirochetes were found to be limited both in terms of replication and migration to the tick salivary glands [41].

Expanding upon this idea, other outer surface proteins were determined to be visible to the immune system during infection with *B. burgdorferi*, and thus were utilized in active immunization experiments. The most utilized surface protein after OspA is OspC, a protein that is used in a range of canine Lyme vaccines. OspC is upregulated on the spirochete surface during tick feeding and early in infection

(approximately 10 days post-transmission), and as such, antibodies directed against OspC target the pathogen in the host. To evaluate protection of OspA, OspB, OspC, and OspD, mice were immunized with recombinant versions of each of the proteins prior to challenge with live *B. burgdorferi* [91]. From this study, Probert et al. [91] found that of these antigens, only OspD was not capable of generating an antibody response, demonstrating the utility of OspC as another LD vaccine candidate. Although OspC is expressed in the tick salivary glands, it has not been shown to function as a transmission blocking vaccine [91]. Although these outer surface proteins are protective, the variability of OspC and to a lesser degree OspA will make the generation of a globally relevant vaccine more challenging than it would be if a more conserved antigen was utilized [92-97].

LYMErix™: a licensed, human Lyme vaccine

In the history of LD, one vaccine has been licensed for use in humans. This vaccine, LYMErix™ (SmithKline Beecham), was based on the lipoprotein outer surface protein A (OspA) and licensed in 1998 by the FDA. The vaccine was released in December 1998 and voluntarily removed from the market February 2002. Notably, another OspA vaccine candidate was also generated in the late 1990s, ImuLyme™ (Pasteur Mérieux Connaught), and made it to phase III clinical trials. Pasteur Mérieux Connaught, however, opted not to apply for licensure for unspecified reasons [98, 99]. A comparison of LYMErix™ and ImuLyme™ can be found in Table 1-2.

Table 1-2: Characteristics of human LD vaccines at Phase III clinical trials

Name	Company	FDA License	*Borrelia* Antigen	Adjuvant	Efficacy *	Location	Citations
LYMErix™	SmithKline Beecham	Yes	Full length, lipidated OspA from strain Z S7	Lipidation, Aluminum Hydroxide	76%	IM	[100, 101]
ImuLyme	Pasteur Mérieux Connaught	No	Full length, lipidated OspA from strain B31	Lipidation only	92%	IM	[99, 102]

*Efficacy is after 3 doses of vaccine.

Both vaccines were administered intramuscularly at a dose of 30 µg and a frequency of 0, 1, and 12 months with required annual boosts.

OspA was selected as the antigen of these vaccines because of its role in the life cycle of *B. burgdorferi*, as well as its relative conservation across the LD causing *Borrelia* species when compared to other outer surface proteins, such as OspC [16, 41, 103, 104]. Immunization with a lipidated version of OspA in conjunction with an aluminum hydroxide adjuvant (LYMErix™ only) was found to generate a long-lasting antibody response that targeted *B. burgdorferi* within the tick. The maintenance of an elevated level of these anti-OspA titers in the host is a necessity due to the mechanism of action in targeting the spirochetes within the tick vector, and may account for the 76% efficacy of the vaccine [41, 105-107]. The vaccine worked because the ticks take up the antibodies and complement factors as well as blood while feeding. Thus, these factors could kill spirochetes within the tick gut, and prevent infection with *B. burgdorferi* [108, 109]. As such, using an OspA based vaccine requires frequent boosting to maintain titers high enough to induce killing of the spirochetes in the tick vector.

While SmithKline Beecham did not give a specific reason for the removal of the LD vaccine from the market, low sales were likely the cause. Use of the vaccine fell after suggestions that the vaccine caused an autoimmune response resulting in inflammation like that seen during infection. It was thought that this reaction was due to the similarity between OspA (amino acids: 163-175), the immunodominant T cell epitope in a subset of patients post vaccination, and human lymphocyte function associated antigen (LFA)-1 (amino acids: 332-340) [110, 111]. These peptides bind certain major histocompatibility complex (MHC)-II alleles. *HLA-DRB1*0401* and *HLA-DRB1*0404* in particular were found to bind $OspA_{163\text{-}175}$, and would be expected to bind to the LFA-1 autoantigen as well, causing an autoimmune response and antibiotic refractory Lyme arthritis. The binding of OspA to these alleles caused the production of a Th1 cytokine environment, and could cause the resulting joint damage. These alleles have also been linked to rheumatoid arthritis, however, this linkage has not been clearly demonstrated *in vivo* for *B. burgdorferi* [80, 111-113]. Studies in OspA vaccinated hamsters demonstrated this link, prompting investigation into the human reports, but no linkage between homology and autoimmune response resulting from vaccination was shown in humans [114, 115]. As such, public perception is another hurdle that a vaccine for LD must be able to overcome.

Vaccines after LYMErix™: multivalent and chimeric molecules

OspA based vaccines were not abandoned with the demise of LYMErix™. Before the generation of LYMErix™, Golde et al. [95] found that the OspA antigen was protective as a vaccine against tick challenge, with some cross protection against strains

of *Borrelia* carrying marginally heterogeneous OspA proteins. However, even in 1995, it was known that OspA varied between serotypes and strains. In order to generate a broadly protective vaccine that would be applicable in the US as well as in Europe and Asia, a multivalent OspA vaccine would have to be generated, or a more conserved antigen would have to be utilized [95]. Further, other outer surface antigens have been tested for utility as Lyme disease vaccine candidates, and have demonstrated even more heterogeneity. Similar to OspA, OspC has been through trials in various formulations, and faces some of the same challenges as OspA. As described previously, OspC is present during mammalian infection and shows a greater amount of heterogeneity than OspA. The lack of conservation between OspC epitopes is one of the contributing reasons as to why OspC was not the primary target of Lyme vaccine studies. Consistent with this idea, Earnhart and Marconi evaluated the OspC epitopes phylogenetically and found that to generate a complete, broadly protective vaccine against LD using the OspC antigen, 34 epitopes would be required [93]. The C-terminal region of OspC has been found to be more conserved than the protein as a whole, and as such, is likely being targeted in future vaccine studies. Although there are difficulties with these antigens, it is likely that these antigens are still targeted as candidates due to the extensive safety profiles completed for each of these molecules due to previous use in veterinary and human vaccines.

Taking into consideration the high amount of variability in currently targeted antigens, present LD vaccine research is moving forward through the utilization of multimeric and chimeric molecules to generate a protection against a broad range of

Lyme disease causing spirochetes. Advances using this approach include one such vaccine developed by Comstedt et al. [116]. To generate protection, this vaccine includes 6 of the most clinically relevant OspA antigens. The utilized antigens include 3 coming from varied OspA serotypes of *B. garinii*, and one epitope each from *B. burgdorferi*, *B. afzelii*, and *B. bavariensis*. Additionally, these OspA antigens have been formulated to lack the region of potential homology by using the *B. afzelii* sequence for this region of the protein, likely removing the concern for autoimmune responses [116, 117]. This vaccine candidate has gone through Phase I/II clinical trials, where it was determined by Wressnigg et al. to be safe and immunogenic. Phase III clinical trials will be pursued with the intention of evaluating efficacy [97]. This candidate is being pursued by Valneva and is now referred to as VLA15 [118].

OspC has also been studied as a candidate for multivalent vaccine development. To this end, chimeric tetravalent and octavalent OspC anti-Lyme vaccine candidates have been evaluated [47, 96]. Through this work, Earnhart et al. has demonstrated immunogenicity of the antigen constructs in the murine model with potential for antibodies to recognize spirochetal surface antigens. Both in the tetravalent and octavalent formulations, antibodies specific to each spirochete included in the formulation have been detected in immunized animals.

Although the outer surface proteins have promise as LD vaccine candidates, the utility of a novel and conserved antigen that does not require the use of multivalent antigens is still clear. Another concern for LD vaccines based on the outer surface proteins is public perception due to the negative publicity LYMErix™ received.

Additionally, the antigens utilized are from the most common genospecies responsible for LD infection, however, there are other pathogenic *Borrelia* that are not accounted for by this formulation. These formulations thus do not offer complete protection from all LD spirochetes. This concept is particularly important when considering the recent discovery of *B. mayonii* [3].

In addition to the multivalent OspA and OspC vaccines, several other antigens are being evaluated as LD vaccine candidates, including: BBA52, BBK32, and DbpA. A multicomponent vaccine was generated with the idea of using the protection from BBK32, DbpA, and OspC in tandem. From this study, the differences between single, double, and triple component vaccines are clearly seen, as each change had a distinct effect on amount and isotype of antibody production as well as on protection. Changing antigen amounts during immunizations were not seen to yield a linear change in protection, but rather, changing concentrations of any or all members of the multicomponent vaccine had an effect on immune response. For example, decreasing DbpA compared to the other components yielded lower bacterial recovery than using all three antigens in the same amounts. As such, further studies are required to better understand the complex response of the immune system to dose and antigen combination in order to optimize the protection of this multicomponent construct [119].

Veterinary Lyme disease and vaccines

Dogs become infected with *B. burgdorferi* through the bite of an infected *Ixodes* tick, particularly due to their propensity to move between the outdoors and inside the home. Once infected, dogs may develop symptoms of ranging severity, including:

lethargy, fever, joint swelling and intermittent lameness, and in some cases nephrological symptoms that can end in renal failure and death [120, 121].

Presently there are 5 distinct vaccines against *B. burgdorferi* produced by 4 different companies that are licensed for use in canids by the United States Department of Agriculture. Additionally, there are combination vaccines produced by Boehringer Ingelheim Vetmedica, Inc. that consist of antigens against *B. burgdorferi*, *Leptospira interrogans*, and a range of viral illnesses including canine distemper, adenovirus type 2, coronavirus, parainfluenza, and parvovirus [122].

Table 1-3: Characteristics of presently licensed canine LD vaccines

Name	Company	*Borrelia* Antigen	Adjuvant	Frequency	Citations
Duramune Lyme ®	Boehringer Ingelheim Vetmedica, Inc.	Bacterin; Strains: 2	Proprietary	0, 2-3 weeks. Annual boosts.	[42, 45, 124, 125]
Nobivac® Lyme	Merck Animal Health (Formerly Schering-Plough)	Bacterin; Strains: 2	Oil	0, 2-4 weeks. Annual boosts.	[43]
LymeVax®	Zoetis (Formerly Pfizer and Fort Dodge)	Bacterin; Strains: 2	Aluminum hydroxide	0, 2-3 weeks. Annual boosts	[44]
Recombitek® Lyme	Merial Limited	Recombinant: Lipidated OspA	OspA Lipidation	0, 2-3 weeks. Annual boosts.	[46]
VANGUARD ® crLyme	Zoetis	Chimeric: OspA (epitopes: 1) OspC (epitopes: 7)	Aluminum hydroxide	0, 3 weeks. Annual boosts.	[96, 123]

All listed vaccines are administered at 1 mL per dose administered subcutaneously.

The trend towards using multivalent and recombinant antigens to prevent Lyme disease has also been applied to veterinary vaccines. In the last quarter of 2015, Zoetis

released a recombinant, multivalent LD vaccine, VANGUARD ® crLyme. VANGUARD ® crLyme contains heptavalent OspC formulated with a single OspA antigen, allowing the vaccine to target *B. burgdorferi* within the vector (OspA) or mammalian host (OspC) [123]. More details on VANGUARD® crLyme and other available veterinary vaccines available in 2017 can be found in Table 1-3.

Although domestic cats are less often thought of as animals that may present with LD, it has been shown experimentally that cats can become infected with *B. burgdorferi.* LD in cats generally presents with minimal symptoms, however may include fever, lethargy, and lameness [120, 126].

To the contrary, horses may become infected with LD and develop symptomatic illness. Horses may present with lethargy, weight loss, uveitis, lameness, muscle tenderness, hyperesthesia, as well as neurological complications including facial nerve paralysis and meningitis [127, 128]. Chang et al. [129] demonstrated in 1999 that vaccination of ponies with OspA was protective from subsequent challenge with *B. burgdorferi*. It is therefore possible to utilize an LD vaccine to protect equids from LD infections, and could be particularly useful in endemic areas [129]. However, no LD vaccines are currently licensed for use with horses.

In cattle, LD may present with fever, swollen and stiff joints, lameness, weight loss, and may result in abortion in pregnant cows [130, 131]. There have not been any attempts to vaccinate cattle against LD specifically, rather, the anti-tick vaccine approach has been utilized in conjunction with acaricides to prevent tick infestation of livestock. The effect ticks have on production animals include: damage to skin, weight

loss, decreased milk production, induction of abortion, death, and transmission of a range of tick borne diseases (TBDs).

Vector targeted alternatives

Vector targeted vaccines are not a novel approach to TBDs. Notably, the Cattle Fever Tick Eradication Program's efforts targeted the one host ticks *Rhipicephalus (Boophilus) microplus* and *R. (B.) annulatus* in the US. The cattle fever ticks vector the protozoan parasites *Babesia bovis* and *B. bigemina*. Upon transmission, these parasites can cause death in up to 90% of naïve, susceptible cattle. Another TBD of concern transmitted by cattle fever ticks is *Anaplasma marginale* that can also cause lethargy, weight loss, and death. Due to an integrated approach that encompasses the use of acaricides, vaccination, and tightly monitored quarantine zones, these ticks have been limited in the US to the southern border of the US and Mexico [132, 133]. As resistance to acaricides and ivermectin increases, vaccination may take a more prominent role in the control of tick vectors, particularly as the eradication program faces a resurgence of CFTs in 2017 [134, 135].

The anti-tick vaccines TickGardPlus and Gavac™ are based on the BM86 antigen from *R. (B.) micrplus* ticks, and are used to control cattle fever ticks (CFTs). BM86 is a glycoprotein found on epithelial cells in the tick gut lumen. Immunization of cattle with the BM86 antigen has been shown to decrease the endocytic activity of gut cells and overall fitness of the female tick [136, 137]. Taken together, immunization of cattle with BM86 based vaccines decreases the number of ticks and therefore potential exposure and transmission of TBDs, as well as minimize acaricide resistance and

environmental contamination thus contributing to the cattle fever tick eradication effort. Although the BM86-based vaccine can control species outside of *R. (B.) microplus*, including *Boophilus decoloratus* and *Hyalomma dromedarii*, the vaccine is not effective against all tick species. BM-86 based vaccines show no effect against *Amblyomma cajennense, Amblyomma variegatum, Rhipicephalus appendiculatus* [138, 139].

Table 1-4: Selected tick vaccine antigens currently under investigation

Antigen	Tick	Class	Result	Citation
Bm86	*R. microplus*	Gut epithelium; Glycoprotein	Impaired feeding; reduced survival; decreased egg mass; decreased larval infestations	[137, 142]
Serine protease inhibitor 19 (AAS19)	*A. americanum*	Salivary protein; Serpin	Impaired feeding; reduced survival; reduced oviposition	[143, 144]
Ixodes ricinus serpin-2 (IRS-2)	*I. ricinus*	Salivary protein; Serpin	Impaired feeding; decreased weight	[20, 144, 145]
Tick histamine release factor (tHRF)	*I. scapularis*	Salivary Protein; Immunoactive	Impaired feeding; decreased transmission of *B. burgdorferi*	[29]
Subolesin	*I. scapularis*	Homeostasis	Impaired feeding; reduced survival; reduced weight; reduced oviposition; decreased pathogen acquisition	[146-149]
Aquaporin	*R. microplus*	Homeostasis	Reduced tick survival; reduced molting; reduced ticks per animal; reduced tick weight	[150-152]
Ferritin 2	*I. ricinus*	Homeostasis	Reduced number of ticks per infestation; reduced weight; reduced fertility	[153, 154]
64P	*R. appendiculatus*	Salivary Protein; Cement	Reduced infestation; increased mortality; impaired feeding; decreased egg mass	[155, 156]

Additionally, experiments utilizing *Ixodes* orthologs of BM86 did not prove successful to inhibit feeding or oviposition of *I. ricinus* on immunized rabbits. It is therefore likely that this approach would not be successful for *I. scapularis* control, and

therefore have no impact on transmission of LD [140]. More promising approaches to an anti-tick vaccine that could apply to *I. scapularis* utilize a range of tick antigens, including salivary proteins, gut proteins, and proteins involved in homeostasis and regulation. Some of the targeted tick antigens, such as the TSLPI and TROSPA are used with the primary focus of inhibiting transmission or infection of ticks with *B. burgdorferi* rather than specifically targeting vector longevity [16, 24]. In contrast, there are a range of proteins being evaluated as vaccines that have effects on the tick vector in terms of ability to take a full blood meal or reproduce [141]. A few examples of these targets include proteins involved in tick homeostasis and immune evasion, as described in Table 1-4.

Although the eradication effort focused on the protection of livestock, the CFT has other host in the US, including white tailed deer and exotic animals such as nilgai and red deer. As such, the ticks may be maintained even if cattle are removed from infected areas and treated for ticks, thus posing a risk for reinfestation of cattle. Deer and exotic animals in pens may be treated similarly to cattle, however, free range animals pose more of a challenge for the elimination of ticks. These animals may be treated using feeders 2- or 4-poster device, that features ivermectin treated corn as a bait source and permethrin infused rubbing posts [133, 157, 158].

Wildlife vaccination using oral bait

A bait-vaccination approach similar to that utilized for rabies has been used to decrease LD prevalence in reservoir hosts and thereby decrease transmission to humans. Bait based vaccination is useful for LD in particular due to the lack of a human vaccine.

In 1991, Fikrig et al. demonstrated that oral recombinant OspA (rOspA) vaccination using OspA expressing *E. coli* was sufficient to protect mice from *Borrelia* challenge [159]. From this knowledge, Tsao et al. [160] demonstrated that the natural reservoir for *B. burgdorferi, P. leucopus* (white-footed mouse), could be immunized with OspA derivatives using subcutaneous inoculation resulting in a modest decrease in the number of infected *I. scapularis* nymphs in the test area (Connecticut) [160]. Building on this idea, Gomes-Solecki et al. [161] developed a method of bait delivery, and in laboratory experiments were able to show that mice immunized with OspA based bait vaccines were able to resist infection with *B. burgdorferi*, and even decreased pathogen load in the vector [161]. In 2011, further studies found that the OspA based bait vaccine could be optimized for use in the field and generate a year-long response in the white-footed mouse against *B. burgdorferi* [162]. A field study in New York then demonstrated a time dependent decrease in *I. scapularis* nymphal infection of 76% after 5 years of treating with bait vaccine [163].

Although there is promise with bait-based vaccination of reservoir hosts to decrease transmission to humans, the utilization of a bait program requires large quantities of vaccine, manpower to distribute bait, and cannot guarantee complete elimination of the pathogen. Other animals are capable of maintaining *B. burgdorferi* infection that will not be targeted by the vaccine, and not all mice will ingest the vaccine bait. For this reason, there is still a need for a human vaccine to increase the likelihood of protection from LD infection in humans. A program utilizing the reservoir targeted vaccine could reduce the pathogen in the wild in endemic areas, but people in endemic

areas that lack vaccine coverage would still be at risk. This method also would likely not be employed in areas that have lower prevalence of *Borrelia*, leaving people at risk in these areas as well.

Reverse vaccinology

The concept of reverse vaccinology began when the approach was used to identify novel antigens against *Neisseria meningitidis* serogroup B, a bacterium that had been problematic to vaccinate against due to antigenic variability and similarity to human proteins. Pizza et al. [164] performed whole genome sequencing of *N. meningitidis* serogroup B strain (MC58) to determined potential vaccine candidates using the reverse vaccinology approach. To identify a candidate antigen, outer membrane vesicles were targeted due to the ability to generate antibody response and protection data showing that the vesicles were able to prevent *N. meningitidis* infection. To this end genes were amplified, cloned, expressed, selected and screened in the murine model for protection. Proteins were then evaluated for protection as determined from sera by determining antibody response and bactericidal activity [164]. Today, reverse vaccinology starts with a known genome, proceeds with *in silico* analysis and antigen predictions, and ends with vaccine candidates for screening.

Comparative genomics may serve as a starting point for identifying conserved proteins between related organisms. The genome of *B. burgdorferi* strain B31 was published in 1997 and was updated in 2000 [165, 166]. With this information, a comparison of the genomes of *B. burgdorferi* and the distantly related spirochete *Treponema pallidum* was performed by Subramanian et al. in 2000. Using this approach,

conserved genes between *Borrelia* and *Treponema* were identified, and these genes are likely to be conserved across the family Spirochaetaceae. In this analysis, a family of proteins with particular interest to the identification of a vaccine candidate was described.

To develop a novel vaccine against *B. burgdorferi*, the target product profile must be considered. LYMErix™ required 3 doses over 1 year to generate an efficacy of 76% in individuals aged 15 – 70, and was potentially responsible for generating inflammation and cross-reactivity in humans [101]. Therefore, the ideal qualities of a new LD vaccine include: safety in humans and animals, >76% efficacy in only 2 doses, affordable to produce, strong IgG antibody response, generation of immunologic memory, and capability to be scaled up for affordable large scale production. Minimally, the vaccine must still be safe for use in humans and animals, achieve an efficacy of greater than 76% after 3 doses, generate a strong IgG and memory antibody response, and have potential to be produced commercially in a cost-efficient manner. LYMErix™ was on the market in 1998, and the cost for the series consisting of 3 doses at 0, 1, and 12 months with annual boosts was approximately $60 per dose [167, 168]. The recombinant OspA antigen used in LYMErix™ was produced on a large scale in *E. coli* because the antigen was a bacterial lipoprotein that could be produced efficiently by the *E. coli* expression system, and likely would still be a viable expression systems for future LD vaccine constructs [169, 170] However, because the OspA antigen was lipidated, expression was lower comparatively to non-lipidated constructs [171]. New approaches to LD vaccines would ideally cost less than LYMErix™, either due to

decreased number of immunizations, or more cost effective production methodology or expression platforms.

Utilizing the reverse vaccinology approach to select a protein, and eventually an antigenic peptide epitope, makes it possible to put together a "shopping list" of characteristics required for a new LD vaccine candidate [172]. These characteristics include conservation across the genus *Borrelia* in an effort to generate protection across both the US and Eurasian strains of *B. burgdorferi* sensu lato complex: extracellular exposure as potentially indicated by the presence of transmembrane domains and signal peptides, and predictions of functional domains likely to be involved in colonization and dissemination within the mammalian host. Following *in silico* analysis, these characteristics will be confirmed *in vitro* if demonstrated in a previously uncharacterized protein, and further analysis to determine regions of antigenicity will be performed both using *in silico* analysis and *in vivo* experiments [172].

To accomplish these goals, the ideal result of this work would be the discovery of a peptide antigen capable of preventing transmission of LD from tick vector to mammalian host. A peptide antigen is readily producible and amenable to formulation through a range of methods including linking to carrier molecules, scaffolding, and immunization with immunostimulatory molecules and adjuvants.

The VWFA domain containing proteins

The vonWillebrand Factor A (VWFA) domain protein family is found broadly in eukaryotes, bacteria, and archaea [173]. The localization and function of VWFA domain containing proteins differs across this range of organisms, however, the VWFA domain

containing proteins maintain Rossmann structural folds and the ability to coordinate divalent cations. This ion interaction takes place due to the presence of a non-contiguous sequence – the metal ion dependent adhesion site (MIDAS) motif [174]. VWFA domain containing proteins are found in a range of capacities in eukaryotes, bacteria, and archaea (as reviewed by Whittaker and Hynes [175]). In mammals, the VWFA domains are found in integrins, extracellular matrix proteins, and the vonWillebrand factor, a multimeric glycoprotein [176, 177]. In prokaryotes, many VWFA domain containing proteins have unknown functions, but some function as chelatases and through binding to mammalian host proteins. In addition, intracellular VWFA domain containing proteins are found in prokaryotes and eukaryotes, and function in a range of cellular functions, including DNA repair and protein degradation [173, 178].

An analysis performed by Subramanian et al. [179] demonstrated the 43% of the genomes of *Treponema pallidum* and *B. burgdorferi* were orthologous. Of the genes highlighted in this analysis, the VWFA domain containing genes were identified as a newly identified protein family in the spirochetes. In *B. burgdorferi*, 4 VWFA domain containing genes were identified on the bacterial chromosome: *bb0172, bb0173, bb0175,* and *bb0325* [173, 179]. Although these genes were identified in the comparative genome analysis, the *Borrelia* VWFA domain containing proteins were not found to be highly similar to the VWFA proteins in *Treponema* [179].

Further, Subramanian et al. [179] also highlighted the similarity of the *B. burgdorferi* VWFA domain containing proteins to the *Plasmodium* thrombospondin-related adhesive protein (TRAP), and idea that was further confirmed through

alignments with *Borrelia* protein sequences [59, 179]. TRAP acts through adhesion to mammalian liver cells, and thus is reasonable to expect that similar proteins of *B. burgdorferi* could facilitate a similar function [180]. As such, BB0172 was characterized by Wood et al. [59] and shown to be exposed on the surface of *B. burgdorferi* and to bind integrin α3β1. It is of interest, therefore, to also characterize BB0173 in order to further expand on the interactions of *B. burgdorferi* with its environment. Further, characterization of BB0173 may yield another candidate protein for use in a Lyme disease vaccine. BB0175 contains the VWFA domain, however, does not contain any putative transmembrane domains, and as such, likely is not surface exposed. BB0325 also lacks putative transmembrane domains and is not found in the same highly conserved region of the chromosome that *bb0170 – bb0176* are located in. For these reasons, BB0175 and BB0325 will not be evaluated further in this work as potential vaccine antigens.

Spirochaetal and host cells interact through protein binding events to facilitate the migration of *B. burgdorferi* within the mammalian host away from the tick bite to areas of long term survival, such as the skin, joints, heart and bladder. These host-pathogen interactions are required for the pathogenicity of *Borrelia*, particularly as the pathogen responds to changes in temperature, pH, oxygen, and the structural environment through differential gene expression [181-184]. With regards to the endothelial cells, *B. burgdorferi* has been shown to bind the extracellular matrix (ECM) components: fibronectin, laminin, collagens (type I, III, and IV), and integrins, among other components, through *Borrelial* proteins such as BBK32, BBA33, ErpX, P66,

BBA07, BB0172 respectively [59, 183-191]. Due to the role of BB0172 and the similarities between BB0172 and BB0173, it is probable that BB0173 is involved in colonization of dissemination in the mammalian host due to the highly-conserved nature of the protein, the presence of a VWFA domain with MIDAS motif, and the *Bacteroides* aerotolerance domain.

Taken together, the common themes of the VWFA domain containing proteins are the ability to coordinate divalent cations and facilitate protein binding to either assemble a protein complex or to bring cells into contact. Further, the VWFA domain containing genes are highly conserved across the genus *Borrelia*, and as such, have potential to serve as anti-Lyme vaccine candidates. The overall goal of this work is to identify a highly-conserved antigen derived from *B. burgdorferi* that can be used to develop a novel anti-Lyme disease vaccine for use in humans and companion animals. The VWFA domain containing proteins of *B. burgdorferi* are prime targets for anti-Lyme vaccine development due to their conservation in pathogenic *Borrelia* and their potential to function during times that are key for mammalian infection: colonization and dissemination. The VWFA domain containing protein BB0173 will be further investigated to determine potential for use as an anti-Lyme disease vaccine antigen.

Central hypothesis and specific aims

Based on the literature, my central hypothesis is that a novel, highly conserved vaccine candidate for LD can be identified with the ability to induce protective immunity in the murine model and that could potentially act as an alternative to the presently employed heterogenous surface proteins.

To evaluate this hypothesis experimentally, the following specific aims will be carried out using *in silico, in vitro,* and *in vivo* methodologies.

Aim I: Characterize vonWillebrand factor A-domain containing protein BB0173 from *Borrelia burgdorferi* and determine potential for use as a vaccine candidate.

Aim II: Evaluate the protection against Lyme disease induced by the surface exposed protein BB0172 and derived peptides in the murine model.

Aim III: Develop delivery methods that will enhance immunogenicity of candidate vaccine peptides in the murine model for Lyme disease.

The objective of this work is to identify a novel vaccine candidate derived from a vonWillebrand factor A containing protein that is highly-conserved in *B. burgdorferi*. This work is significant because current vaccine development has focused on using complex, multimeric antigens derived from variable surface targets to generate protection against *B. burgdorferi* s.l. pathogens. As such, a novel candidate may pave the

way for a safer and more effective LD vaccine for use in companion animals. Further, the development of a novel antigen could also advance the development of a human LD vaccine.

CHAPTER II

EVALUATION OF BB0173, A MEMBRANE PROTEIN COMPONENT OF AN AEROTOLERANCE MEDIATED GENE COMPLEX

Introduction

B. burgdorferi has a fragmented genome composed of a linear chromosome and more than 20 extrachromosomal elements [165]. Genes required for virulence are often localized in the extrachromosomal elements of the bacteria. In contrast, the linear chromosome contains the majority of housekeeping genes required for bacterial replication and homeostasis in the tick and mammalian environments, and thus are often required for the basic survival of the spirochete. Due to stabilizing selection, the chromosomal genes of *B. burgdorferi* also demonstrate low levels of genetic variability, unlike the virulence genes found on the extrachromosomal elements (as reviewed by Brisson et al. [192]). As a result of these general characteristics of stability and necessity for survival of the pathogen, chromosomal genes are ideal targets for LD vaccine development.

Comparative genome analysis between *Treponema* and *Borrelia* identified a range of conserved genes between the spirochetes [179]. Using this methodology, genes coding for *bb0172* and *bb0173* were identified on the linear chromosome that are highly conserved across pathogenic *Borrelia* and have a potential role in pathogenesis of *B. burgdorferi* due to the presence of the VWFA domain and transmembrane domains

contained within the same gene. Further, *bb0170* and *bb0173* contain a domain identified originally in the aerotolerant anaerobe *Bacteriodes fragilis*, the *BatI* (*Bacteriodes* aerotolerance) complex [193]. This conserved genomic region has also been described in *Rhizobium leguminosarum* and *Leptopsira interrogans*, although no definite function has been determined [193, 194]. Previous studies indicate that BB0172 has a functional VWFA domain and MIDAS motif capable of biding integrin α3β1, and is inserted into the outer membrane. Further *bb0172* is differentially expressed, and found only during the transition between tick vector and mammalian host. Due to the similarities between *bb0172* and *bb0173,* it is worthwhile to further evaluate the localization, expression, and function of *bb0173* in regards to the survival and pathogenicity of *B. burgdorferi* in order to better evaluate the antigenic potential of these genes for vaccine design.

Attempts to understand the signaling and gene expression in *B. burgdorferi* have utilized a global analysis of genes in a variety of conditions mimicking the tick and mammalian host. Differential gene expression may be an indicator of the physiological role of the protein, as upregulation will occur when the differentially expressed gene product is required. In global analyses of *B. burgdorferi* gene expression, expression changes in *bb0173* have been noted. In 2003, Ojaimi et al. [36] used whole genome arrays to evaluate the effect of temperature on gene expression in *B. burgdorferi.* In this study, BB0175 was found to be upregulated at 37ºC compared to 23ºC, a result that was confirmed by Tokarz et al. [35] in 2004 a study on the effect of blood and temperature on *B. burgdorferi* gene expression. Building on this understanding of gene regulation,

Rogers et al. [195] studied the function of the two-component signaling response regulator 1 (Rrp1) and hybrid histidine kinase-response regulator (Hpk1). In these studies, microarray analysis was used to determine expression changes in between wildtype *B. burgdorferi* or *B. burgdorferi* lacking *rrp1*. In this study, expression of 140 genes was found to be altered by the absence of *rrp1*, including *bb0170, bb0173, bb0174, and bb0175.* To evaluate the change in expression, a ratio of wildtype/mutant hybridization values were used, demonstrating that the ratios for *bb0170* and *bb0173* were greater than 100, while *bb0174* presented a much lower ratio (27) and *bb0175* was found to be very low (<5). Genes under the control of *rrp1* are expected to be induced in the tick condition (25ºC, pH 7.6), and as such, the low ratio for *bb0175* agrees with the previous literature showing *bb0175* upregulation under at 37ºC [35, 36, 195].

In order to better understand the contribution of *bb0170 – bb0176* genes to the survival and pathogenicity in *B. burgdorferi* and other *Borrelia* species, BB0173 will be evaluated and characterized. BB0173 is a conserved hypothetical protein that contains several predicted transmembrane regions, a VWFA domain and a metal binding motif. Through evaluation beginning with an *in silico* analysis and progressing to *in vitro* DNA and protein studies, the membrane topology of this protein is explored in order to evaluate its cellular localization and potential function. Taken together, the investigation of these genes will enhance the understanding of the biology of *B. burgdorferi*, and may lead to an increased ability to target the pathogen using novel therapeutics.

Materials and methods

Growth conditions of Borrelia burgdorferi

DNA used for experiments was extracted from *Borrelia burgdorferi* B31 A3 strain, as described in Table 2-1. Cells were grown in BSK II with 6% inactivated normal rabbit serum (iNRS) and 1% CO_2 at either pH 6.8 and 37°C (mammalian, 37°C/pH 6.8) or pH 7.6 and 25°C (tick, 25°C/pH 7.6). Additionally, *B. burgdorferi* grown under shifted conditions between tick and host were started under tick growth conditions [59]. Once the culture reaches a density of 2–3 × 10^7 spirochetes/ml a subculture is started and grown under mammalian conditions until reach a density of 5 × 10^7 spirochetes/ml. Low oxygen conditions were generated using Oxyrase® for Broth (Mansfield, Ohio) at 0.025 mL Oxyrase® per 1.0 mL BSK II media. If no growth condition was specified, cells were grown in BSK II pH 7.6 at 32°C with 6% iNRS and 1% CO_2.

RNA and genomic DNA purification for detecting bb0173 transcript by PCR

RNA was extracted as previously described [59, 196, 197]. Briefly, *B. burgdorferi* cultures were grown to a density of 2–3 × 10^7 spirochetes/ml under the shifting conditions outlined above. RNA was extracted by re-suspending the bacterial pellets with 0.2 ml RNA-Bee (Tel-Test, Inc., Friendswood, TX) for every 10^6 cells. Following extraction with chloroform, RNA was precipitated with isopropanol, washed with 75% ethanol, air dried, and re-suspended in RNase-free water. To remove contaminating DNA, the RNA was treated twice with DNase I at 37°C for 45 min. Then the total RNA was quantified spectrophotometrically, and reverse transcribed to cDNA

using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). From *B. burgdorferi* cultures growing under tick-feeding conditions (pH 6.8 and 37ºC) and regular growing condition (pH 7.6, 32ºC), genomic DNA was obtained by general phenol:chloroform extraction.

Table 2-1: Bacterial strains and plasmids used in this study

Bacterial Strain or Plasmid	Genotype	Source
Borrelia burgdorferi B31A3	cp9^{-}, wild type	Rocky Mountain Labs [200]
E. coli strains		
OneShot Top10	Cloning host; F^{-} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL*(Str^{r}) *endA1 nupG*	Invitrogen
Rosetta(DE3)pLysS	Expression host; F^{-} *ompT hsdS*$_{B}$(r$_{B}^{-}$ m$_{B}^{-}$) *gal dcm* (DE3) pLysSRARE (Cam^{R})	Novagen
Plasmids		
pCS1-5	pCR2.1(*bb0173*$_{T}$)	This study
pCS1-9	pET23a(*bb0173*$_{T}$)	This study

Gene expression

RNA, cDNA, and genomic DNA (positive control) samples from each growing condition were used to detect when *bb0173* was expressed. A 501-bp fragment of *bb0173* was amplified using primers BB0173cDNA-F (*B. burgdorferi* nucleotides 175811-175860 and BB0173cDNA-R (*B. burgdorferi* nucleotides 175334-175357) (Table 2-2). Primers specific to the *flaB, ospC,* and *p66* genes were also included as controls for the temperature and pH shift as previously described [59, 198, 199]. PCR products were separated on 0.8% agarose gels and imaged using the Bio-Rad Gel Doc^{TM} XR system.

To evaluate the potential contributions of the Bat domains in BB0173 and BB0175, cultures were grown to late log phase under microaerophilic (standard oxygen with 1% CO_2) or low oxygen conditions, and transcripts of *bb0170 – bb0176* were analyzed. Low oxygen conditions were generated by including Oxyrase® for broth. Expression from genes *bb0170 – bb0176* was evaluated by conventional PCR to qualitatively evaluate a change in gene expression.

Computer-assisted analysis of BB0173 transmembrane regions

Putative insertion of hydrophobic regions (HR) from BB0173 proteins was predicted using the ΔG Prediction Server v1.0 using standard parameters combined with subsequent detection of the lowest apparent free energy differences (ΔG_{app} values) ([201]; http://dgpred.cbr.su.se/). Models of tertiary structure were generated using template 4jdu.1.A, an aerotolerance related membrane protein, using SWISS-MODEL [202-204].

Cloning of putative transmembrane regions

For the membrane insertion of isolated BB0173-segments, HR1 (residues 7 – 25), HR2 (residues 55 – 77), HR3 (residues 163-185) and HR4 (residues 310 – 328) fragments were independently amplified and introduced into the modified *E. coli* leader peptidase (Lep) sequence from the pGEM1 plasmid [205] using the *Spe*I/*Kpn*I sites. After an overnight ligation, constructs were electroporated into TOP10 cells. Positive clones were selected on ampicillin plates (100 µg/ml). The primary sequence of each construct was confirmed by DNA sequencing

Alternatively, we prepared templates for the *in vitro* transcription of the truncated *BB0173* mRNA with a 3´-glycosylation tag. BB0173 truncated constructs were obtained by using forward primers that include the T7 promotor sequence at the 5' end. The 3´ reverse primers were designed to have approximately the same annealing temperature as the 5` forward primer, contained an optimized glycosylation C-terminal tag followed by tandem translational stop codons, TAG and TAA, and annealed at specific positions to obtain the desired polypeptide length as previously described [206]. Primers described in Table 2-2.

Agarose gels (2%) were used to verify PCR product size then samples were cleaned using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI). After an overnight ligation, constructs were electroporated into TOP10 cells. Positive clones were selected on ampicillin plates (100 µg/ml) and verified by sequencing (Eton Biosciences, San Diego, CA). After sequencing confirmation, clones found to be in frame with Lep protein were selected for use in the *in vitro* transcription-translation experiments. *E. coli* strains and plasmids used in this study are found in Table 2-1.

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Table 2-2: Oligonucleotide primers used in this study

Primer Pair	RS	Sequence (5'→3')	Application
bb0173T-NdeI-F	NdeI	ACG CCA TAT GGC TTT AGC AGG TCC TTC	Amplification of *bb0173* for cloning into pCR2.1 and expression vector pET23a
bb0173T-XhoI-R	XhoI	ACG CCT CGA GTA GTA TCT CTT TTA AG	
bb0173cDNA-F(nt249-272)		GAA GAT GAT ACA TCT TAG TGC TGG	Amplification of bb0173 cDNA from RNA
bb0173cDNA-R(nt725-749)		CTT CCC TGA TAA AAT TTT CCA GAT	
bb0173TM1-SpeI-F	SpeI	ACG CAC TAG TGG AGG ACC AGG AAA TGA GCC TTT ATA TTT G	Amplification of the first putative *bb0173* transmembrane sequence in frame with the Lep construct
bb0173TM1-KpnI-R	KpnI	ACG CGG TAC CCC TCC TGG TCC CTT TAT CTT GCC TCC TCT	
bb0173TM1-2-SpeI-F	SpeI	ACG CAC TAG TGG AGG ACC AGG AGA TTA TAG ATT AA TTT G	Amplification of the second putative *bb0173* transmembrane sequence in frame with the Lep construct
bb0173TM2-KpnI-R	KpnI	ACG CGG TAC CCC TCC TGG TCC AAC TGA AGG ACC TGC TAA AGC	
bb0173TM2b-SpeI-F	SpeI	ACG CAC TAG TGG AGG ACC AGG ACT AGA TGA TAT TTA TAT TAT G	Amplification of the third putative *bb0173* transmembrane sequence in frame with the Lep construct
bb0173TM2b-KpnI-R	KpnI	AGC CGG TAC CCC TCC TGG TCC AGC CTC AGA ATG CTT TAA ATG	
bb0173TM3-SpeI-F	SpeI	ACG CAC TAG TGG AGG ACC AGG AGA TAT TTA TAA AGA ATT TTT AG	Amplification of the fourth putative *bb0173* transmembrane sequence in frame with the Lep construct
bb0173TM3-KpnI-R	KpnI	ACG CGG TAC CCC TCC TGG TCC CTC TTT TAA GAA AAT TTT TG	
bb0173-NcoI-F.1	NcoI	ACG CCC ATG GAT GTT AAC ATT TAA TGA G	Common amplification start site for truncated insertion constructs
bb0173-50aa-KpnI-R.1	KpnI	ACG CGG TAC CTT AAG TTT TAA AGA G	Amplification of the first 50 amino acids of *bb0173* for truncated insertion constructs
bb0173-150aa-KpnI-R.1	KpnI	ACG CGG TAC CCT ATC TGT TAT AGG CAC TAC TAT TG	Amplification of the first 150 amino acids of *bb0173* for truncated insertion constructs
bb0173-250aa-KpnI-R.1	KpnI	ACG CGG TAC CTC TTT AAA ACT TCC CTG ATA AAA TTT TCC	Amplification of the first 250 amino acids of *bb0173* for truncated insertion constructs
bb0173-FL-KpnI-R.1	KpnI	ACG CGG TAC CGC TAG TAT CTC TTT TAA G	Amplification of full length *bb0173* for truncated insertion constructs

In vitro transcription-translation

The BB0173 Lep-derived constructs were transcribed and translated using the TNT SP6 Quick Coupled System (Promega, Madison, WI). The reactions contained 75 ng of DNA template, 0.5 µl of [^{35}S]Met (5µC$_i$), and 0.25 µl of microsomes (tRNA Probes) were incubated for 90 min at 30 °C. The translation products were ultracentrifuged (100,000 *g* for 15 min) on a sucrose cushion, and analyzed by SDS-PAGE. The bands were quantified using a Fuji FLA-3000 phosphoimager and Image Reader 8.1j software.

For the proteinase K protection assay, 2 µl of proteinase K (1 mg/ml) was added to the sample, and the digestion reaction was incubated for 15 min on ice. Before SDS-PAGE analysis, the reaction was stopped by adding 1 mM phenylmethanesulfonyl fluoride (PMSF).

For EndoH (New England Biolabs, Beverly, MA) treatment, 1 µl of 10X Glycoprotein Denaturing Buffer, 1 µl of 10X GlycoBuffer, 1 µl of EndoH and 7 µl of H_20 were added to make a 10 µl total reaction volume and incubated for 1 h at 37°C with 0.1 mU of EndoH. The samples were analyzed by SDS-PAGE.

Expression and purification of rBB0173$_T$

The first two transmembrane domains of the BB0173 protein were excluded during cloning, in order to enhance the ability and viability of *E. coli* cells used to express *bb0173*. This N-terminally truncated version of *bb0173* will be referenced in this paper as *bb0173$_T$* (Figure 2-1B). The construct was generated using primers summarized in Table 2-2 by amplifying the gene from *B. burgdorferi* B31A3 genomic DNA and

introduced restriction enzyme sites *Nde*I (5') and *Xho*I (3') prior to introduction into pCR 2.1 – TOPO™ (Invitrogen™ LifeTechnologies®) following manufacturer's recommendations. Positive clones were confirmed by sequencing (Eton Biosciences, San Diego, CA, USA) and sub-cloned into the expression vector pET23a (Novagen, Madison, WI) using *Xho*I and *Nde*I restriction sites [59]. The plasmid constructs containing inserts of expected sizes were sequenced and used to transform the *E. coli* expression host.

Truncated recombinant BB0173 (rBB0173$_T$) with a C-terminal 6 × histidine tag was overexpressed by inducing the *E. coli* strain containing pET23a-*bb0173$_T$* with 1 mM IPTG for 3 hours and purified as described [59]. Fractions with the highest concentration of rBB0173$_T$ were combined and dialyzed against a buffer consisting of 50 mM sodium phosphate and 300 mM NaCl (pH 7.4; Slide-A Lyzer™ G2 dialysis cassette; Thermo Scientific, Waltham, MA) prior application to Amicon® Centrifugal Filters (EMD Millipore, Billerica, MA) to concentrate the protein. Quantification of protein concentration was achieved using a bicinchoninic acid (BCA; Pierce/Thermo Scientific, Waltham, MA) assay. Aliquots of the protein were stored at -80ºC until further use.

Generation and purification of polyclonal antibodies against BB0173

Antibodies against BB0173 were generated to detect this *Borrelial* protein in immunoblot assays. Chickens were utilized as model for the generation of specific antibodies, and were housed at the Texas A&M University poultry farm. A 30-amino acid peptide (BB0173$_{pep}$) was generated (Peptide 2.0 Inc., Chantilly, VA) from the region found within the large loop predicted to contain the VWFA domain and just

beyond the Metal Ion Dependent Adhesion Site (MIDAS) motif (Figure 2-1B). $BB0173_{pep}$ was rehydrated using 50% ethanol and was used to immunize chickens in parallel with $rBB0173_T$ in order to compare antibody response, sensitivity, and specificity. Each hen (n=3 per antigen) received 50µg of either the truncated protein or the peptide using equal parts protein and TiterMax™ Gold adjuvant (Sigma-Aldrich, St. Louis, MO). Chickens were immunized intramuscularly through the breast at days 0, 14, and 28 to allow for the generation of a sufficient memory antibody response. From days 35 to 45, eggs were collected daily and frozen at -20ºC until antibodies were purified from the yolk as previously described [207]. Antibodies were determined to recognize both the truncated antigen as well as full length BB0173 in *Borrelial* whole cell lysates via ELISA and western blot (data not shown). For this study, detection was carried out using $BB0173_{pep}$ specific antibodies.

Protease treatment of B. burgdorferi

Proteinase K degradation of proteins exposed to the extracellular environment was conducted using *B. burgdorferi* cells grown at 32°C in BSK II pH 7.6 following previously standardized protocols [59]. Briefly, *B. burgdroferi* B31 A3 strain cells were washed in Hank's Balanced Salt Solution (HBSS) containing 5 mM $MgCl_2$ and 50 mM sucrose to enhance membrane stabilization. After washing, a whole cell lysate aliquot was separated, washed further, and stored at -20°C until use. The rest of the cells were then treated with 0, 10, 20, 50, 100, or 200 µg Proteinase K and incubated at 37°C for 30 minutes. After incubation, PMSF was added to each sample at a final concentration of 1

mM to stop Proteinase K activity. Cells were then washed in supplemented HBSS containing 1 mM PMSF. Treated cells were stored at -20°C until use.

Detergent phase partitioning

Triton X-114 phase partitioning was conducted using *B. burgdorferi* B31 A3 grown at 32°C in BSK II pH 7.6, pelleted and washed in HBSS. Cells were treated with 1% Triton X-114 in HBSS and incubated at 4°C overnight with gentle agitation. Cells were then centrifuged at 8,000 x *g* and the pellet containing the protoplasmic cylinders (PC) was saved. The supernatant was treated with 2% then 10% Triton X-114. The detergent (DT) and aqueous (AQ) phases were washed in HBSS then precipitated with 10 fold volume of ice cold acetone, stored at -20°C overnight and pelleted as previously described [208]. The supernatant was discarded and samples were stored at -20°C until use.

Protein resolution and detection

Both Triton X-114 and Proteinase K treated samples were analyzed using SDS-PAGE and immunoblot analyses. In both cases, SDS—12% PAGE gels were used to separate proteins from treated or untreated whole cell lysates from *B. burgdorferi*. After protein separation, gels were either stained or transferred to membranes for immunoblot. Gels for visualization were treated with either Coomassie brilliant blue in the case of Proteinase K treatment or Silver Stain Plus (Bio-Rad Laboratories, Inc., Hercules, CA) for Triton X-114 treated samples. For immunoblot analysis, gels were transferred to PVDF membranes (Hybond-P; GE Healthcare, Piscataway, NJ) as previously described [59]. The PVDF membranes were blocked overnight at 4ºC in Tris-buffered saline

containing 0.2% Tween 20 (TBS-T) and 10% skim milk. After blocking, membranes were probed with chicken anti-$BB0173_{pep.}$

Primary control antibodies included: OMPs OspC and P66, and the cytosolic proteins superoxide dismutase A (SodA, cytosolic), oxidative stress regulator (BosR, periplasmic), and Flagellin B (FlaB, periplasmic) [196]. OspC, VlsE, and P66 were utilized to determine proteinase K activity on outer membrane proteins, while BosR and FlaB served intracellular controls. OspC (OMP) and FlaB (periplasmic) were used to as controls for the Triton X-114 detergent phase separation assay. [209]. Blots were developed following incubation with appropriate dilutions of HRP-conjugated secondary antibodies and detected using ECL western blotting reagents (GE Healthcare, Piscataway, NJ) as previously described [67, 196, 197, 209]. All gels and blots were imaged using a ChemiDoc ™ Touch Imaging System (Bio-Rad Laboratories, Inc. Hercules, CA)

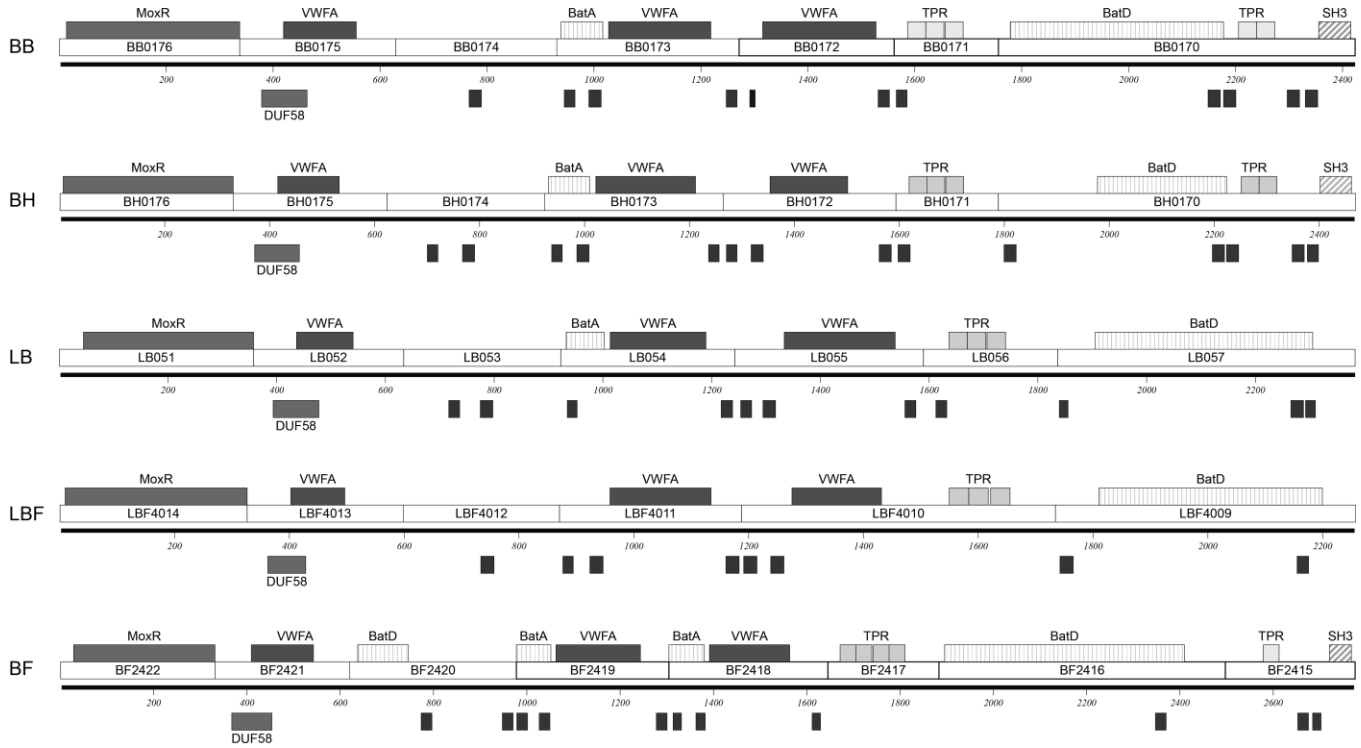

Figure 2-1: Organization and conservation of BB0173.

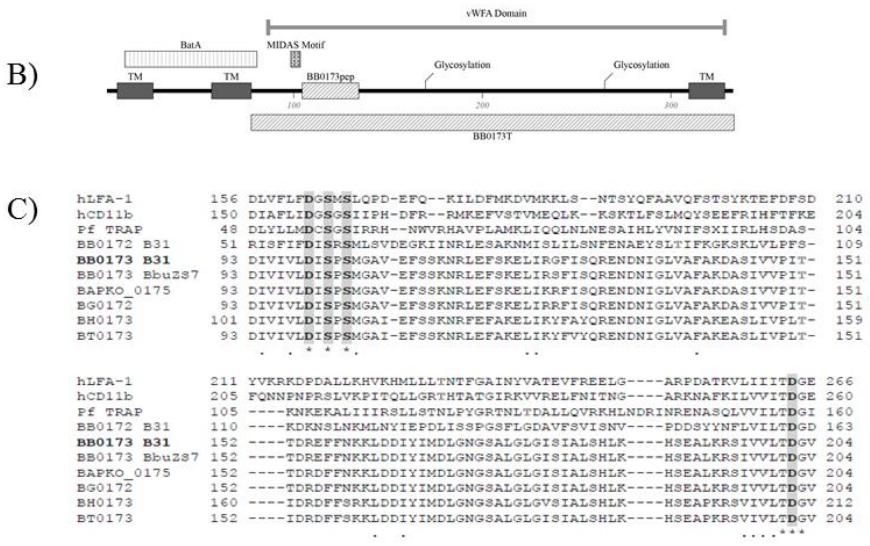

Figure 2-1, continued. (A) Schematic demonstrating the similarity between the Bat region of *Borrelia burgdorferi* (BB), *Borrelia hermsii* (BH), *Leptospira interrogans* (LB), *Leptospira biflexa* (LBF), and *Bacteroides fragilis* (BF). Note the similarities across BB0172 through BB0176. **(B)** Map demonstrating the pertinent domains of BB0173. The map demonstrates the three transmembrane domains (amino acids: 7-25, 57-77, 310-328), VWFA domain (amino acids: 87-328), MIDAS motif (amino acids: 99-103), BatA domain (amino acids: 9-86), and N-glycosylation sites (amino acids 170-172, 265-267). Additionally, a 30-mer peptide is denoted, $BB0173_{pep}$, which was used to generate chicken anti-BB0173 antibodies. $BB0173_{T}$, the truncated BB0173 protein, was also used to generate chicken anti-BB0173 antibodies. **(C)** Clustal W (v1.83) alignment of *B. burgdorferi* B31 BB0173 (bold) against homologues in *B. burgdorferi* ZS7 (BB0173 BbZS7) *Borrelia garinii* (BG0172), *Borrelia afzelii* (BAPKO_0175), and the relapsing fever species *Borrelia hermsii* (BH0173) and *Borrelia turicatae* (BT0173). Alignments are also made to *B. burgdorferi* B31 BB0172 (BB0172 B31), which was found to be very similar in sequence and topology. There is also homology seen to *Plasmodium falciparum* membrane protein TRAP (Pf TRAP) as well as to the human adhesins LFA-1 (hLFA-1) and CD11b (hCD11b). Conserved residues corresponding to the MIDAS motif are highlighted, including the DXSXS as well as the threonine (T) required for MIDAS function.

Results

Homology of B. burgdorferi aerotolerance mediating genes

Upon investigating proteins surrounding *bb0173* on the *B. burgdorferi* linear chromosome, other similarities appeared between *bb0170* to *bb0176* and similar regions of *B. fragilis, R. leguminosarum, L. interrogans, and L. biflexa* [210]. The domains are highly conserved in each of these organisms, including the VWFA, Bat, SH_3, transmembrane domains, TPR repeats, a domain of unknown function (DUF58), and the presence of a MoxR type ATPase, as seen in Figure 2-1A. It has been well documented that MoxR ATPases are found near VWFA domain containing proteins [211], and has been found to be the case in the *Borrelia* species evaluated in this paper. Further, *bb0170* appears to be a fusion protein with homology to both *bf2416* and *bf2415*, consolidating the BatD, TPR, and SH_3 domains into a single protein in *B. burgdorferi* and *B. hermsii*. Although the *B. fragilis* genome encodes duplicates of the BatA (*bf2419* and *bf2418*) and BatD domains (*bf2420* and *bf2416*), these domains are only predicted to be present singly in this region of the genomes of both *Borrelia* and *Leptospira.* Interestingly, spirochete *Treponema* has VWFA domain containing proteins that are much less similar to the VWFA domain containing proteins of *B. burgdorferi* [59, 179].

Protein features of BB0173

Initially, *bb0173* was predicted to contain four transmembrane domains, in addition to the BatA and VWFA domain containing the MIDAS motif. The arrangement of the features present in BB0173 after analysis is seen in Figure 2-1B, showing the

predicted transmembrane domains, aerotolerance domain, VWFA domain, MIDAS motif, glycosylation sites, and regions utilized for antibody production. Moreover, alignments with other VWFA domain containing proteins make a strong case for a functional MIDAS motif, when comparing conserved residues DXSXS and the downstream conserved location of aspartic acid (T**D**G motif) known to be required for coordination of metal ions (Figure 2-1C).

Expression of Bat-like genes

Prior to studying the protein, the expression conditions of *bb0173* were assessed. From the cellular mRNA levels, we evaluated the expression of *bb0173* under tick growth conditions as well as during conditions shifted from tick to mammalian conditions. As shown in Figure 2-2A, the expression of *bb0173* was observed at both ambient temperature as well as during mammalian conditions. Controls for the PCR are shown in Figure 2-2B. The differentially regulated *ospC* is expressed only during mammalian conditions, and the constitutively expressed *flaB* and *p66* are present under both tick and mammalian conditions, as expected.

Due to the presence of Bat domains and the localization of BB0172 and BB0173 to different membranes, evaluation of oxygen levels on gene transcripts was performed. *B. burgdorferi* cultures were grown under standard or low oxygen conditions, and *bb0170 – bb0176* were evaluated for gene expression changes between the two environments. Qualitative evaluation of expression of *bb0170-bb0176* was performed using conventional PCR (Figure 2-3). BB0172 is known to be expressed only during shifting temperature conditions, and no amplification was observed when growing *B.*

burgdorferi at constant 32ºC and pH7.6, regardless of the presence or absence of oxygen in the culture conditions. Notably, several genes appear upregulated under low oxygen conditions, including *bb0170, bb0174,* and, to a lesser extent, *bb0171*. No major changes in the level of expression of *bb0173* were observed by conventional PCR.

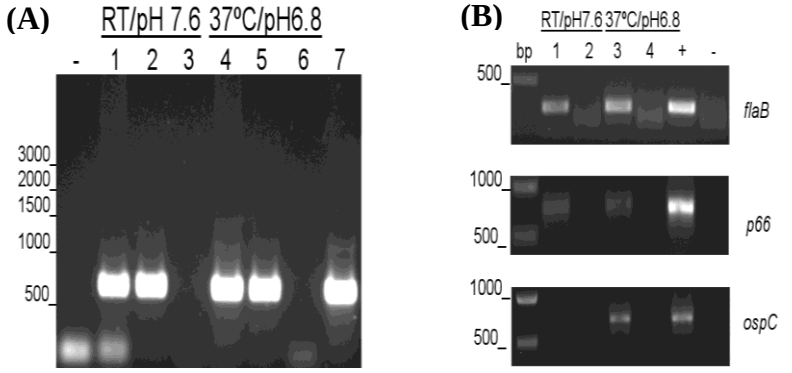

Figure 2-2: Expression of bb0173 cDNA upon temperature shift. *B. burgdorferi* B31A3 strain was grown under tick conditions to late log phase then shifted to mammalian conditions before collection of mRNA. The purified mRNA was reverse transcribed to cDNA, and PCR was performed to detect *bb0173, flaB, p66*, and *ospC*. Water was used as a negative control (-). **(A)** RNA samples were tested for DNA contamination in lanes 3 and 6. Genomic DNA was run in lanes 2 and 5 and served as the positive control. In lanes 1 and 4, cDNA samples were evaluated. To confirm functionality of primers, a second genomic DNA sample was applied in lane 7. **(B)** The same shifting conditions were used to generate DNA samples as previously. cDNA samples are in lanes 1 and 3, RNA in lanes 2 and 4, and genomic DNA is labeled as (+). Negative control is water, as above. On the left of the figure, the DNA ladder is shown and sizes are denoted in basepairs.

Insertion of BB0173 hydrophobic regions into ER-derived microsomal membranes

To identify the hydrophobic regions (HRs) of the human BB0173 amino acid sequence was parsed to test the performance of the ΔG Prediction Server. Given the amino acid sequence, this algorithm predicts the corresponding apparent free energy

difference, ΔG_{app}, for insertion of each hydrophobic region into the ER membrane by means of the Sec61 translocon [201, 205]. Figure 2-4A shows the predicted ΔG_{app} values for the hydrophobic regions predicted. The negative ΔG_{app} value for the HR1, HR2 and HR4 regions predict a TM disposition, whereas the positive values computed for HR3 predicts that this sequence does not integrate into ER membrane.

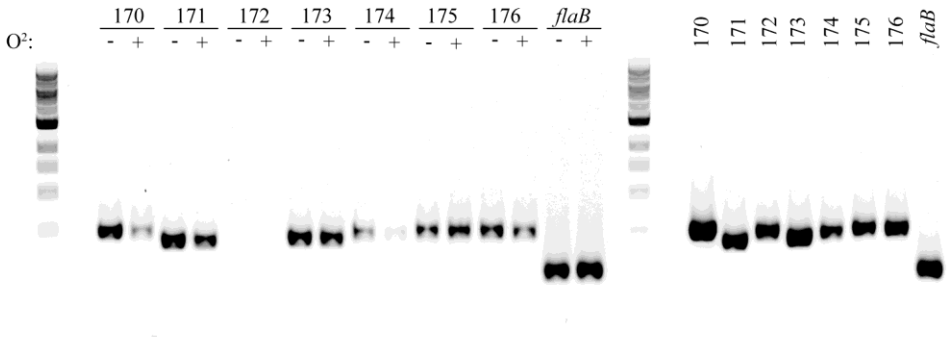

Figure 2-3: Expression of *bb0170 – bb0176* under decreased oxygen conditions. Gene expression of the Bat-like genes is compared under atmospheric oxygen and decreased oxygen conditions. Qualitative expression of each gene under both conditions as determined by PCR with *flaB* included as positive control. Gene expression under each condition is evaluated on the left, with controls for each PCR reaction using genomic DNA is shown on the right. All negative controls using water lacked bands (not shown).

To test these predictions, we assayed the membrane insertion capabilities of these HRs using an *in vitro* experimental system based on the *E. coli* inner membrane protein leader peptidase (Lep) [205], which accurately determines the integration of TM helices into ER membranes. Lep consists of two TM segments (H1 and H2) connected by a cytoplasmic loop (P1) and a large C-terminal domain (P2) (Figure 2-4B), and inserts into ER-derived rough microsomal membranes (RMs) with both termini located in the

lumen. The analyzed segment (HR tested) is engineered into the luminal P2 domain and is flanked by two acceptor sites (G1 and G2) for *N*-linked glycosylation. Single glycosylation (i.e., membrane integration) results in a molecular mass increase of 2.5 kDa relative to the observed molecular mass of Lep expressed in the absence of microsomes (Figure 2-4B, left). A molecular mass shift of 5 kDa occurs upon double glycosylation (i.e., membrane translocation of the HR-tested) (Figure 2-4B, right). This system has the obvious advantage that the insertion assays are performed in the context of a biological membrane.

Translation of Lep chimeric constructs harbouring the BB0173 regions predicted by the Δ*G* Prediction Server (Figure 2-4A) resulted mainly in single-glycosylated forms for HR1, HR2 and HR4 regions (Figure 2-4C, lane 2,5 and 11), except for HR3 containing construct (Figure 2-4C, lane 8). In this last case, translation products were found mostly triple-glycosylated. It should be mentioned that BB0173 sequence includes a native potential *N*-glycosylation site at position 187, i.e. within HR3 region (see Figure 2-5A), adding an *N*-glycosylation motif that would be modified only if this region is not inserted into the lipid bilayer. (Figure 2-4C, lane 8). These results were confirmed by proteinase K (PK) treatment. Digestion with PK degrades membrane protein domains located exclusively towards the cytosol, while membrane-embedded or lumenally exposed domains are protected. As expected, Lep chimeras bearing HR1, HR2 and HR4 regions were sensitive to PK digestion (Figure 2-4C, lanes 3, 6 and 12). However, Lep constructs containing HR3 sequence were partially resistant to the protease treatment

due to its luminal P2 localization (Figure 2-4C, lane 9, arrowhead, expected size ≈33.5 kDa).

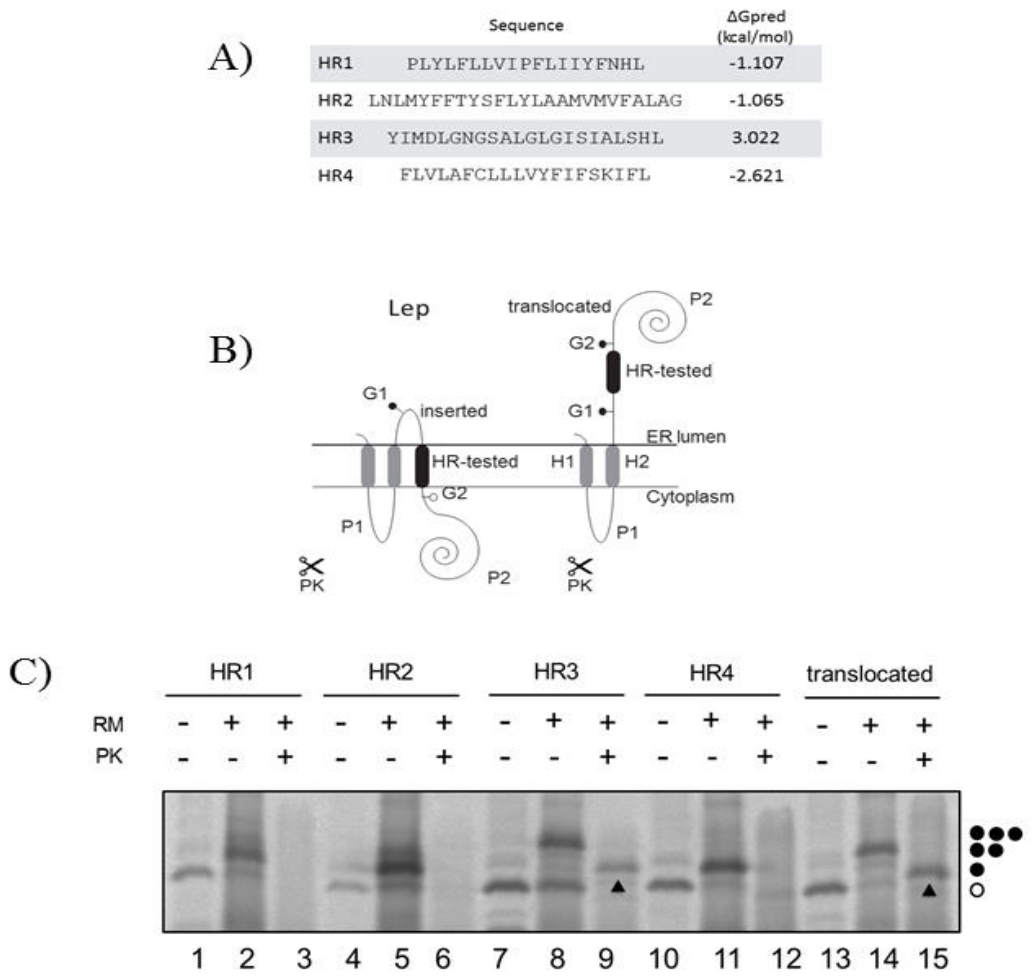

Figure 2-4: Insertion of hydrophobic regions of BB0173 into membranes. (A) Schematic representation of the Lep construct used to report insertion of hydrophobic regions of BB0173 into endoplasmic reticulum membranes. The TM segment under investigation (HR-tested) was introduced into the P2 domain of Lep, flanked by two artificial glycosylation acceptor sites (G1 and G2). Recognition of the tested sequence as a TM domain by the translocon machinery results in the location of only G1 in the luminal side of the ER membrane, preventing G2 glycosylation (left). The Lep chimera will be doubly glycosylated when the sequence being tested is translocated into the lumen of the microsomes (right). **(B)** *In vitro* translation of different Lep constructs containing BB0173 HR1 (TM1), HR2A (TM2A), HR3 (TM2B) and HR4 sequences in the presence (CON), absence (SIN) of membranes and in the presence of membranes and proteinase K (PK). **(C)** Table with the hydrophobic regions (HR) detected in BB0173, their sequence and predicted DG.

Membrane insertion and topology of BB0173 into the ER membrane

To experimentally map the membrane insertion and topology of BB0173 protein, we prepared a series of polypeptide truncates containing an added C-terminal glycosylation tag (NST), which has been proven to be efficiently modified in the *in vitro* translation system [206].

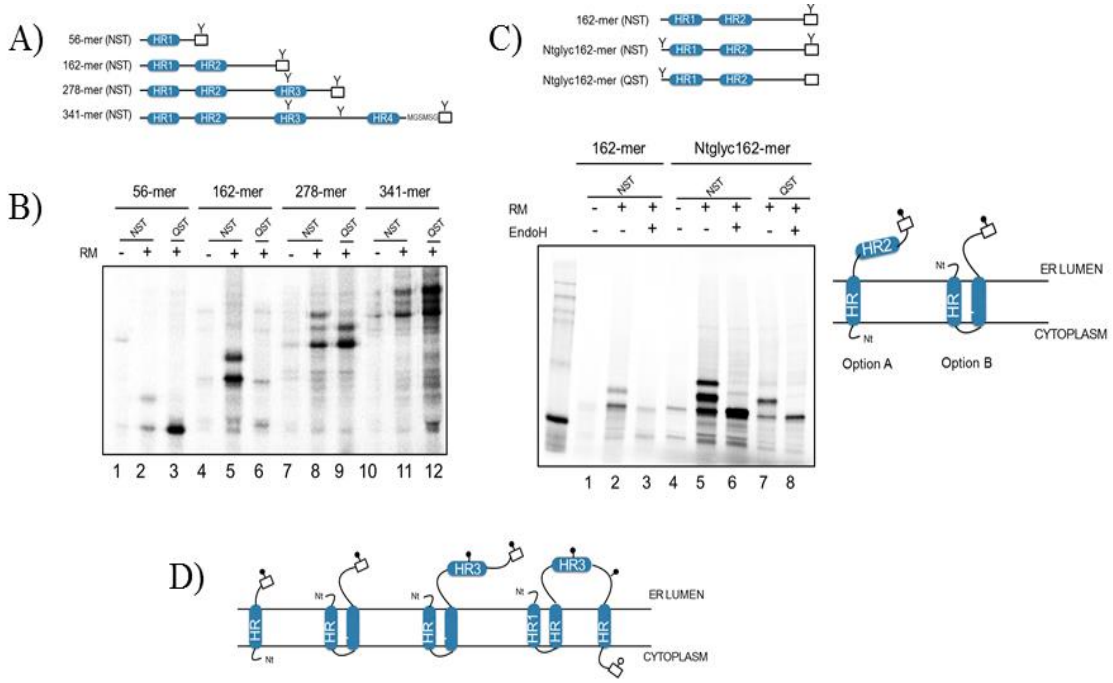

Figure 2-5: Transmembrane domain insertion of BB0173. (A) Cloned constructs for detection of membrane insertion of sequential putative transmembrane domains evaluated via *in vitro* translation. **(B)** *In vitro* translation of the different BB0173 constructs containing BB0173 HR1, HR1 and2 (HR2Ct), HR3 with 2 glycosylation sites, one on HR3 and one on the C-terminus (HR2BNStT), HR3 with 2 glycosylation sites, one on HR3 and one before the C-terminus (HR2BAmpli274) and HR4CT which comprises the BB0173 full length sequences and 3 glycosylation sites on H#, before HR4 and on the C-terminus. The experiment was done in the presence (+), absence (-) of reticulocyte membranes (RM) and in the presence of a glycosidase (QST). (C) Schematic of BB0173 topology as determined by *in vitro* translation studies.

The constructs used are shown in Figure 2-5A. As shown in Figure 2-5B, translation products containing the N-terminal 56 residues of BB0173 sequence, including the first predicted TM segment (HR1) plus an optimized glycosylatable C-terminal tag (56-mer NST), were efficiently singly-glycosylated in the presence of microsomal membranes (Figure 2-5B, lane 2). The nature of these higher molecular weight polypeptide species was analysed by translating the first 56 residues with a C-terminal tag that includes a non-acceptor site for N-glycosylation (56-mer QST), rendering the elimination of the higher molecular mass band (Figure 2-5B, lane 3), confirming the sugar source of their retarded electrophoretic mobility and suggesting 56mer insertion into the microsomal membrane with an N-terminal cytoplasmic orientation (see Figure 2-5D for a scheme).

Truncated 162-mer polypeptides, which include the first two HR (Figure 2-5B, lane 5), were efficiently glycosylated (45 ± 4% of glycosylation), depicting two possible situations. Either the glycosylation observed could be obtained as result of the HR2 translocation (Figure 2-5C, option A), or a topology inversion of HR1 could lead to the insertion of HR1-HR2 and the translocation of the C-terminal tag (Figure 2-5C, option B). To distinguish between both situations, an N-glycosylation acceptor site was introduced at the N-terminus of BB0173 (position 5), creating construct NtglycBB0173. Translation of 162-mer NtglycBB0173 with an N- glycosylation acceptor site as a C-terminal tag rendered singly- and doubly-glycosylated forms (Figure 2-5c, lane 5). The presence of double-glycosylated forms suggests the inversion of the HR1 and the subsequent insertion of HR2 into the ER membrane (Figure 2-5C, option B). When a

non-acceptor (QST) site as C-terminal tag was used, a singly-glycosylated forms were observed, consistent with the N-terminal translocation of the polypeptide chain (Figure 2-5C, lane 7).

The insertion of the third predicted TM segment (HR3) was tested by translating a 278-residue truncation with the same C-terminal glycosylatable tag (278-mer). As mentioned before, wild type BB0173 carries a potential glycosylation site at position 187 (see Figure 2-5A). In case of translocation across the microsomal membrane, both Asn187 and the added C-terminal glycosylation tag should be modified rendering doubly-glycosylated forms. Translation of construct 278-mer produced singly (41%) and double (59 %) glycosylated forms indicating that predominantly HR3 is translocated (Figure 2-5B, lane 8). When the same chimera was translated with a C-terminal tag harbouring a non-acceptor site (QST), only singly glycosylated forms were detected (Figure 2-5B, lane 11), confirming native Asn187 glycosylation.

Finally, the insertion of the predicted HR4 was analyzed by translating full-length *bb0173* gene (341-mer). It should be noted that wild type BB0173 carries another glycosylation site at position 273 (see Figure 2-5A). Translation of C-terminal tagged full-length constructs either with an acceptor (NST) or a non-acceptor (QST) glycosylation sites rendered double glycosylated forms (Figure 2-5B, lanes 14 and 15), indicating that HR4 is efficiently inserted. Overall, these results evidenced that BB0173 protein inserts into the ER membrane with Nt-cytosol/Ct-lumenal orientation, where HR1, HR2 and HR4 are truly transmembrane segments (Figure 2-5D).

Cellular localization of BB0173 within B. burgdorferi

With combined knowledge, both from the predicted model of BB0173 insertion into the membranes and expression conditions, we evaluated the actual cellular localization of BB0173 within *B. burgdorferi* cells using both a protease protection assay and detergent phase separation assay.

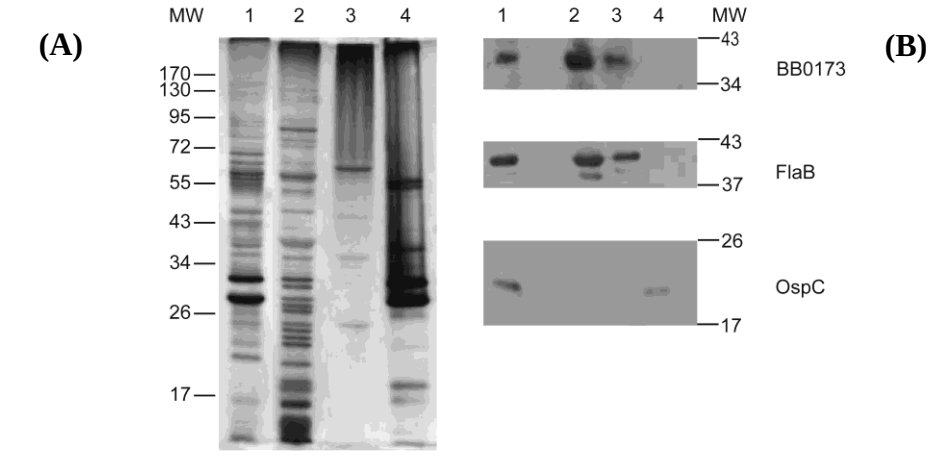

Figure 2-6: Localization of BB0173 to the aqueous and inner membrane fractions after treatment with detergent. *B. burgdorferi* cells disrupted using the detergent Triton X-114 separated into three distinct fractions, the aqueous (AQ), protoplasmic cylinders (PC), and detergent (DT) phases. The phases were separated using SDS-12% PAGE and either stained using Silver Stain Plus (Biorad) **(A)** or were transferred to a PVDF membrane and probed using anti-BB0173$_T$ and a secondary anti-chicken HRP-conjugated antibody **(B)**. Controls for outer membrane and inner membrane proteins were OspC and FlaB.

The Triton X-114 detergent phase separation assay evaluated localization of proteins within the cell to either the inner or outer membrane or to the cytoplasm. This separation method was used to evaluate BB0173 localization from cells grown at

32°C/pH 7.6, which were then silver stained to evaluate equal loading (Figure 2-6A). Using western blotting with chicken-anti-BB0173, the protein was detected in both the AQ phase and PCs, and no band was seen in the DT phase (Figure 2-6B). Control proteins were observed in the expected fractions based on their described localization (Figure 2-6B) including: OspC (DT and PC) and FlaB (AQ and PC). Therefore, these results suggest that BB0173 is associated to the inner membrane of *B. burgdorferi*.

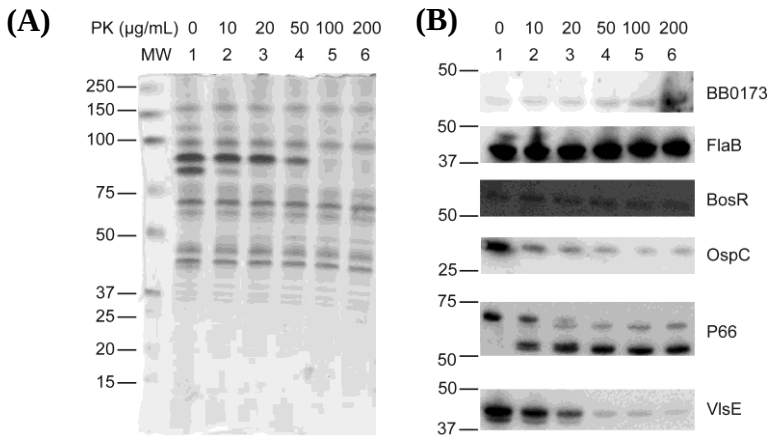

Figure 2-7: Protection of BB0173 from protease degradation. Surface proteins of *B. burgdorferi* are degraded by serine protease Proteinase K (PK). Whole cell lysates were treated with doses ranging from 0 to 200 µg/mL PK prior to separation using SDS-12% PAGE. Gels were either visualized using Coomassie blue staining **(A)** or transferred to a PVDF membrane and probed with antibodies **(B)**. BB0173 was detected using anti-BB0173$_{pep}$ and anti-chicken HRP-conjugated antibody. Controls for PK mediated degradation and cell integrity during treatment included intercellular protein BosR and periplasmic protein FlaB, as well as outer membrane proteins OspC, VlsE, and P66.

In order to confirm the intracellular localization of BB0173, degradation of extracellularly-exposed proteins of *B. burgdorferi* was performed using the protease Proteinase K treatment protocol. After treating *B. burgdorferi* cells grown at both

25°C/pH 6.8 and 37°C/pH 7.6 with Proteinase K at a concentration of 200 μg/mL, no apparent change in size was observed in the band corresponding to BB0173, nor did any smaller bands become apparent. To ensure more accurate results, a titration of Proteinase K ranging from 0 to 200 μg/mL was used to treat *B. burgdorferi* cells. Samples were Coomassie blue stained for equal loading (Figure 2-7A) prior to blotting for proteins (Figure 2-7B). In each experiment, regardless of concentration of Proteinase K used, bands corresponding to OM anchored proteins P66, OspC and VlsE observed a decrease in visualization with treatment. Moreover, periplasmic FlaB and intracellular BosR were unaffected. BB0173 was seen to be unaffected by protease treatment.

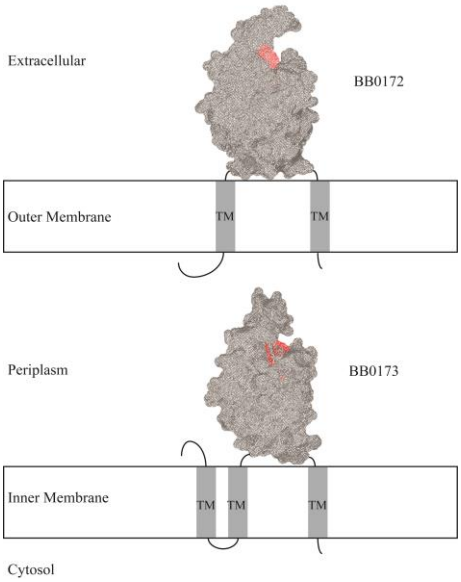

Figure 2-8: Localization of the tertiary structures of BB0172 and BB0173 within *B. burgdorferi*. Models of the tertiary structures of BB0172 and BB0173 were generated and superimposed onto either the inner or outer membrane as predicted from localization studies.

Cellular localization model

There is a likelihood that these proteins function together due to the gene arrangement, cellular localization, and presence of domains with potential for protein:protein interaction. In Figure 2-8, tertiary structures have been predicted for each protein, taking into consideration exposure of the VWFA domain and MIDAS motif.

Discussion

On the *B. burgdorferi* linear chromosome, *bb0172, bb0173, bb0175,* and *bb0325* were identified as genes encoding for proteins containing VWFA domains. Within *B. burgdorferi*, BB0173 is only the second VWFA domain containing protein to be characterized, the other being BB0172 [59]. Based on the similarity between predicted motifs of BB0172 and BB0173, expression, localization, and ultimately functions were expected to be quite similar. However, our studies revealed that there are key distinctions between BB0172 and BB0173.

BB0172, was determined to be a VWFA domain-containing protein that contains two transmembrane helices with extracellular exposure [59]. In contrast to BB0172, we demonstrated that BB0173, a VWFA domain-containing borrelial protein, is anchored to biological membranes through three TM helices. Further, we have demonstrated that the first TM segment functions as a signal sequence that must emerge from the ribosome to bind the signal recognition particle and thereby enable nascent BB0173 targeting to the membrane.

The potential role of BB0173 is definitively unique from BB0172, which functions through the binding of mammalian integrin $\alpha_3\beta_1$ [59]. However, the localization of BB0173 strictly within the cell coupled with the extracellular function of BB0172 could support each other. The MIDAS motif is seen to be accessible to interaction partners in both cases, albeit to the host environment in the case of BB0172 and to the periplasm of *B. burgdorferi* in the case of BB0173. Partial support for this idea comes from the presence of the TPR-like sequences found in BB0170 and BB0171, which have been suggested by Tang et al. to play a role in protein complexing of the BatI-like proteins [210, 212]. In addition to the known ability of BB0172 to bind human integrin $\alpha_3\beta_1$, differential gene regulation of the Bat-like genes is seen both under shifting conditions of pH and temperature, as well as after exposure to varied oxygen levels [59].

BatI-like genes have also been noted in other spirochetes, such as *Leptospira interrogans* and *Treponema denticoloa* [179, 193]. In each of these cases as well as in *B. burgdorferi*, the VWFA domain containing proteins are found to be associated with a methanol dehydrogenase regulatory (MoxR) ATPase Associated with Diverse Cellular Activities (AAA). These genes have been suggested to function together as an operon system, although there is no clear function associated to the operon [193, 194, 210, 213]. In *Rhizobium leguminosarum*, it has been observed that cells with defects in these genes demonstrated envelope and cell morphology changes. This operon is referred to as complex media growth deficient (*cmdA-cmdD*) [194, 214]. It is interesting to note that both *R. leguminosarum* and *B. burgdorferi* encounter stark changes of environment, with

both moving from conditions of higher oxygen conditions, (*Rhizobium:* free living, *Borrelia*: tick) to decreased oxygen conditions (*Rhizobium*: plant host, *Borrelia*: mammalian host) [194, 214, 215]. Interestingly, *Rhizobium* DNA has also been detected in tick microbiome studies and as such, the environments may be more similar than previously thought [216]. This may also act as an interface between these two bacteria that could allow for gene sharing events.

Bearing the ideas of differential gene expression, indicating a specific cellular function, and the possibility of these genes functioning as a complex, a few ideas for the function of these genes exist. One idea, proposed in relation to both *B. fragilis* and *L. biflexa*, is the potential for these genes to protect the organisms from oxidative stress [193, 210]. This idea is particularly attractive in *B. burgdorferi*, which lacks in traditional mechanisms to combat oxidative stress [217]. Taking into consideration the ability to differentially regulate protein expression, namely BB0172, and the lack of mechanisms to deal with oxidative stress, it is possible that these proteins work together to subdue effects of oxidative damage, although this hypothesis has not been specifically proven to be the case in either *Bacteroides* or *Leptospira*. This may occur through signaling after the recognition of the mammalian environment and consequent transportation causing the generation of a periplasmic environment rich in reducing power [193, 210].

The second functional prediction for these genes was proposed by Vanderlinde et al., in which *R. leguminosarum* cells with mutations of these genes demonstrated defects in envelope integrity when stressed [194]. In relation to *B. burgdorferi*, this idea may

also be a feasible function of the gene complex. Compared to *Escherichia coli* or other gram-negative organisms, the cell wall of *B. burgdorferi* lacks LPS, potentially causing the membrane to be more sensitive to physical stressors [218]. As such, these genes may play a role in supporting cell wall function, particularly in the tick during the blood meal or mammalian infection. As the tick vector engorges, the spirochete must survive a drastic increase in pressure before migrating to the salivary glands and being transmitted to the host [219]. After transmission, gene expression must again change to support the spirochete as it colonizes the host, avoiding the protective mechanisms of the immune system, and disseminates through the tissues [33, 36, 220].

Evaluation of hypothetical proteins of organisms such as *B. burgdorferi*, pathogens that are presently not well understood, is a worthwhile endeavor to identify potential targets for diagnostics, prevention, and treatment of disease. Lyme disease, in particular, is an important consideration, as missing the window for treatment can cause a lifetime of ongoing symptoms and prevention or enhanced treatment could change the outcome for these individuals [221, 222]. Particularly due to their highly-conserved nature, elucidation of the function of the *bb0170 – bb0176* gene complex transcends spirochete biology, and can apply broadly to a wide range of bacteria. To better understand the function of *bb0173,* qPCR to evaluate the expression changes of *bb0173* under changing conditions will be performed in a future study. Additionally, pull down assays to determine if the proteins are binding to each other or other proteins of *B. burgdorferi* could help illuminate the purpose of these genes. Conservation of these genes across such a wide variety of bacteria implies that these genes likely impart a

function useful for survival. As such, an understanding of the roles of such proteins may facilitate enhanced detection, prevention, and treatment options for Lyme disease as well as other infectious diseases.

CHAPTER III

IMMUNIZATION WITH A *Borrelia burgdorferi* BB0172-DERIVED PEPTIDE PROTECTS MICE AGAINST LYME DISEASE

Introduction

Lyme disease (LD) is the most prevalent arthropod-borne infection in the United States with 30,831 cases of LD reported to the Centers for Disease Control and Prevention (CDC) in 2012. A significant increase in the number of reported cases has been observed in the past few years, classifying LD as a re-emerging infection. *Borrelia burgdorferi*, the causative agent of Lyme disease, is transmitted to humans through the bite of infected *Ixodes* ticks [13, 223-225]. This pathogen is maintained in nature through a very complex enzootic cycle in which small mammals and birds serve as reservoirs [226-228]. This pathogen is accidentally transmitted to humans and companion animals where it causes disease. The ability of this spirochetal pathogen to colonize mammals is dependent on its ability to rapidly alter gene expression in response to highly disparate environmental signals following transmission from infected ticks [17, 229-233]. Consequently, a lot of interest has been devoted to the study of proteins differentially expressed in the tick and the mammalian host as a way to identify potential targets for vaccine development. One of the first targets identified using this approach

was the borrelial outer surface protein A (OspA) which was the target in the only licensed human Lyme vaccine, LYMErix (SmithKline Beecham) [234]. In the arthropod tick, the OspA protein is expressed by *B. burgdorferi*, adhering to the tick receptor for OspA (TROSPA) located in the tick mid-gut [16]. Upon tick feeding, OspA is down regulated allowing the bacteria to migrate from the tick mid-gut into the salivary glands and from there into the mammalian host [16, 103, 235]. Taking this into account, the OspA-based vaccine induced high antibody levels in laboratory animals as well as in humans and consequently conferred protection by blocking the transmission of *B. burgdorferi* from the tick to the mammalian host [41, 107, 236]. Despite the fact that this vaccine showed good protection in phase III human clinical trials, the company voluntarily discontinued the distribution of this vaccine [111, 234, 237, 238]. This was due to a number of reasons including a significant reduction in the vaccine demand, the appearance of adverse reaction to the vaccine, the complicated immunization protocol with periodic boosts to maintain high antibody titers and age limitations [234, 238-240].

This vaccine formulation has been used to develop vaccines administered to wild life (small rodents in particular) to lower *B. burgdorferi* burden in the mammalian reservoirs and the tick vectors, thus reducing the risk for human infection [160, 161, 241-243]. In addition, the OspA-based vaccine has been used in veterinary medicine for some time (Nobivac® Lyme from Merk Animal Health; LymeVax® formulated by Fort Dodge and Recombitek® Lyme y Merial) to prevent Lyme disease in dogs [43, 44, 46, 244-246]. Unfortunately there is no Lyme vaccine currently available for use in humans and horses.

Other differentially expressed proteins such as BBA52, OspC, BBK32 and DbpA, have been evaluated as potential vaccine targets [47, 53, 93, 96, 119, 247]. However, none of these have been tested in human or veterinary clinical trials. Nevertheless, these target proteins are not optimal vaccines for differentiating infected from vaccinated animals (DIVA vaccines) since both immunized and infected animals respond to these antigens [248-251].

In our study, we have selected the chromosomally encoded membrane-associated protein BB0172 of *B. burgdorferi* to develop a DIVA vaccine. We have previously shown that BB0172 [59] inserts into the Borrelia outer membrane and through its von Willebrand Factor A domain (VWFA) binds to the human integrin $\alpha_3\beta_1$. BB0172 is expressed only when shifting *B. burgdorferi* cultures growing at room temperature and pH7.6 (unfed tick conditions) to 37ºC/pH6.8 (fed tick conditions). In addition, BB0172 is not expressed in cultures adapted to either of the conditions and furthermore is not recognized by serum from infected animals nor animals immunized with the full length protein [59]. Thus, a conserved domain in the VWFA-domain of BB0172 could be an excellent candidate for developing a DIVA vaccine due to the highly conserved nature of BB0172 among *B. burgdorferi* sensu lato complex genospecies which cause LD in Europe and the US [59]. In this study, we designed a series of short peptides from the VWFA domain of BB0172 and conjugated them to KLH as potential vaccine candidates. We immunized C3H/HeN mice with each one of the peptides following conventional immunization protocol. Our first goal was to identify the most antigenic peptide, therefore, safety of each one of the peptides was evaluated as well as the protective

response they induced in the murine model of Lyme disease. Our second goal was to determine the potential of these peptides to protect against Lyme disease in the murine model, using the tick challenge as the natural way of disease transmission, and elucidate the role of antibodies and T cells in protection against Lyme disease.

Materials and methods

Ethics statement

All animal experiments were done following the Texas A&M University IACUC approved animal use protocol #2010-124. Texas A&M has adopted the "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training," and complies with all applicable federal, state, and local laws which impact the care and use of animals.

Borrelia burgdorferi strains and growing conditions: identification phase

B. burgdorferi B31 A3 (*Bb*) virulent isolate was used throughout this study. In order to obtain an antigenic profile similar to that observed in the natural infection, we grew this bacterium at room temperature (RT) and pH 7.6 to mimic the unfed tick conditions. Once the cultures reached a cell density of $1\text{-}2\times10^7$ spirochetes/ml a subculture was transferred to 37ºC, 1% CO_2, and pH 6.8 mimicking the conditions in the tick upon feeding. To run the ELISA tests using whole cell lysates, *B. burgdorferi* was grown in 500ml cultures shifted from RT/pH 7.6 to 37ºC/pH 6.8 and 1% CO_2. After cultures reached a cell density of $3\text{-}5\times10^7$ spirochetes/ml, cells were harvested, washed three times with HBSS buffer (HyClone, Thermo Scientific Inc.), quantified, and lysed

using 0.1mm glass beads in 2ml screw cap tubes in a BeadRuptor 24 (Omni International, Inc). After the lysis cycle, the glass beads were sedimented by quick centrifugation and the supernatants were stored at -20ºC in 1ml aliquots until use in the ELISA assays. For the needle infection experiments, *Bb* cultures were similarly prepared. The bacterial cultures were shifted from RT/pH 7.6 to 37ºC/pH 6.8 and reaching a density of 3-5×10^7 spirochetes/ml prior to being harvested, washed three times with HBSS buffer, and re-suspended in HBSS containing inactivated normal rabbit serum (50:50, *v:v*). The cultures were then quantified and diluted to the appropriate cell density (10^3 or 10^5 spirochetes/ml).

Peptide design

The BB0172 antigen is a *B. burgdorferi* membrane protein, poorly immunogenic in the murine model of Lyme disease [59]. Four peptides within the VWFA domain of BB0172 were designed considering their probability of being exposed to the external environment and distance from a potential internal glycosylation site. The peptides have been designated by the letters A through D (pepA, PepB, pepC and PepD). Peptides were synthetized at Peptide 2.0 Inc. (Chantilly, VA) at 98% purity and conjugated to Keyhole Limpet Hemocyanin (KLH) to ensure immunogenicity. The same peptides were synthetized without conjugation to KLH for *in vitro* T-cell and ELISA assays.

Immunization protocol: identification phase

The protective immunity elicited by each one of the KLH-conjugated BB0172 peptides (A, B, C or D), was evaluated in mice. Groups of 6-8 week old female C3H/HeN mice (n=12) were inoculated subcutaneously with each one of the KLH-

conjugated peptide at a dose of 50µg/mouse formulated in TitterMax® Gold (*v:v*, Sigma-Aldrich, St. Louis, MO) at days 0, 14 and 21 (Figure 3-1). A group of six mice similarly inoculated but with adjuvant alone served as the negative controls. One week after the last boost and prior to challenge, four mice per group were euthanized and sampled to evaluate the antibody levels in serum and T-cell proliferation in draining lymph nodes and spleens. Samples from the heart and tibiotarsal joint from these mice were evaluated histologically to rule out possible side effects due to the antigen administration. Mice were infected by needle inoculation one week after the last boost as described below.

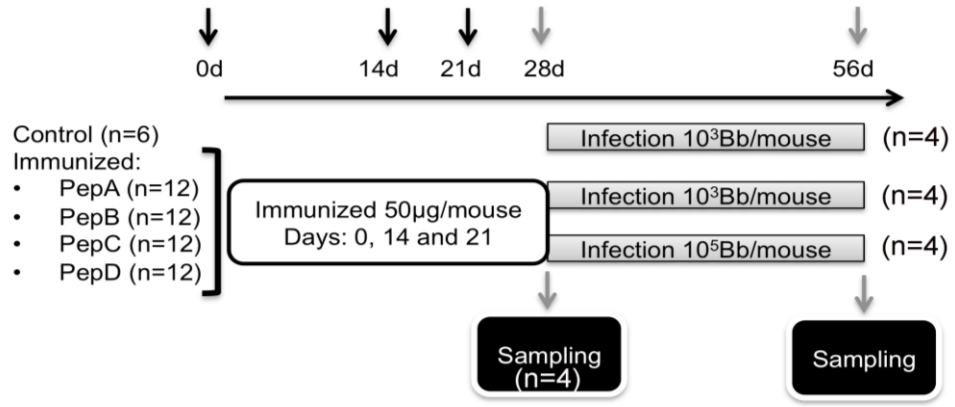

Figure 3-1: Schematic representation of the target identification phase. C3H/HeN mice were immunized with peptides derived from the VWFA domain of BB0172 (A, B, C and D) conjugated to KLH and administered at 50µg/mouse with equal volume of TiterMax® Gold (Sigma-Aldrich) at days 0, 14, and 21. Four weeks post-priming, 4 mice per treatment were sampled to evaluate vaccine safety and antibody levels to each one of the peptides used. The other eight mice were infected with either 10^3 (n=4) or 10^5 (n=4) spirochetes/mouse. Four weeks post-challenge, mice were euthanized and blood collected to determine antibody levels. Tissues were sampled to determine bacterial burden by growth and qPCR as well as to determine any pathology by histology.

B. burgdorferi challenge protocols: identification phase

To determine which peptide elicited protection in the murine model of Lyme disease, four mice per immunized group were challenged by subcutaneous needle inoculation with 10^3 (low) or 10^5 (high) *Bb*/mouse 28 days post-priming as described above (Figure 3-1). The challenge doses used correspond to 10× and 1000× the infectious dose-50 (ID_{50}), respectively. Control mice were infected with only one dose, 10^3 spirochetes/mouse (10× ID_{50}).

To evaluate protection, the mice were euthanized 28 days post-challenge and blood samples were collected to evaluate antibody levels. Skin, spleen, inguinal lymph nodes, heart, bladder and tibiotarsal joint were collected from each mouse for bacterial recovery in BSK-II media complemented with 6% inactivated normal rabbit serum and incubated at 32ºC and 1% CO_2. Five days post inoculation cultures were blind passed to prevent inhibition of bacterial growth by tissue degradation. Blind passaged cultures were incubated at 32ºC and 1% CO_2 for 15 days before evaluating bacterial growth by dark field microscopy [252]. One piece of heart and a tibiotarsal joint were collected for histopathology. Finally, a piece of skin, a small piece of spleen, one inguinal lymph node and one joint were collected for evaluation of bacterial burden by qPCR as previously described [209]. All animal experiments were conducted following the Institutional Animal Care and Use Committee and the Biosafety committee recommendations.

Histopathology: identification phase

Mouse tissues were collected 4-weeks post-priming and 4 weeks after challenge as described above. Tissues were fixed in 10% buffered formalin, processed for routine

histopathology, paraffin embedded, sectioned and stained with H&E. The tibiotarsal bones and joints were decalcified in 10% EDTA prior to being processed for histopathology. A board-certified pathologist blindly evaluated all tissues. Inflammation in selected tissues were scored from 0-4 based on the following scale: normal = 0 (no inflammation), minimal =1 (one small foci of inflammation), mild = 2 (2-5 foci of inflammation with increased numbers of inflammatory cells), moderate = 3 (multifocal inflammation with significant number of inflammatory cells), and severe =4 (multifocal to diffuse, with more than 30% of section infiltrated with inflammatory cells) [253].

Enzyme linked immuno-sorbent assay: identification phase

Sera from immunized mice (0, and 4 weeks post-priming) as well as from animals immunized and then challenged (4 weeks post-challenge) were evaluated for IgG and IgM levels by ELISA. 96-well MaxiSorb® plates (Nunc, Thermo Scientific, Ltd.) were coated overnight at 4ºC with either 500ng/well of each one of the BB0172 peptides or with the whole cell lysate of *B. burgdorferi* A3 strain (10^7 *Borrelia*/well) grown at RT/pH7.6 and shifted to 37ºC/pH 6.8 as described above. Carbonate buffer pH9.1 was used for coating the ELISA plates and after coating, the plates were washed three times in Phosphate Buffered Saline (PBS) containing 0.2% Tween 20 (PBS-T) and blocked for 2 hours at room temperature in PBS-T containing 3% Bovine Serum Albumin (BSA). Blocked plates were washed three times in PBS-T and mouse serum samples were added in duplicates and in 2-fold serial dilutions ranging from 1:100 to 1:102,400 in PBS-T containing 1% BSA. Plates were incubated for 1 hour at room temperature and unbound primary antibodies were removed by washing plates three

times in PBS-T. Secondary anti-mouse HRP conjugated antibody was added to the plates at 1:3000 dilution in PBS-T containing 1% BSA. After washing, plates were incubated with OPD (*o*-phenylenediamine dihydrochloride) color substrate following manufacturer recommendations (Pierce, Thermo Scientific, Ltd). After a 20-minute incubation in the dark, plates were read at a wavelength of 450nm and analyzed by using the BMI LABTECH OMEGA plate reader and software. All samples were evaluated in triplicates.

B. burgdorferi growing conditions: efficacy phase

Bb B31 A3 virulent isolate was also used throughout this section of the study. Culture conditions were the same as described above. In addition, *Bb* used for *in vitro* infection of *Ixodes scapularis* nymphs was grown in BSK-II media pH 7.6 and 1% CO_2 until cultures reached a cell density of 2×10^7 spirochetes/ml.

Immunization protocol: efficacy phase

The same immunization protocol as described in the target identification phase above was used in the efficacy study (Figure 3-4A). PepB was used to immunized mice (n=12) since it was the only peptide that conferred protection in the target identification phase. PepD (n=12) served as an internal negative control since it did not confer protection and in addition, a control group receiving adjuvant only was also included (n=12). Vaccine safety was evaluated at 8 and 12 weeks post-priming (Figure 3-4A). Protection was evaluated 12-weeks post-priming following challenge using *Bb*-infected ticks (Figure 3-4A). Four-weeks post-challenge, the mice were euthanized and protection and safety were evaluated as described below.

Passive transfer protocol

To evaluate the role of antibodies and lymphocytes in the protection induced against Lyme disease in mice immunized with PepB, we conducted passive transfer studies in which groups of donor mice (control, PepB and PepD) were immunized following the immunization protocol described above (Figure 3-4B). Twelve weeks post-priming, the mice were euthanized, and blood and spleens were collected. Splenocytes were isolated from each of the groups as well as serum following procedures described elsewhere [254], and pooled splenocytes and serum were passively transferred to recipient mice (Figure 3-4B). Recipient mice were divided into 6 groups (n=10). Three groups were inoculated with 300µl/mouse of serum samples from control, PepB or PepD-immunized mice, whereas the other three groups were similarly inoculated but with 4×10^7splenocytes/mouse from control, PepB, or PepD-immunized mice, respectively. Forty-eight hours post-transfer, all the mice were challenged by needle inoculation with either a low or a high dose of *B. burgdorferi* as described below. Four weeks post-infection, the mice were euthanized and protection evaluated (Figure 3-4B).

B. burgdorferi challenge protocols: efficacy phase

The protection elicited by the peptides B and D in the murine model of Lyme disease was evaluated by challenging the mice with infected *I. scapularis* ticks (Figure 3-4A) 12-weeks post-priming.

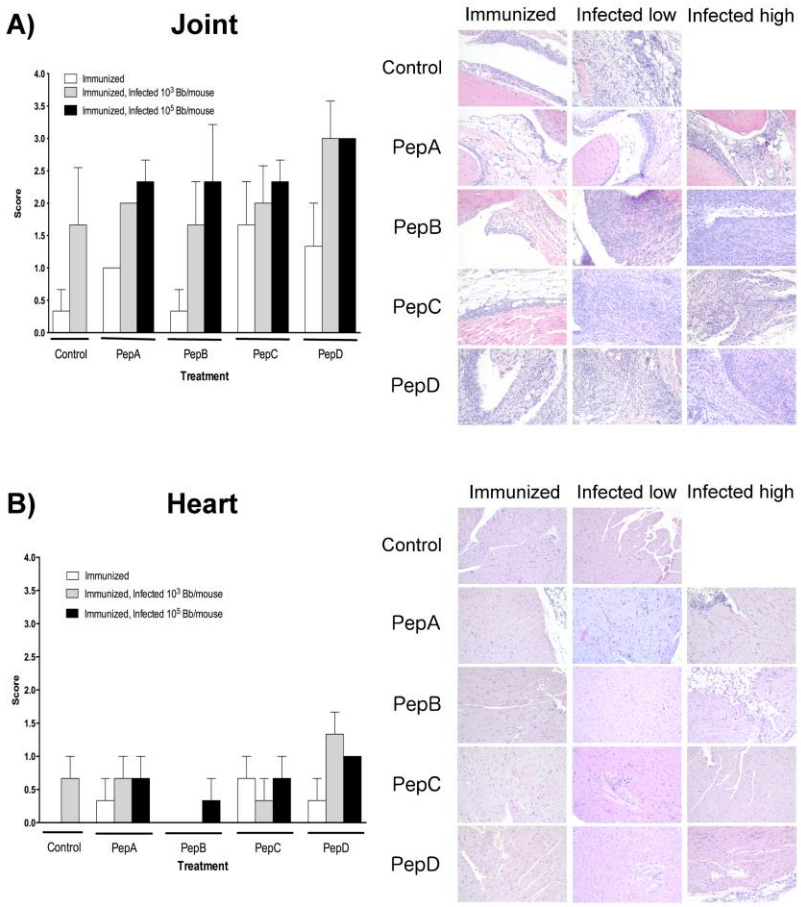

Figure 3-2: Average inflammation in tibiotarsal joint and heart after challenge. Representative histological images of the average level of inflammation observed in each treatment group (control, pepA, pepB, pepC, and pepD) after immunization and/or infection with the low (10^3 spirochetes/mouse) or high (10^5 spirochetes/mouse) doses in the tibiotarsal joint **(A)** and the heart **(B).**Tissues were histologically evaluated at four weeks post priming, as well as four weeks post needle inoculation. Average scores for areas of inflammation were classified as 0= none; 1= minimal; 2= mild; 3= moderate; 4= severe. Peptide B induces minimal inflammation in hearts and tibiotarsal joints after administration in the mouse model for Lyme disease. Of all the peptides evaluated after immunization, only peptide B showed inflammation comparable to the negative control group in both heart and joints. Similar results were observed after infection with low doses of *B. burgdorferi*. Images were captured using an Olympus BX41 microscope at 200X magnification. Average ± SD are presented in the graphs.

To conduct this study, naïve *I. scapularis* nymphs were purchased from the Oklahoma State University Tick Laboratory. Briefly, nymphs were desiccated for 4 days at 79% relative humidity (RH) in a chamber, followed by dipping the ticks in a suspension of 10^8 spirochetes/ml for 45 minutes. After the 45 minute infection, the ticks were washed and placed in the same 79% RH chamber for 3 days in order to improve attachment of the nymphs to mice [255]. Prior to the challenge, a group of 10 ticks was used to evaluate the level of infection with *B. burgdorferi* by quantitative real time PCR (qPCR). Immunized C3H/HeN mice were infested with 5 infected nymphs per mouse and housed in wire bottom cages following standard operational procedures. Ticks were left to feed on mice until repletion.

The challenge protocol described in the target identification phase was used to evaluate the protection conferred by passive transfer of specific serum or adoptive transfer of splenocytes (Figure 3-4B). In both needle and tick challenge, mice were euthanized 28 days post-infection and blood samples were collected to evaluate antibody levels. Skin, spleen, inguinal lymph nodes, heart, bladder and tibiotarsal joint were collected from each mouse for bacterial recovery in BSK-II media as previously described [252]. One ear, a piece of liver, one kidney, a piece of heart and a tibiotarsal joint were collected for histopathology. Finally, a piece of skin, a small piece of spleen, one inguinal lymph node and one joint were collected for evaluation of bacterial burden by qPCR as previously described [209].

Enzyme linked immuno-sorbent assay: efficacy stage

Sera from immunized mice (0, 4, 8, and 12 weeks post-priming) as well as from animals immunized and then challenged (4 weeks post-challenge) were evaluated for IgG and IgM levels by ELISA as described above.

Histopathology: efficacy phase

Mouse tissues were collected after immunization (4, 8, and 12 weeks post-priming) and 4 weeks after challenge as described above. Tissues were processed in the same way as described in the target identification phase above. A board-certified pathologist blindly evaluated all tissues, and inflammation in selected tissues was scored from 0-4 as described above.

T-cell proliferation assay

Priming of *Bb* PepB-specific T-cell responses was tested by proliferation assays using cells isolated from the lymph nodes or spleens as previously described [256, 257]. Two months post-immunization, single cell suspensions were isolated from pooled lymph nodes or spleens from four mice immunized with the KLH-PepB conjugate or from three control mice. Proliferation assay was conducted using 5×10^5 cells/well in triplicate-wells of 96-well plates in a total volume of 100 µl of complete medium containing different doses of PepB (0.01, 0.1, 1, 2.5, 5, or 10 µg/ml). The positive control was 1.25µg/ml concanavalin A (conA), whereas medium alone served as a negative control. In addition, whole cell lysates of *B. burgdorferi* B31 A3 isolate was included in this assay (serial dilutions as above). The cells were cultured for 72 hours at 37°C with 5% CO_2 then labeled with 0.25 µCi of ^{3}H-thymidine for 6 hours, collected

using an automated cell harvester (Tomtec). The incorporated ^{3}H-thymidine was counted with a liquid scintillation counter. The incorporation of ^{3}H-thymidine by the proliferating lymphocytes was presented as mean counts per minute (cpm) of triplicate wells.

In a second experiment, cells were isolated from the lymph nodes and spleens from mice 3 months post-immunization with the KLH-PepB conjugate and proliferations assays were conducted as above. Naïve mice and mice immunized with KLH-PepD conjugate served as controls. The positive control was 1.25µg/ml conA, whereas medium alone served as a negative control. In addition, whole cell lysates of *B. burgdorferi* B31 A3 isolate was also included in this assay. The cultures were labeled and processed as above.

Statistical analysis

Bacterial recovery from tissues was analyzed using the Two-way ANOVA to determine significant differences in between treatments. Quantitative real time PCR data were analyzed using the Mann Whitney U test to determine differences in the bacterial burden determined in each group compared with the control group. In addition, antibody levels were also analyzed utilizing a Two-way ANOVA with the Bonferroni multiple comparison test, in which all groups were compared to the control group. All tests and graphics were performed using Prism 6.0d (GraphPad Software, Inc.).

Results

BB0172 Peptide B protects mice against Lyme disease after needle challenge

Potential vaccine candidates were identified in the target identification phase of the study. In this phase, safety and efficacy of four putative antigens were evaluated in the murine model in order to identify the most promising vaccine candidate that would then progress to the efficacy phase of experiments. In the efficacy phase, the most protective and safest candidate identified in phase one will be more thoroughly characterized as a Lyme disease vaccine candidate in the murine model.

The *B. burgdorferi* chromosomally encoded BB0172 protein has been shown by our laboratory to be a membrane protein containing a VWFA domain exposed to the extracellular milieu [59]. In our efforts to obtain specific antibodies against this protein we observed that BALB/c mice or C3H/HeN mice could not raise specific antibodies to the full-length BB0172 protein (Esteve-Gassent, personal observation).

Table 3-1: Peptide B protects after needle inoculation of $10\times ID_{50}$ in the murine model of Lyme disease.

Strain and dose	No. of tissues positive/No. of tissues tested							No. animals infected/ No. animals tested
	Skin	Spleen	Lymph node	Bladder	Heart	Joint	All sites	
Control								
10^3 spirochetes/mouse	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4
PepA								
10^3 spirochetes/mouse	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4
10^5 spirochetes/mouse	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4
PepB								
10^3 spirochetes/mouse	0/4	0/4	0/4	0/4	0/4	0/4	0/24	0/4***
10^5 spirochetes/mouse	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4
PepC								
10^3 spirochetes/mouse	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4
10^5 spirochetes/mouse	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4
PepD								
10^3 spirochetes/mouse	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4
10^5 spirochetes/mouse	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4

* Denotes statistically significant differences (* *P* value <0.05; ** *P* value < 0.01; *** *P* value < 0.001) when compared with the control group

After analyzing the amino acid sequence of the VWFA-domain in BB0172, peptides A, B, C and D with B-epitope qualities were designed and conjugated to KLH. Following immunization of groups of mice at days 0, 14, and 21, immune protection was tested by challenging the mice 28 days post-priming by needle inoculation of some mice with low dose ($10\times ID_{50}$) and some mice with high dose of ($1000\times ID_{50}$) borrelial cells/mouse. Twenty-one days post-infection, the mice were euthanized and blood and tissues were collected to evaluate antibody responses, bacterial load, and histopathology. *B. burgdorferi* was recovered from tissues (skin, spleen, inguinal lymph node, bladder, heart and joint) from all treatment groups except from tissues collected from mice immunized with PepB and challenged with $10 \times ID_{50}$ (Table 3-1). Evaluation of bacterial burden in the tissues from PepB vaccinees by q-PCR revealed low to undetectable infection levels (data not shown).

Vaccine or *B. burgdorferi*-induced inflammation in joints and heart was evaluated by histological analysis of these tissues collected 4 weeks post-priming and 4 weeks post-infection, respectively (Figure 3-2). PepB-vaccinees developed a minimal inflammation in the tibiotarsal joint similar to the background inflammation observed in the control non-immunized group (Figure 3-2A).

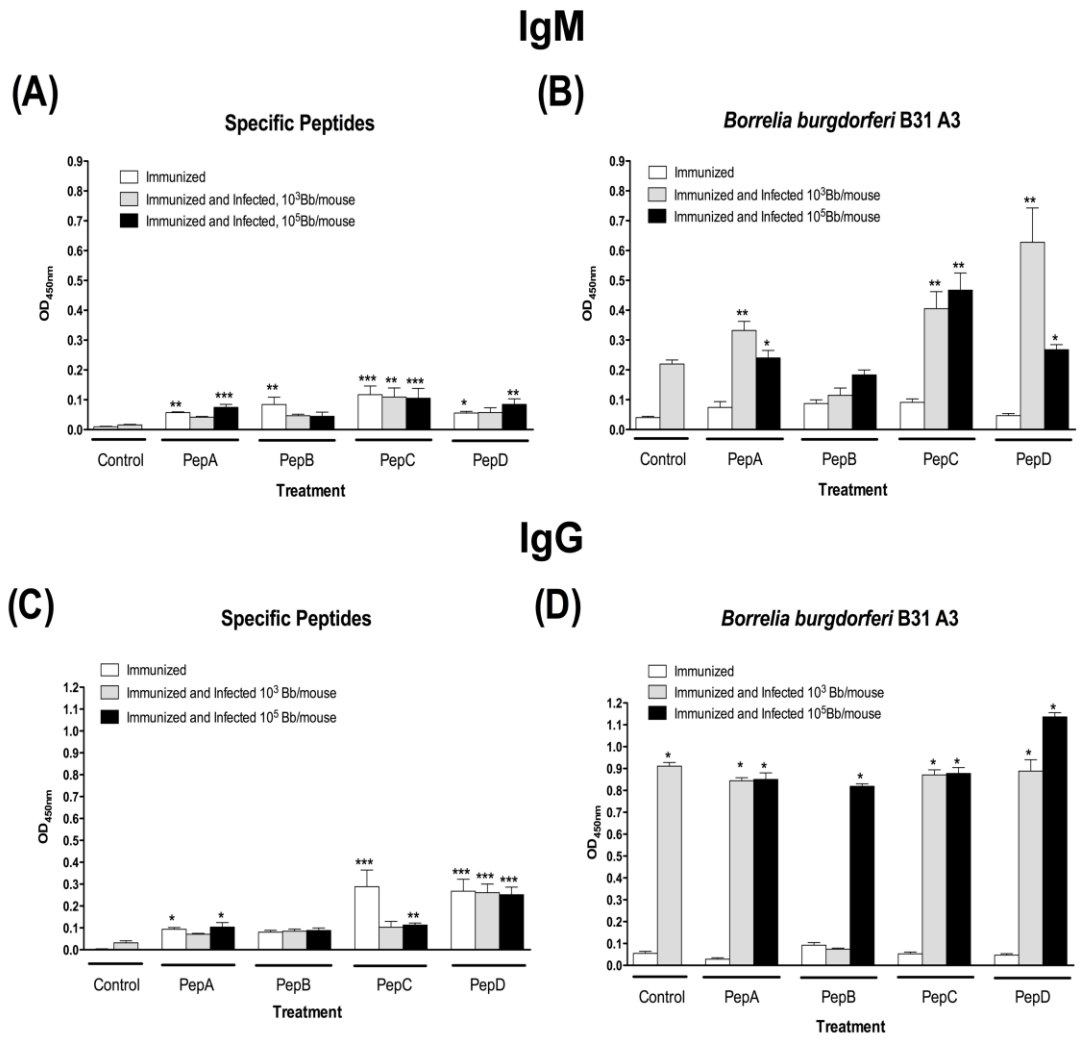

Figure 3-3: Low IgM and IgG antibodies were detected 4-weeks post priming in all groups. Antibody levels were evaluated 4-weeks post-priming as well as 4-weeks post needle infection. (**A**) Peptide-specific IgM antibodies. (**B**) *B. burgdorferi*-specific IgM antibodies. (**C**) Peptide-specific IgG antibodies. (**D**) *B. burgdorferi*-specific IgG antibodies. * Denotes statistically significant differences (* *P* value <0.05; ** *P* value < 0.01; *** *P* value < 0.001) when compared with the control group.

Peptides C and D vaccinated mice had moderate to severe inflammation in the tibiotarsal joint after immunization (Figure 3-2A). Severe inflammation was observed in mice challenged with 1000 × ID_{50} with mice immunized with PepD having the most severe tibiotarsal joint inflammation among the groups tested. Histological evaluation of the heart revealed that PepB vaccinees had no signs of inflammation after immunization and after low dose challenge (Figure 3-2B). PepD treatment induced the highest inflammation in the heart as was observed in the joints.

Evaluation of antibody responses showed that peptide specific IgG and IgM were relatively low in all groups regardless of the treatment received, with slight increase in antibody levels after immunization with peptides C and D (Figure 3-3A and C). In addition, the presence of *B. burgdorferi* specific antibodies was very low in all groups after the immunization schedule was completed. Nevertheless, *B. burgdorferi* specific antibody titers (Figure 3-3B and D) were significantly amplified in all groups after challenge infection, except for the IgG levels in the PepB vaccines challenged with the low borrelial dose. Moreover, serum cross-reactivity in between peptides was not observed (data not shown).

Since PepB induced the best protection, immunization and needle challenge was repeated two more times and similar results were observed with no recovery of bacteria from tissues of mice challenged with 10^3 Borrelia/mouse. Consequently, PepB and PepD (which showed no protection) were selected for the subsequent studies using tick infection to evaluate protection (Table 3-1).

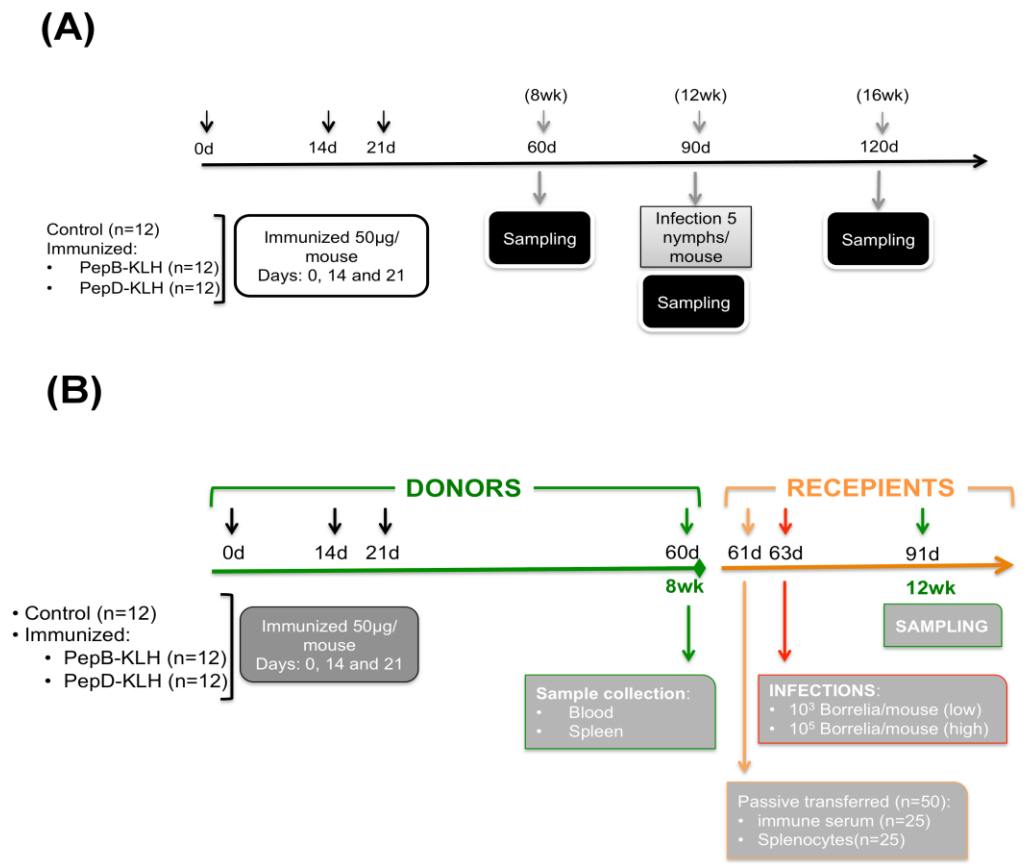

Figure 3-4: Summary of the study design. **(A)** Schematic representation of the efficacy study. C3H/HeN mice were immunized with peptide B or D derived from the VWFA domain of BB0172 conjugated to KLH and administered at 50 µg/mouse with equal volume of TiterMax® Gold (Sigma-Aldrich) at days 0, 14 and 21. Eight weeks post-priming, a subgroup of mice (4/treatment) were sampled to determine antibody levels and pathological side effects. Twelve weeks post-priming a second subgroup of mice (4/treatment) were euthanized and sampled for antibody levels in blood, T-cell activity (from draining lymph nodes and spleens) and tissue damage. At the same time, a final group of 4 mice/treatment was infected by tick challenge, utilizing 8 infected *Ixodes scapulars* nymphs/mouse (containing around 150 *Borrelia*/nymph). Sixteen weeks post-priming mice were euthanized and protection evaluated by determining bacterial recovery from tissues as well as bacterial burden, tissue damage and antibody levels in blood. **(B)** Schematic representation of the passive transfer experiment conducted during phase II. Donor C3H/HeN were immunized with peptide B or D administered at days 0, 14, and 21. Eight weeks post-priming, donor mice were euthanized and blood and spleens were collected. Serum and splenocytes were isolated and passively transferred to recipient mice. Two-days after transfer mice were infected with either a low (10^3 spirochetes/mouse) or a high (10^5 spirochetes/mouse) dose of *B. burgdorferi* B31 by subcutaneous inoculation. Four weeks post-challenge mice were euthanized and protection was evaluated.

BB0172 Peptide B–specific antibody titers peak 8-weeks post-priming

Once a suitable target antigen was determined in phase one, a second efficacy phase was performed to specifically evaluate the immune response and protection induced by the target antigen, PepB. Mice were immunized with PepB as described above (Figure 3-4A) and blood samples for serum were collected at the time of priming, 4, 8, and 12-weeks post-priming. PepB-specific IgG antibodies peaked at 8-weeks post-priming and decreased to levels closer to basal at 12-weeks post-priming (Figure 3-5A). The negative control mice immunized with peptide D had a small IgG antibody peak 4-weeks post-priming (Figure 3-5B) and this outcome was similar to the result observed in the previous screening experiment (Figure 3-3).

In addition, none of the serum samples from immunized mice reacted with *B. burgdorferi* whole cell lysates. Twelve weeks post-priming, the antibody levels had reduced to basal levels and mice were challenged by applying 5 *B. burgdorferi* infected ticks per mouse. Four weeks post-challenge, all the mice were euthanized and blood samples were collected. PepB-immunized mice had the highest peptide-specific IgG antibody levels as well as the anti-*B. burgdorferi* IgG levels (Figure 3-5C). Notice that the antibody titers were also significantly higher in the PepB-immunized group starting at 4-weeks post-priming with maximum titers of 102,400 observed at 8-weeks post-priming (Figure 3-5A). Four weeks post-tick challenge, PepB-immunized mice showed peptide-specific IgG titers of 6,800 significantly higher than those observed in naïve infected mice and PepD-immunized mice (Figure 3-5C). Similar results were observed when *Bb*-specific IgG titers were evaluated.

The PepB vaccinees had IgG antibody titers of 4,266 post-challenge, which was significantly higher than the titers observed in the naïve infected mice and the PepD vaccinees post-challenge mice (925 and 1,925 respectively). In addition, IgM antibody levels remained very low throughout this experiment (data not shown) as observed in the previous study (Figure 3-3A and B). PepD-specific IgM antibodies increased slightly after tick challenge (Figure 3-5D), whereas PepB-specific IgM antibodies remained at basal level similar to the results observed in the earlier study (Figure 3-3A and B). Protective IgG antibody levels specific for PepB started peaking at 4-weeks post priming, reaching maximum levels at 8-weeks post-priming.

In addition, we measured the levels of IgG1 and IgG2a in the PepB-immunized group at 4, 8, and 12-weeks post-priming as well as at 4-weeks post-tick challenge (16 weeks). As observed in Figure 3-5E, IgG1 titers were significantly higher than IgG2a with a peak at 12 weeks post-priming (307,200 and 78,400 respectively). Four weeks post-challenge, IgG1 titers (23,200) remained similar to those observed at 12-weeks post-priming (38,400) and were significantly higher than levels measured for IgG2a at the same time points (850, 12-weeks post-priming and 1,250 post-challenge). This observation suggests that the immune response after immunization and tick infection skewed towards Th2.

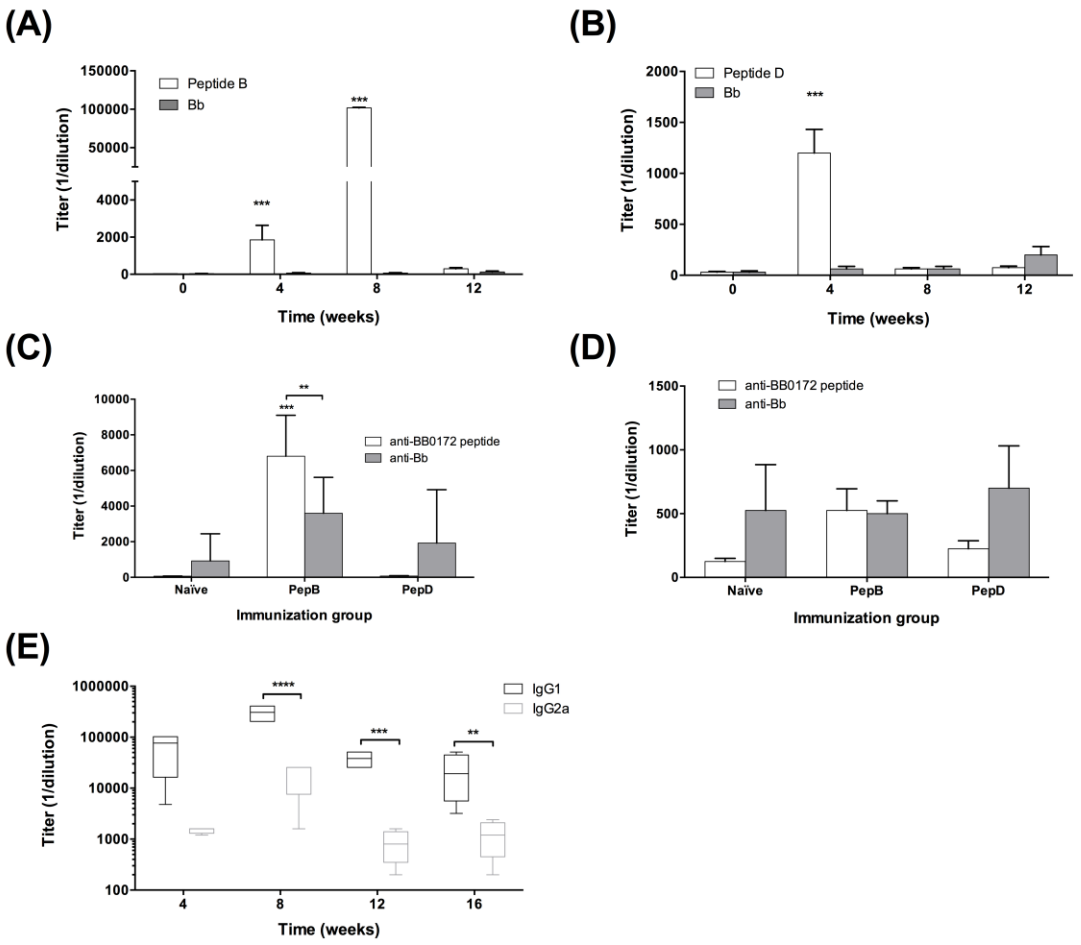

Figure 3-5: Peptide B-specific antibodies peaked 8-weeks post immunization and were significantly stimulated 4 weeks post-tick infection. (**A)** IgG antibodies specific to Peptide B (open bars) and *B. burgdorferi* (grey bars) at weeks 0, 4, 8, and 12 post-priming. (**B**) IgG antibodies specific to Peptide D (open bars) and *B. burgdorferi* (grey bars) at weeks 0, 4, 8, and 12 post-priming. IgG (**C**) and IgM (**D**) antibody levels specific to each of the BB0172 peptide (open bars) and *B. burgdorferi* (gray bars) in control and animals immunized with either Peptide B or D 4-weeks post tick infection. **(E)** IgG1 and IgG2b antibody levels after 4, 8, 12 and 16 weeks post-priming with PepB. Titer represented in parenthesis. * Denotes statistically significant differences (* *P* value <0.05; ** *P* value < 0.01; *** *P* value < 0.001) when compared with the control group.

BB0172 Peptide B partially protects against Lyme disease after tick challenge

After tracking antibody responses in the mice immunized with PepB, the mice were challenged to determine whether or not the high antibody levels could protect mice

against Lyme disease. At 12-weeks post-priming, mice were housed individually in wire bottom cages and challenged by applying 5 infected *I. scapularis* nymphs with an average of 100 *B. burgdorferi* cells per nymph. The mice were euthanized 4 weeks after ticks were applied. Analysis of skin, spleen, inguinal lymph node, bladder, heart and tibiotarsal joint tissues showed that PepB-immunized group had a significantly lower percent of positive cultures, compared with control and PepD-treated mice (Figure 3-6E). Importantly, the outcome from this challenge study using the tick infection model, a 50% vaccine efficacy was achieved. In addition, the bladders of the PepB-immunized mice had lower less bacterial load when compared with the control and PepD-immunized groups (Figure 3-6E).

Furthermore, the bacterial burden in skin and spleens of mice immunized with PepB were significantly lower compared with the control group (Figure 3-6A and B). Lymph nodes and joints had very low bacterial burden in both immunized groups regardless of the peptide used (Figure 3-6C and D). Overall, the PepB-immunized mice had the lowest bacteria burden following challenge using infected ticks suggesting that this is a good candidate for the development of a Lyme disease vaccine of use in veterinary medicine.

Peptide B–specific antibodies are responsible for protection against Lyme disease

To determine whether the protection observed in the PepB-immunized mice was due to the high antibody titers or the cellular immune response, donor mice were immunized with PepB, PepD, or adjuvant only (control). When the peptide-specific antibody titers peaked at eight-weeks post-priming (Figure 3-7A, peptide B: 100,000;

peptide D: 300; control: 50) serum and splenocytes from each donor group were transferred to recipient mice, and then challenged 48hr after transfer. PepB-specific antibodies protected mice challenged with low doses of *B. burgdorferi* B31 ($10 \times ID_{50}$) while no protection was observed in the other groups (Figure 3-7B). In addition, splenocytes from PepB-immunized mice conferred partial protection, which suggests a protective role of splenocytes (Figure 3-7C).

Analysis of bacterial burden in different tissues of the recipient mice showed that animals that received anti-PepB specific antibodies had very low bacterial numbers in tissues, specifically skin and spleen, compared to the control group or the anti-peptide D treated group (Figure 3-7 D-G). The mice that received splenocytes had higher bacterial burden than those that received antiserum. Moreover, mice that received splenocytes from PepB vaccinees had the lowest bacterial burden in lymph nodes and joints, compared to those that received splenocytes from the control and PepD-immunized mice (Figure 3-7 F and G). No difference in bacterial burden was observed between treatments in the skins and spleens (Figure 3-7 D and E). These results suggest the relevance of specific antibodies to block colonization.

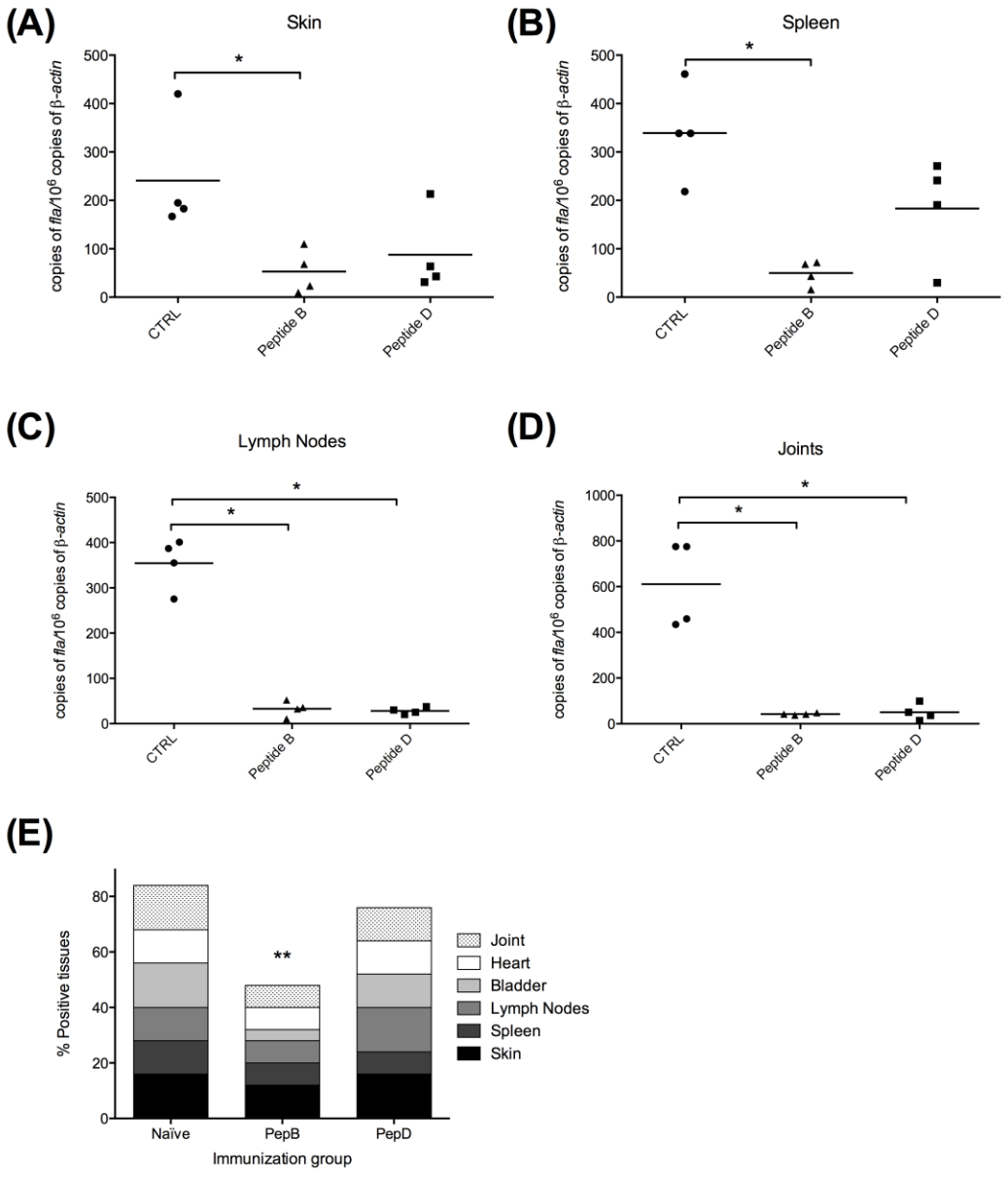

Figure 3-6: Peptide B induces partial protection in mice infected by using the tick model. Bacterial burden in tissues was significantly lower in animal immunized with Peptide B especially in skin (**A**) and spleen (**B**). Lymph nodes (**C**) and Joints (**D**) show lower bacterial burden in both Peptide B and D immunized mice. Nevertheless, the bacterial recovery in cultures (**E**) was significantly reduced in mice receiving the Peptide B formulation compared with Peptide D or the control group. * Denotes statistically significant differences (* *P* value <0.05; ** *P* value < 0.01) when compared with the control group.

BB0172 peptide derived antigens are safe when injected subcutaneously in C3H/HeN mice

The safety and tolerability of PepB immunogen was evaluated in C3H/HeN mice by histological evaluation of tissues at 4, 8, and 12 weeks post-inoculation. Most significant inflammation was mainly observed after infection with high bacterial doses, regardless of the vaccine candidate used (Figure 3-2). This was observed consistently throughout the challenge experiments with high dose *B. burgdorferi* infection. Only minimal myocarditis and synovitis were observed in mice after immunization with PepB, as described above. Furthermore, no histological changes or areas of inflammation were observed in additional tissues evaluated at 8 and 12 weeks post-priming (skin, heart, tibiotarsal joint, liver and kidney, data not shown). Taken together, in the murine model, the BB0172 PepB antigen was shown to be particularly safe. As such, further studies with PepB should be performed in other animal models in order to demonstrate the safety of this vaccine candidate.

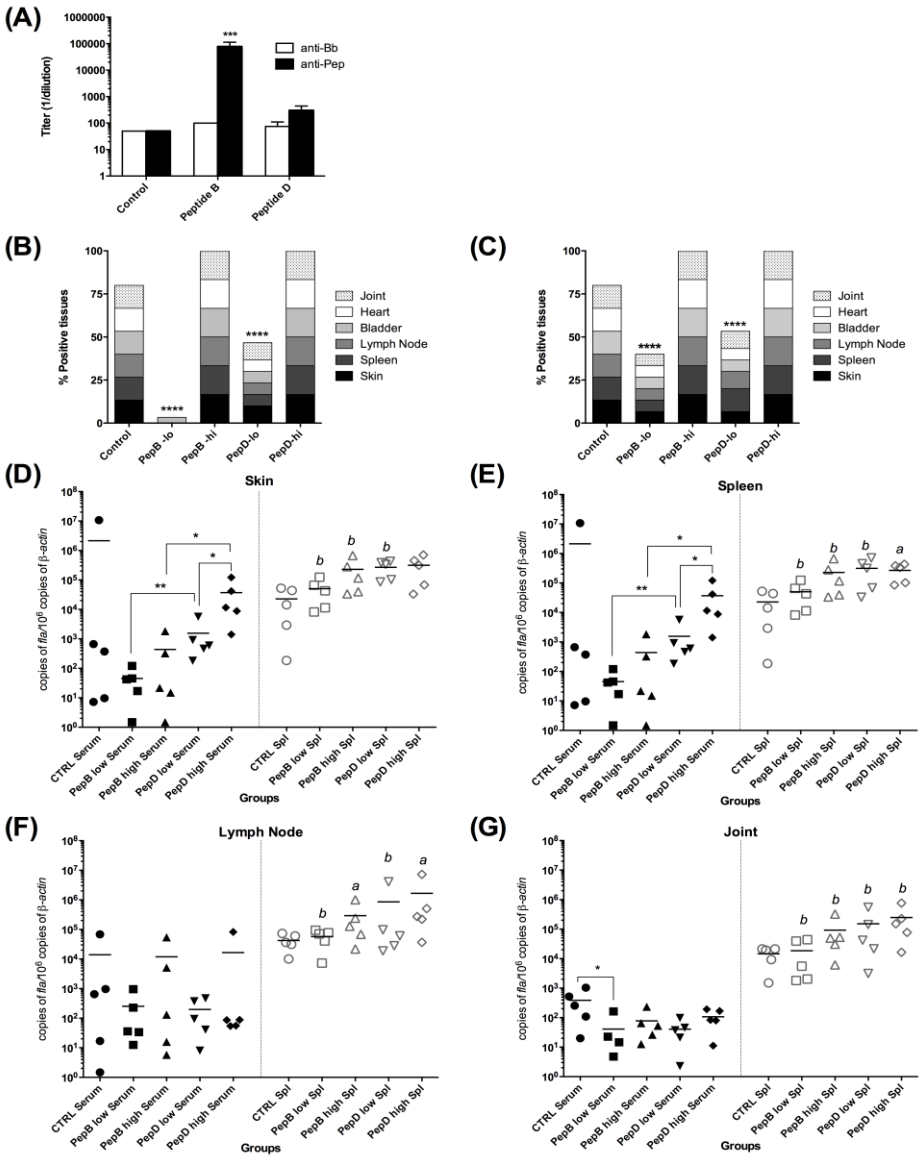

Figure 3-7: Peptide B-specific antibodies confer protection against *B. burgdorferi* infection. Antibody titers of control, peptide B-immunized, and peptide D-immunized animals. Open bars represent the anti-*B. burgdorferi* titers and black bars represent the peptide-specific antibody titers in each group (**A**). Bacterial recuperation from tissues of animals infected after passively transferring peptide specific serum (**B**) or splenocytes (**C**) to naïve mice. Bacterial burden was evaluated by qPCR in skin (**D**), spleen (**E**), lymph nodes (**F**), and joints (**G**). Bacterial recuperation from tissues and quantification was done 21 days-post infection. * Denotes statistically significant differences (* *P* value <0.05; ** *P* value < 0.01) when compared within the passive transfer treatment, while ***a*** (*P* value <0.05) and ***b*** (*P* value < 0.01) denote significant differences in between animals receiving serum or splenocytes from the same treatment (peptide B or peptide D).

T-cell response

PepB-specific T-cell responses in mice immunized with the KLH-PepB conjugate or the control KLH-peptide D conjugate was tested by proliferation assays using cells isolated from lymph nodes or spleens. At 8 weeks post-priming, significant PepB-specific T-cell responses and PepD-specific T cell responses were detected in the cells isolated from the lymph nodes draining the immunization sites but not in splenocytes (Figure 3-8A). This outcome was rather unusual given that primed antigen-specific T cells were also expected to be detected in the spleen. However, at 12 weeks post-priming, no PepB-, PepD-, nor *B. burgdorferi* B31 A3-specific T-cell responses were detected in the lymph nodes (Figure 3-8B) or splenocytes (Figure 3-8C). The cells from these tissues responded well to conA mitogen suggesting that the cells were healthy (Figure 3-8B and C).

Discussion

Currently there is no commercial LD vaccine available in the market to protect humans, and hence we primarily rely on other preventive measures to control the incidence of this disease, especially in endemic areas. A number of vaccine candidates have been studied and tested in the mouse model for Lyme disease as well as in wild life [160, 161, 241-243], in an effort to control the spread of this disease. Most of the approaches used in the last few years are based on the outer-membrane lipoproteins OspA and OspC [43, 44, 46, 244-246], together with a few novel antigens such as BBA52 [53, 247].

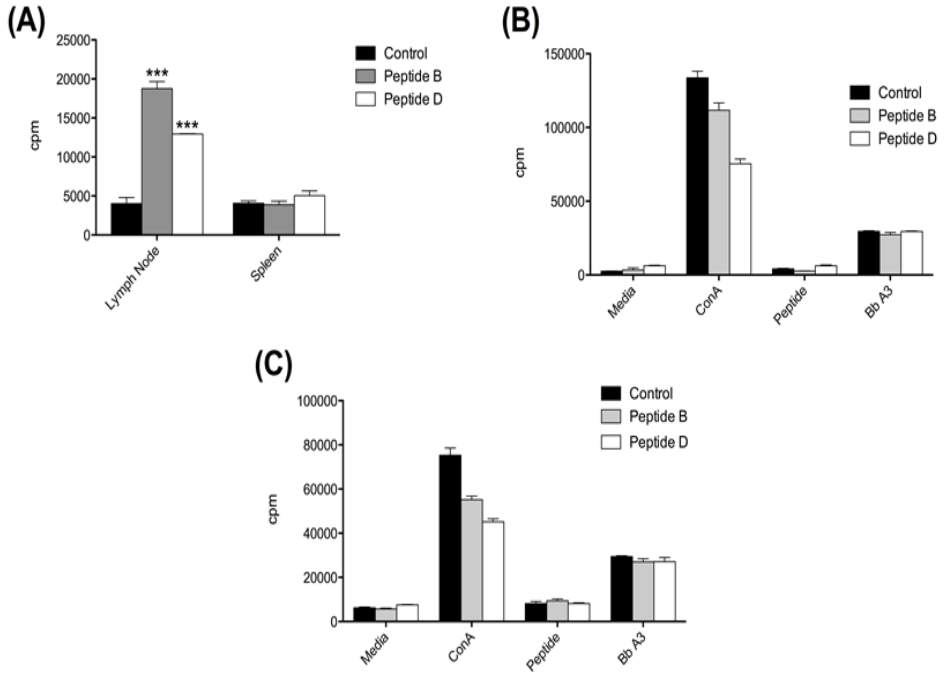

Figure 3-8: Proliferation assay of T-cells isolated from lymph nodes and spleens of mice immunized with peptide B, peptide D and controls. (A) Proliferation assay at 8 weeks post-priming. Notice the high activity of cells isolated from lymph nodes of immunized compared with the control mice without any stimulation of the cultures. Proliferation assay of cell isolated from lymph nodes (B) and spleens (C) at 12 weeks post- priming. Concavalin A (ConA) was used as positive control for stimulation of the cell cultures. Specific peptides and *B. burgdorferi* B31 A3 strain whole cell lysates were used as test antigens to stimulate the cultures. Mean ± standard error of the mean, is represented for each lymphocyte proliferation measured. * Denotes statistically significant differences (* *P* value <0.05; ** *P* value < 0.01) when compared with the control group.

Dogs and horses have been identified as sentinels for Lyme disease across the US [258-260]. Under this scenario, and since Lyme disease affects both humans and companion animals, the development of a DIVA vaccine (differentiating infected from vaccinated animals) will be of great value in the control of Lyme disease utilizing a global health approach [261-264]. The DIVA vaccine strategy will not only improve our diagnostic capabilities, but also helps us in the prevention of Lyme disease in companion

animals and in the reduction of reservoir competence. Therefore, a new Lyme DIVA vaccine can significantly impact the prevention of LD in humans and animals.

Our previous studies have identified BB0172, a chromosomally encoded borrelial protein anchored to the outer membrane through two hydrophobic domains [59]. In addition, BB0172 is conditionally expressed and has been shown to bind to integrins α3β1 *in vitro* as it is relatively conserved among Borrelia species [59]. Therefore, we hypothesized that this protein could be an effective vaccine candidate due to both its function as an adhesin and the fact that sera from naturally infected animals did not react to this protein in ELISA and immunoblot assays [59].

Consequently, we developed a number of short peptides conjugated to a hapten (KLH). Our results showed that mice immunized with the PepB formulation were protected against infection with pathogenic *B. burgdorferi* administered by injection at low infectious doses. These results supported the hypothesis that PepB could be a strong vaccine candidate to prevent Lyme disease. In addition, no inflammation was observed in hearts and joints from animals receiving this vaccine formulation, even after infecting with low doses of *B. burgdorferi*. Very low peptide-specific antibody titers were observed in this first screening experiment. After infection, only high *B. burgdorferi* specific antibody titers were generated in all groups except in animals immunized with PepB and infected with low borrelial doses.

Following low dose *B. burgdorferi* challenge, PepB conferred the highest vaccine efficacy (100%) compared with the other peptides tested, and therefore was selected as a potential DIVA vaccine antigen. We also selected PepD as a negative

control as it is a peptide from the same protein but it did not confer protection. In our studies, the antibody titers for PepB consistently increased during the weeks following the immunization schedule, peaking at 8-weeks post-priming. We evaluated the protection acquired after vaccination by exposing the mice to *B. burgdorferi* through the natural route of infection. Using infected *I. scapularis* ticks directly after the antibody levels returned to basal level, we consistently observed that PepB-immunized mice were significantly protected against infection.

Passive transfer of sera from PepB-immunized, but not from PepD-immunized mice, to naïve recipients conferred protection upon challenge. This suggested that antibodies play a role in protection, an outcome which is consistent with previous demonstrations that anti-*B. burgdorferi* antibodies play a significant role in protection [162, 265-268]. Analysis of PepB-specific antibody isotypes revealed IgG1 dominance, suggesting a Th2-type immune response, which was consistent with previous findings [269-271]. Adoptive transfer of splenocytes from PepB-immunized, but not from PepD-immunized mice, conferred partial protection. This outcome could have been due, in part, to the presence of PepB-specific antibodies secreted by memory B cells in splenocytes. If the presence of memory B-cells was responsible for the partial protection, it is not clear why the cells did not undergo recall upon challenge, but it was notable that no PepB-specific T cells were detected in spleens by proliferation assay. In addition, PepB-specific splenocytes were transferred to recipient mice through intravenous administration while *B. burgdorferi* was administered by subcutaneous needle injection. The discrepancy in administration of both splenocytes and the infectious agent could

explain why the B-cells injected did not generate enough antibodies to neutralize *B. burgdorferi* after infection. Under these circumstances, B-cells will tend to migrate to the spleen while the borrelial cells will prefer the draining lymph nodes, skin and joints [48, 200, 225]. The disparity in tissue tropisms may account for the discrepancy in the results observed, where passive transfer of PepB-specific antibodies induce protection, and the transfer of PepB-specific splenocytes did not [272]. Additional studies are needed to define the role played by T cells in protection.

Further studies need to be done in order to improve the protection, mostly by improving the delivery method as well as the hapten/adjuvant with which this antigen is administrated. In particular, delivery of the vaccine antigen utilizing viral particles [273, 274], as well as the use of microneedles [275-278] for the delivery of vaccines can significantly improve the immune response and consequently protection after both needle and tick infection. In addition, by using transdermal inoculation we will be stimulating the cell types that most likely will be the encountered by the bacterium after the tick bite [277, 278].

Taken together, an improved DIVA vaccine will significantly impact the prevention and control of Lyme diseases as well as its surveillance since it will be compatible with currently available tests for the detection of Lyme diseases in animals such as IFA, ELISA and immunoblot assay (in particular, the C6 base technology (IDEXX laboratories Inc)), without the necessity of developing further tests to detect infected animals. With the vaccine antigen PepB, regular ELISA tests can differentiate which animals have been vaccinated (react to PepB antigen only) from those that have

been infected (react to *B. burgdorferi* extract only), and also those that had received the vaccine and are undergoing infection (react to both PepB and *B. burgdorferi* extract in ELISA), making PepB a suitable candidate for the development of a DIVA vaccine.

CHAPTER IV

DESIGN AND IMMUNOGENICITY OF A SCAFFOLDED PEPTIDE ANTIGEN

Introduction

There is a range of methods and combinations employed to enhance the immune response against a peptide antigen based vaccine. The use of carrier proteins like KLH and CRM-197, immunostimulatory molecules such as IL-1 and CpG motifs, and adjuvants such as aluminum hydroxide and emulsions may all contribute to an enhanced immune response as opposed to using a small peptide alone. Formulation of the vaccine candidate may also decrease the amount of peptide required to generate a protective immune response [279].

In order to enhance the immunogenicity of the peptide antigen, PepB will be substituted into a protein scaffold. This scaffolded antigen will be used in conjunction with an adjuvant with the goal of increasing the protective antibody immune response by increasing the size of the peptide. Further, this method will still be in line with the ideal target product profile of using a recombinant protein vaccine candidate with potential to be scaled up using commonly utilized protein expression and purification methods.

Two *B. burgdorferi* proteins were considered to be PepB scaffolds. Each protein has been evaluated individually in the murine model, however, have proven not to be protective when utilized alone. Nevertheless, each protein was capable of generating a strong humoral immune response upon immunization. Ideally, by combing these

proteins with the protective epitope, PepB, a stronger PepB immune response will be stimulated. Further, neither scaffold protein will limit the DIVA functionality of PepB, making it possible to continue surveillance of companion animals. The scaffold proteins investigated in this study are VlsE and BBA34, and they are introduced in more detail below.

One of the most important antigens present during LD infection is the 31 kDa Variable major protein-Like Sequence, Expressed (VlsE). This surface exposed lipoprotein is immunodominant, and the conserved region, C6, is present both in *B. burgdorferi s.s.* and in Eurasian strains, making VlsE an ideal candidate for detection in diagnostics [68]. The role of VlsE in *B. burgdorferi* is thought to be immune evasion for two reasons: VlsE is highly immunogenic and undergoes antigenic variation through recombination with 15 silent cassettes found upstream of the expression site [69, 280]. The result of these two functions is that much of the host antibody response is directed against a protein that changes as infection progresses, causing misdirection of the immune system limited control of the pathogen [281, 282].

VlsE plays an important role in the detection of LD. To confirm infection with LD in the laboratory, patients undergo a two-tiered testing protocol. The C6 ELISA is used in the first tier of testing, that consists of identifying host antibodies to either the VlsE C6 23-mer peptide or to whole-cell *Borrelia* lysate [82]. To use VlsE as a scaffold, it is important to eliminate the C6 epitope to conserve the ability to detect naturally infected patients as opposed to vaccinated individuals, allowing the serological detection

of a natural *B. burgdorferi* infection to remain relevant even in VlsE-scaffold vaccinated individuals.

B. burgdorferi contains one peptide transport system of the ABC family type that bears similarity to the *Escherichia coli* oligopeptide permease (Opp) transport system. BBA34 is one of 5 oligopeptide permease A homologs in *B. burgdorferi* and is denoted as OppA5. The *bba34* gene is located on linear plasmid 54 (lp54), while OppA1-3 are found on the linear chromosome, and OppA4 is found on circular plasmid 26. BBA34 was found to be upregulated in spirochetes in fed ticks and the mammalian host.

Studies done by Raju et al. [67] implicate that BBA34 functions in the transport of molecules like acetate and bicarbonate rather than heptapeptides, as is performed by OppA1-3. Further, the putative binding domain of BBA34 bears homology to outer surface protein P66, a porin capable of binding integrins [64].

Interestingly, BBA34 and OppA4 were both predicted to be exposed to the periplasmic environment, a prediction that Raju et al. [67] confirmed for BBA34. As such, BBA34 is a lipoprotein that anchors to the outer membrane, but is exposed to the *B. burgdorferi* periplasm. This is useful for application as a scaffold protein, as intact *B. burgdorferi* will not be recognized by antibodies generated against BBA34. For this reason, it will be possible to determine strength and duration of antibody responses specifically against the BBA34 scaffold or PepB both before and after challenge with *B. burgdorferi*, allowing for the quantification and comparison of the scaffolded construct to previous PepB-KLH studies. For comparison, the control proteins BBA34 (no substitution) and OspC will be utilized.

Materials and methods

Cloning and expression of scaffolded antigen

The scaffolded BBA34:PepB (BP) construct was synthesized in the expression vector pET23a by Genscript (Piscataway, NJ). Upon receipt, the plasmid was electrotransformed into Rosetta™(DE3)pLysS *E. coli* (Novagen, Madison, WI) and stored at -80°C. BP was overexpressed at 37°C using 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours. The cells were pelleted immediately after overexpression, and were disrupted via sonication for a total sonication time of 10 minutes in lysis buffer (20 mM imidazole; pH 7.4) in the presence of the protease inhibitor cocktail, HALT™ (Thermo Scientific, Inc.). The lysed cells were then clarified by centrifugation, and the supernatants underwent affinity purification with a His60 Ni Superflow resin (Clontech, Mountain View, CA) according to the manufacturer's protocol. Bound proteins were washed with 2 column volumes of wash buffer (40 mM imidazole; pH 7.4) prior to elution. Elution buffer (300 mM imidazole; pH 7.4) was used to elute the bound 6×His-tagged proteins. Fractions were then analyzed using SDS–12.5% PAGE.

Purification of scaffolded antigen

After detection on SDS-12.5% PAGE, fractions containing the highest relative amounts of protein were pooled and concentrated using Spin-X™ centrifugal filters (Corning, Lowell, MA) with a 10 kDa MWCO. Concentrated BP was then applied to a column containing Sephadex G-75 resin for size-exclusion chromatography. Proteins were eluted using immunization buffer. Fractions were analyzed using Bradford reagent

(Bio-Rad, Hercules, CA) detected at a wavelength of 595 nm and fractions with the highest relative concentration of protein were pooled. Pooled fractions were concentrated as previously described and applied to a PD-10 desalting column (GE Healthcare, Piscataway, NJ). Fractions were then analyzed by SDS-12.5% PAGE, and fractions containing the highest concentration of protein were pooled and concentrated as described. A 61.5-kDa (BP) was purified and the protein was stored at 4°C until further use.

Expression of control proteins

Control proteins OspC or BBA34 previously cloned into pET23a and transformed into Rosetta™(DE3)pLysS *Escherichia coli* (Novagen, Madison, WI) before being stored at -80. Proteins were overexpressed at 37°C using 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 hour (OspC) or 3 hours (BBA34). The cells were pelleted immediately after overexpression, and were disrupted via sonication for a total sonication time of 10 minutes in lysis buffer (20 mM imidazole; pH 7.4) in the presence of the protease inhibitor cocktail, HALT™ (Thermo Scientific, Inc., Rockford IL). The lysed cells were then clarified by centrifugation, and the supernatants underwent affinity purification with a His60 Ni Superflow resin (Clontech, Mountain View, CA), according to the manufacturer's protocol. Bound proteins were washed with 2 column volumes of wash buffer (40 mM imidazole; pH 7.4) prior to elution. Elution buffer (300 mM imidazole; pH 7.4) was used to elute the bound 6×His-tagged proteins. Fractions were then analyzed using SDS–12.5% PAGE.

Purification of control proteins

After SDS-12.5% PAGE, fractions containing the highest relative amounts of protein were dialyzed into immunization buffer (50 mM Sodium Phosphate, 300 mM Sodium Chloride, 0.1% Triton X-100; pH 7.4) using Slide-A-Lyzer dialysis cassettes (Thermo Scientific, Inc., Rockford, IL). Dialyzed protein was then applied to Amicon centrifugal filters (EMD Millipore, Billerica, MA) with a 10 kDa MWCO were then utilized to concentrate the proteins. From these purifications, two proteins with weights of 23-kDa (OspC) and 61-kDa (BBA34), were purified and quantified using the Pierce™ BCA Protein Assay (Thermo Scientific, Inc., Rockford, IL). Proteins were stored at 4°C until further use.

Immunization protocol

Mice were immunized either at days 0, 14, and 28 with decreasing antigen dosages (BP Group A (n=3), BBA34 Group A (n=3), OspC (n=2)) or were immunized at days 0, 28 with decreasing antigen doses (BP Group B (n=3), BBA34 Group B (n=3)), as shown in Figure 4-1A. Previous work demonstrated that Adjuplex adjuvant utilized at either 5 or 10% could protective immunity similar to that seen with the commonly utilized TiterMax Gold adjuvant (data not shown). Antigens and controls were diluted in immunization buffer with 5% Adjuplex™ adjuvant. Control mice (n=3) were immunized at days 0, 14, and 28 with 5% Adjuplex™ in immunization buffer only. The immunization schematic shown in Figure 4-1B highlights the dates for immunizations, and also shows that mice are euthanized at 8 weeks days post priming, as antibodies against PepB have been seen previously to peak at this time [283]. Blood and tissues

(joint, heart, kidney, lymph node, spleen, and bladder) were collected. In addition, serum was collected from the blood by centrifugation to evaluate antibody titers by ELISA (described above).

A)

Group	Prime		Boost 1		Boost 2	
	wpp	dose	wpp	dose	wpp	dose
BBA34:PepB-A (BPA)	0	50 µg	2	10 µg	4	5 µg
BBA34:PepB-B (BPB)	0	50 µg	-	-	4	15 µg
BBA34-A (BA)	0	50 µg	2	10 µg	4	5 µg
BBA34-B (BB)	0	50 µg	-	-	4	15 µg
OspC	0	50 µg	2	10 µg	4	5 µg
CTRL	0	50 µg	2		4	-

B)

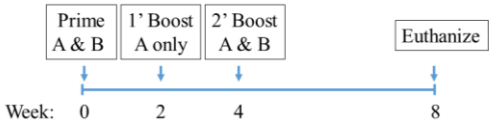

Figure 4-1: Dosing groups and schedule for scaffolded vaccine antigen. (A) Immunization plan for the scaffolded peptide antigens and controls. Two immunization schedules were used for the experimental constructs, differing in number of immunizations and amount of antigen utilized. Group A was administered in 3 doses (50, 10, 5 µg antigen) two weeks apart, and consisted of BBA34:PepB, BBA34, OspC. Group B was 2 doses (50 and 15 µg) administered 4 weeks apart. Control mice received only adjuvant, and were immunized according to the group A schedule. **(B)** Schematic visually describing immunization protocol. Animals were immunized at either 0, 2 and 4 weeks, or at 0 and 4 weeks. All animals were euthanized at 8 weeks post priming.

Enzyme-linked immunosorbent assay

Sera from fully immunized mice was evaluated for IgG antibody titers using the enzyme-linked immunosorbent assay (ELISA). 96-well MaxiSorb® plates (Nunc; Thermo Scientific, Inc. Rockford, IL) were coated either with 500 ng/well of antigen or 10^7 Borrelia/well of *B. burgdorferi* A3 whole cell lysate in coating buffer (15 mM Sodium Carbonate, 35 mM Sodium Bicarbonate; pH 9.1). After coating, plates were

washed 3 times with Phosphate Buffered Saline (PBS) containing 0.1% Tween-20 (PBS-T). Plates were then blocked in PBS-T containing 3% Bovine Serum Albumin (BSA) at room temperature for 2 hours. After blocking, plates were again washed 3 times with PBS-T. Mouse serum samples were applied to the plate in duplicates serially diluted from 1:100 to 1:102,400 in PBS-T containing 1% BSA and incubated for 1 hour at room temperature. Unbound antibodies were removed by washing 3 times with PBS-T prior to application of a 1:3000 dilution of secondary anti-mouse HRP conjugated antibody diluted in PBS-T containing 1% BSA. After incubating for 1 hour at room temperature, unbound secondary antibody was removed by washing 3 times with PBS-T. Plates were developed using the *o*-phenylenediamine dihydrochloride (OPD) color substrate following the manufacturer's recommendations (Pierce; Thermo Scientific, Inc. Rockford, IL). Plates were incubated for 20 minutes at room temperature in the dark before evaluation at a wavelength of 450 nm using the Bio-Tek Synergy™ H1 microplate reader and Gen5™ software (BioTek Instruments Inc., Winooski, VT).

SDS-PAGE gels and immunoblot analysis

B. burgdorferi whole-cell lysates (prepared from cultures grown at RT and pH 7.6) or purified protein aliquots were separated using SDS–12.5% PAGE. The separated proteins visualized by either Coomassie brilliant blue staining or immunoblot analysis. For immunoblot analysis, proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were then blocked overnight at 4°C in Tris-buffered saline containing both 0.1% Tween 20 (TBS-T) and 10% skim milk. Membranes were then probed with mouse anti-PepB polyclonal antibodies or anti-His tag monoclonal

antibodies (GE Healthcare, Piscataway, NJ). Blots were washed 3 times with TBS and secondary HRP conjugated anti-mouse IgG antibody was applied at a dilution of 1:3000 in TBS-T with 10% skim milk. Membranes were washed 6 times with TBS-T then developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ).

B. burgdorferi growing conditions

Bb B31 A3 virulent isolate was also used throughout this section of the study. *B. burgdorferi* was grown in BSK-II media at pH 7.6 with 1% CO_2 at RT until reaching a density of 5×10^7 spirochetes/ml. Cells were then pelleted and lysed for use as whole cell lysates in ELISAs and immunoblots.

Results

Modeling of substitution constructs

Initially, constructs for the VlsE scaffolded constructs were generated. In these models, the PepB antigen was used to substitute for IR6 (VIR6P) or the entire cassette region (VCP), as demonstrated in Figure 4-2A. Further, to screen the potential for surface exposure of the PepB antigen on the construct surfaces, protein structure modeling was performed using PyMOL. The models for VIP and VCP are found in Figure 4-2B, and show that PepB is predicted to be exposed on the surface of the proteins.

Figure 4-3A shows a comparison of BBA34 (top) and the substitution construct that replaces the putative interaction domain of BBA34 with PepB (bottom). The models of the parent BBA34 protein (top) to the substituted BBA34:PepB scaffolded construct (bottom) are found in Figure 4-3B. These models also demonstrate the possibility of PepB exposure on the surface of the scaffolded antigen.

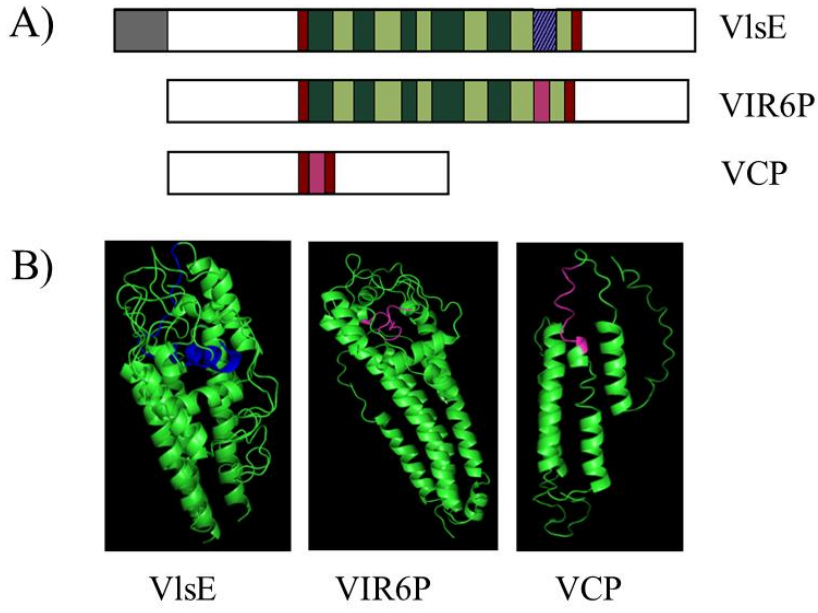

Figure 4-2: Schematic and model of VlsE scaffolded antigens. (A) The arrangement of VlsE is visualized at the top of the figure, denoted VlsE. The lipoprotein leader sequence, which directs surface localization is shown in gray, the unique conserved regions flanking the protein are shown in white. The direct repeats flank the cassette region and are shown in red. Additionally, the cassette region is shown between the direct repeats, with the invariable (light green) and variable regions (dark green) described for each of the 6 sections of the cassette region. The putative vaccine constructs VIR6P and VCP are shown below. In VIR6P, the invariable region 6 (blue) is replaced by PepB (pink). In VCP, the entire cassette region (between direct repeats shown in red) is replaced with PepB. **(B)** Models of immunization constructs. Far left demonstrates VlsE secondary structure. Invariable Region 6 is denoted in blue. The middle shows the predicted secondary structure of VIR6P after PepB (pink) substitution of the IR6 region. On the right is the VCP predicted secondary structure modeled after VlsE, with PepB substitution against shown in pink. All models visualized in PyMOL.

Test expressions of substitution constructs

Once plasmids were cloned into maintenance and expression strains of *E. coli*, the conditions for expression were optimized. In small scale expression experiments (Figure 4-4), it was found that the VlsE based constructs were not highly expressed after 4 hours at 37ºC.

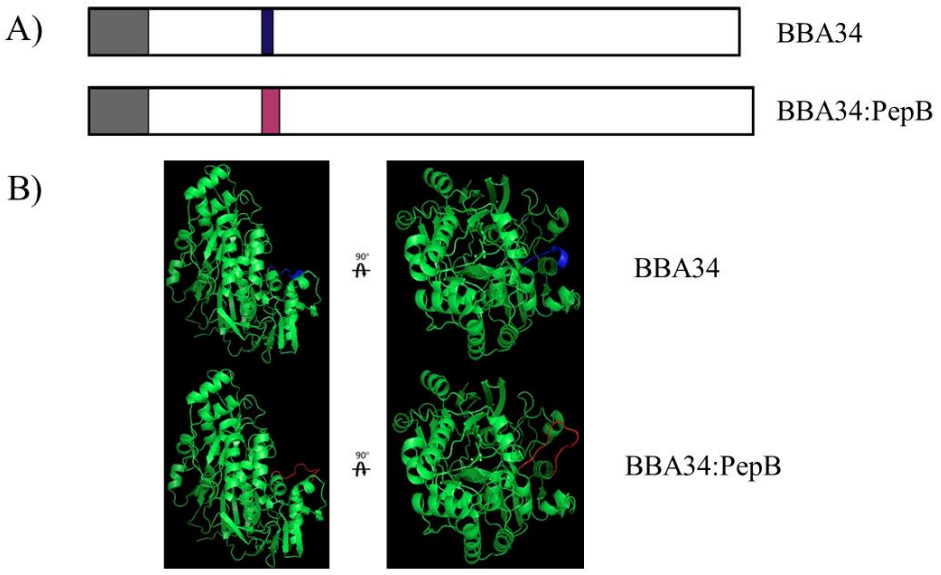

Figure 4-3: Schematic and model representations of the BBA34 based scaffolding constructs. (A) The arrangement of BBA34 is visualized at the top of the figure, denoted BBA34. The lipoprotein leader sequence, which directs surface localization is shown in gray, and a putative interaction domain (ENELDVP), is shown in blue. The scaffolded vaccine antigen is shown below, denoted as BBA34:PepB. In this construct, the putative interaction domain is replaced by the PepB sequence, shown in pink. **(B)** Models of the BBA34 based immunization construct. The top shows native BBA34 with the interaction domain highlighted in blue, shown at 90º rotations. The bottom models show BBA34:PepB and the resultant predicted structural changes in the protein.

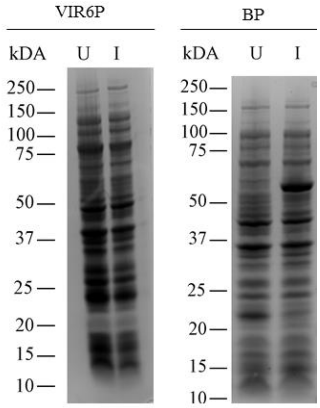

Figure 4-4: Small scale expression of putative vaccine constructs. For both VIP and BP, the uninduced samples (U) were not exposed to IPTG and were incubated for 4 hours at 37ºC, while the induced sample (I) was induced with 1 mM IPTG and incubated for 4 hours at 37ºC. No expression of VIP was visualized (expected: 64.2 kDA; 35 kDa + GST tag), while expression of BBA34:PepB was seen (expected: 62 kDa; 61 kDa + histidine tag).

Small scale expression of the BP constructs under the same conditions were more successful, and a band corresponding to 61 kDa was seen in the induction samples taken after 4 hours in the BP expressing cells. Expression of full length VlsE is notably difficult, although it is possible [284]. Due to these difficulties, expression of VlsE was suboptimal for the protein quantities needed for a vaccine study. BBA34 was more readily manipulated, and as such, was pursued as the protein scaffold for PepB to evaluate the validity of the concept of scaffolding.

Large scale expression of BP

Using the same conditions described for small scale purification, BP was purified on a larger scale. Contrary to the BBA34 and OspC control proteins (Figure 4-5A), immobilized metal ion affinity chromatography (IMAC) and spin column filtration were not sufficient to purify BP (Figure 4-5B, Lane 1). As such, size exclusion

chromatography was used after IMAC and spin column filtration to enhance the purity of BP (Figure 4-5B lanes 2-4).

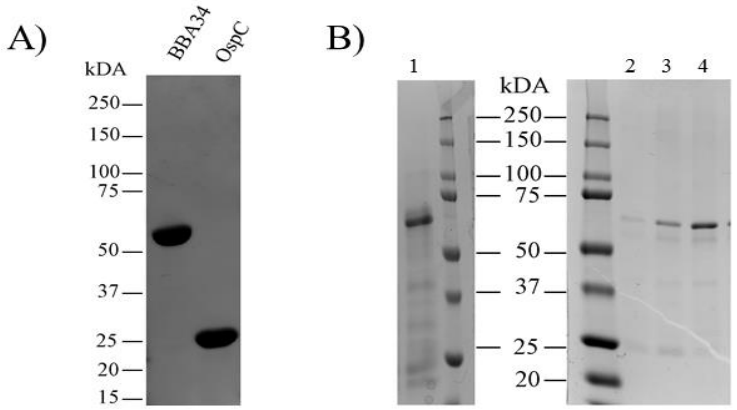

Figure 4-5: Purification of protein antigens. (A) BBA34 and OspC after purification. **(B)** BP purification fraction after IMAC and spin column filtration. Lanes 2-4 represent elution fractions collected after size exclusion chromatography performed on concentrated IMAC elution fractions.

Antibody response against scaffolded PepB

Antibody levels were evaluated for each group by ELISA against either the immunizing antigen or *B. burgdorferi* lysate (Figure 4-6A, B). Sera from each group were capable of detecting the antigen used for immunization, as seen in Figure 4-6A. Further, the animals were only immunized and not challenged, and as such the serum samples did not react strongly against *B. burgdorferi* lysate (Figure 4-6B). Only BP contained the PepB sequence, however, there seemed to be little antibody recognition of the PepB antigen (Figure 4-6C).

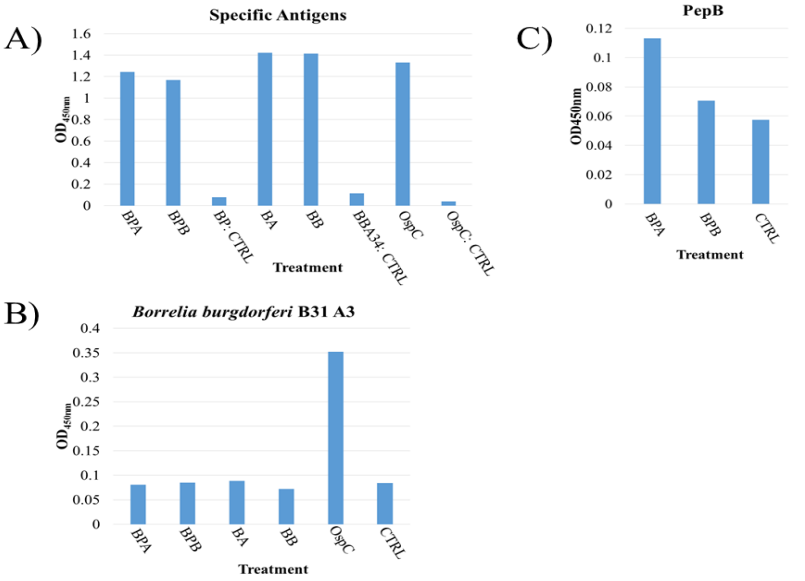

Figure 4-6: Evaluation of specific antibody levels at 8 weeks post priming. (A) Sera from each group was evaluated against the corresponding vaccine candidate. For each group, control serum was evaluated in addition to the immunized groups (antigen:CTRL). **(B)** Antibody levels for each group were evaluated against *B. burgdorferi* B31 A3 lysate. **(C)** BP immunized mice were evaluated for ability to bind PepB.

Discussion

Previously, the 12-mer peptide PepB was shown to be partially protective as a LD vaccine antigen, with 50% efficacy in tick challenge models. In order to improve the immunogenicity of the peptide, this work has addressed increasing the size of PepB by scaffolding the peptide into a protein scaffold derived from *B. burgdorferi*.

Due to the previous complications with human vaccines for Lyme, it is of the utmost importance to utilize antigens and adjuvants that are immunostimulatory without generating adverse reactions. The scaffold utilized, BBA34, has been previously evaluated in the murine model without any notable adverse effects (Dr. Esteve-Gassent, unpublished data). Further, we have used Adjuplex™ (Advanced BioAdjuvants LLC,

Omaha NE), a carbomer-lecithin adjuvant that is free of oils, detergents, and preservatives. Studies have shown that Adjuplex generates antibody titers equivalent to those seen with Aluminum Hydroxide, however lower than Monophosphoryl Lipid [285]. TiterMax Gold® was utilized for the initial efficacy studies for PepB, however, TiterMax lesions to form at the injection site in mice (Brock, personal observation; [286]). For these reasons, Adjuplex was utilized in this study.

Before selecting the final protein scaffold, we cloned and expressed VIP, VCP, and BP. From these studies, it was seen that VlsE based constructs were difficult to clone into competent *E. coli*, and that colonies rendered from cloning did not have the VIP or VCP inserts. Once a clone was identified containing either a VIP or VCP insert, small scale expression results demonstrated that the sequence was either not expressed, not expressed efficiently, or the protein was degraded upon expression for all of the clones. Longer expression conditions, varied IPTG concentrations, and lower expression temperatures were evaluated and did not yield improved expression. As such, efforts shifted to focus on the BBA34 based scaffold.

BBA34 was successfully cloned into competent *E. coli* at a much higher efficiency than that seen with VlsE based constructs, and the presence of the insert was confirmed by sequencing analysis. Small-scale expression showed that BP was expressed successfully upon exposure to 1 mM IPTG for 4 hours at 37ºC (Figure 4-4). However, progression to large-scale purification was not as straightforward as expected, and the construct produced low quantities of protein compared to the BBA34 control construct that was generated previously in our lab. Attempts to reclone BP are underway

with sequence modification to remove the signal peptide that was included in the BP construct, as seen in Figure 4-3. Inclusion of the signal sequence will target protein to the cell membranes, and can cause decreased expression [287]. The amount of protein produced was in line with the expression seen in OspA constructs that also retain an intact signal sequence [171]. However, for the purposes of this study, the clone including the signal sequence was used.

Further, it was determined that protein loss occurred at any step that exposed the BP protein to regenerated cellulose membranes, such as those used in centrifugal concentrator units and dialysis membranes. Further purification using items with polyethersulfone membranes enhanced the retention of BP. Regenerated cellulose products could be used with BBA34 control protein purification, either owing to the absence of PepB, or more likely, due to the quantity of protein. Retention of the lower-expressing BP was detrimental to yield, however, the BBA34 control plasmid is highly overexpressed. It is possible that even if the regenerated cellulose membrane saturated with BBA34, total yield would not be severely affected.

Purification of BP was also substantially more difficult than that of BBA34. To obtain a purer sample than that gained from standard metal-affinity chromatography, pooled BP elution fractions were subjected to size exclusion chromatography (SEC). Utilizing IMAC in conjunction with SEC yielded a purer product than seen previously. Evaluating the post-purification fractions with Bradford reagent revealed the presence of a large singular peak early in the elution fractions (data not shown). However, upon SDS-PAGE analysis, some contaminants persisted (Figure 4-5B). The decreased

comparative purity between BP and BBA34 and this may account for the slight decrease in reactivity of BP against itself and the reactivity of BBA34 against BBA34.

Evaluation of the antibody response reactivity against pure PepB revealed that the scaffolded antigens obscured the recognition of PepB, rather than enhanced the response against PepB. One disadvantage of using small peptide antigens is the lack of reactivity when used independently as a vaccine without other immunostimulators. In this experiment, however, it was shown that the scaffold obscured the recognition and reactivity of PepB, particularly compared to the high antibody response seen when PepB was used in conjunction with KLH [283]. A previous study utilizing PepB linked to an immunostimulatory region of IL-1 administered with TiterMax Gold® adjuvant also demonstrated the same lack of specific antibody response in spite of using smaller and less bulky antigen scaffold (data not shown). As such, using scaffolds to enhance the immunogenicity has not proven successful, and other approaches should be utilized to increase the immunogenicity of PepB.

An alternative to scaffolding is the use of other carrier proteins. The efficacy of PepB was initially described in conjugation with the carrier KLH [283]. KLH has not been utilized in human vaccines. In order to both enhance PepB immunogenicity and to utilize carrier molecules already used in licensed human vaccines, the carrier proteins CRM-197 or tetanus toxoid could be explored as alternatives to KLH. The usage of carrier proteins may be advantageous in the generation of a strong immune response, however, may increase the cost of production in the long-term. Adverse reactions may

occur in a small subset of people due to the utilization of toxoid-based carriers, and as such, eliminating the need for carriers would be ideal [288].

Building on the ideal parameters of the LD vaccine, a new vaccine should be amenable to a simple large-scale production scheme, be capable of reproducibly generating protective immunity in a range of individuals, and be affordable to produce. Therefore, modifying the peptide to synthetically generate a better antigen as well as using that antigen in a multi-copy peptide. It has been shown that multi-copy peptide antigens are capable of generating a better antibody response than that seen with a single copy peptide used in conjunction with KLH [289, 290]. One concern, however, is that linking the antigen to generate the multi-copy peptide may incur the same issues as those seen in the previous scaffolding constructs.

PepB is a highly promising antigen in the development of a novel LD vaccine. Scaffolding approaches have not proven successful to increase the immunogenicity of PepB in the murine model. As such, other novel approaches must be utilized to formulate a successful PepB-based LD vaccine capable of generating a PepB specific humoral immune response in order to enhance the efficacy of PepB.

CHAPTER V

SUMMARY AND CONCLUSIONS

LD is a globally relevant pathogen and is the most prevalent arthropod borne illness in the United States. Due to the discovery of *B. mayonii* and the potentially expanding range of *B. burgdorferi* in the US as well as the increasing resistance to acaricides, it is more important now than ever before to take a novel approach to vaccination against LD. Through this work, the goal of eliminating the need for antigens based on variable proteins requiring chimeric and multivalent constructs is closer to being accomplished. The identification of a highly-conserved novel LD vaccine antigen has the potential to apply to new pathogenic *Borrelia*, as well as the existing infectious species and strains both in the US and in Europe and Asia. This concept makes the novel antigen found through this dissertation more applicable to the global public and therefore more marketable for commercialization.

To this end, the work in this dissertation addressed three main aims to approach the development of a novel vaccine antigen against LD. Initially, the reverse vaccinology approach was utilized to identify regions of the *B. burgdorferi* genome that were highly conserved in pathogenic *Borrelia*, and more broadly, within the family Spirochaetaceae. Selection of the BB0170-BB0176 proteins was enhanced greatly by the work of Subramanian et al. and the analysis performed comparing spirochetes *T. pallidum* and *B. burgdorferi.* Of these, BB0172, BB0173, BB0175, and BB0325 were

predicted to have a VWFA domain, and BB0172 and BB0173 were predicted to have extracellularly exposed domains.

Upon selecting a family of proteins, these previously uncharacterized proteins with similarity to known proteins of other bacteria were analyzed for vaccine potential by looking at a range of characteristics including extracellular exposure, membrane localization and expression to narrow down the search to two potential candidate proteins, BB0172 and BB0173. While BB0173 was predicted initially to be anchored to the outer membrane, *in* vitro analysis demonstrated that BB0173 localized to the inner membrane of *B. burgdorferi.* BB0173 was found to be expressed constitutively in *B. burgdorferi*, and expression did not change under conditions relating to the tick, mammalian, or transitory conditions. In contrast, work by Wood et al. determined that BB0172 was identified to be inserted into the outer membrane and exposed to the extracellular environment. Further, BB0172 was transiently expressed during conditions simulating the transmission from the tick vector to mammalian host, and was found to bind integrin $\alpha_3\beta_1$. Taken together, BB0172 was determined to be a more promising candidate for the development of a novel LD vaccine.

Although immunization with the full-length protein was not found to be protective, peptide antigens were selected from BB0172 using *in silico* prediction techniques by looking at localization in relation to features of the protein and secondary structure. From BB0172, 4 peptide antigens were tested in the murine model to evaluate for safety and efficacy in the murine model. These studies demonstrated that PepB, when conjugated to KLH, had the ability to generate a protective immune response against *B.*

burgdorferi challenge by both needle (low dose challenge) and tick challenge. Further, the determinants of protection were more closely evaluated, and it was found that passive transfer of antibodies generated by PepB immunized mice could protect naïve mice from *B. burgdorferi* challenge and were primarily of the IgG1 isotype that is indicative of a primarily Th2 antibody response. This finding was in agreement with the literature, which has shown that a strong humoral response is sufficient to protect against LD infection upon passive transfer.

Building on the use of PepB as a vaccine candidate, PepB was scaffolded with *B. burgdorferi* proteins to eliminate the need for the previously used KLH carrier protein. Two proteins were considered as scaffold proteins, VlsE and BBA34. Attempts to generate VlsE constructs were unsuccessful, and as such, BBA34 was utilized as a scaffold for PepB. Immunization with the scaffolded antigen, however, did not yield specific antibodies capable of targeting PepB. These findings were in line with a previous study that utilized PepB in conjunction with a small immunostimulatory region of IL-1 and a short linker sequence. This construct was also that was also unable to generate high and specific antibody titers, although there was was less non-PepB protein present. At this point, the future of the PepB-based LD vaccine will involve reformulation of the peptide, either using alternative carrier proteins and adjuvants, or generation of repetitive, synthetic PepB antigens.

Further, the most effective PepB antigen formulation must be tested against *B. afzelii*, *B. garinii*, and *B. mayonii* in addition to *B. burgdorferi*. This work will expound on the idea that using a highly-conserved antigen is capable of protecting against a range

of pathogenic *Borrelia* capable of causing LD both in the US and in Europe and Asia. Upon success with protection against a range of pathogenic *Borrelia*, the logical next step is progression to the canine model for safety and efficacy studies to move the vaccine towards clinical licensure. Within the realm of veterinary medicine, utilizing the PepB based vaccine for horses would be another reasonable and welcome advancement. Long term, however, the goal for the PepB based LD vaccine is licensure and use in humans. A broadly protective human LD vaccine could be employed worldwide and potentially protect the majority of people in the northern hemisphere from LD.

An additional interesting idea of the future of LD prevention is the utilization of an anti-tick vaccine. Building on this idea, the coupling of an anti-tick antigen with PepB has the potential to produce a vaccine that can not only inhibit tick feeding and survivability but also prevent LD transmission in the event of a successful blood meal. This would be a strong advantage in the realm of preventing TBDs and be a welcome safeguard particularly in LD endemic areas.

Taken together, this work has advanced an alternative approach to LD vaccination using new ideas in vaccinology and novel antigens of *B. burgdorferi*. The success found in these studies has potential to advance to clinical phases of development and may one day yield a functional vaccine both in veterinary and human medicine.

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