CHARACTERIZATION OF *Borrelia burgdorferi* OUTER SURFACE PROTEIN CRASP-2 IN A CANINE HOST

Megan Walker

Characterization of *Borrelia burgdorferi* outer surface protein CRASP-2 in a canine host

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Masters of Science in Biology

Megan Walker

May,2013

May, 2013

To the College of Graduate Studies,

We are submitting a thesis written by Megan Walker entitled "Characterization of *Borrelia burgdorferi* outer surface protein CRASP-2 in a canine host." We have examined the final copy of this thesis for form and content. We recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science in Biology.

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Dedication

This thesis is dedicated to my parents. I have an enormous amount of gratitude for the strong foundations of character they helped me develop at a young age. Due to their inexhaustible effort, I take pride in my work, relish in surpassing expectations of others, and strive to make them and others proud. Without this early sculpting of ambition, work ethic, and perseverance, this work would not have come to fruition. For this, words, nor actions, could ever approach to describe the infinite credit they deserve.

Acknowledgements

Austin Peay State University has been fundamental in my talent and ability to complete my education. Funding my Masters education and research has been underappreciated. I am confident the quality education and support I received at Austin Peay State University will serve me well in my future endeavors.

Dr. Gilbert Pitts is also worthy of an acknowledgement for giving me an opportunity to work in a research lab. Over time his trust and confidence in me improved my own self-esteem and my writing skills. I owe him appreciation for understanding many of my personal and professional decisions. My only hope is he is proud to have worked with me despite my feelings of disappointing him more times than not.

Dr. Dailey, Dr. Meisch, and Dr. Schiller have made themselves available to me and have offered advice at times when I felt there were no answers. I appreciate all of their knowledge and feel privileged they would consider helping me.

Lastly, I would like to recognize Dr. Chad Brooks. Dr. Brooks has become more than just a mentor. I feel he has added to my foundational characteristics and has made me a better person and scientist. I value his approval more than others and because of this my own standards have risen to unprecedented elevations. He has reassured and demonstrated to me I am more capable of accomplishment than I once believed. I wish to express my extreme gratefulness for the guidance Dr. Brooks has given me. He has furnished me with convictions to be successful beyond measures I never knew I had.

ABSTRACT

MEGAN WALKER. Characterization of *Borrelia burgdorferi* outer surface protein CRASP-2 in a canine host (Under the direction of DR. CHAD BROOKS.)

Once *B. burgdorferi*, the causative agent of Lyme disease, enters an animal host, the bacteria must contend with an immunological barrier known as the complement cascade. Interestingly, *B. burgdorferi* has adapted a strategy to deal with the complement cascade by deactivating a specific component known as C3b. However, the complement cascade proteins are not identical in all potential animal hosts for *B. burgdorferi* and therefore, the bacterium must have several different complement regulatory proteins. Currently, five complement regulatory-associated surface proteins (CRASPs) expressed by *B. burgdorferi* have been identified. Several of the CRASPs have been characterized to specifically bind to the host-derived complement regulatory protein factor H (FH). Hypothetically, CRASPs bind to FH and inactivate the complement protein C3b on the cellular surface of the bacterium, thus allowing evasion of host complement-mediated lysis. Because not all animal FH proteins are the same, it is suggested the CRASPs behave in an animal host-dependent manner. This study transformed a vector encoding the *CRASP-2* gene, also known as c*spZ*, into a bacterial cell line to produce CRASP-2 protein; however, no protein was ever overexpressed or purified. Further experiments will need to be conducted to optimize protein expression. Work concerning microscopy studies involving the challenge of various strains of *B. burgdorferi* to dog sera, shows B31cF dies when exposed to dog sera and B31MI and B31cF(*flgB::cspZ*) survives. These experiments are in agreement with earlier work. Real-time polymerase chain reaction (RT-PCR) results showed *cspZ* levels to be undetectable.

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CHAPTER I

Introduction

Lyme disease overview

Lyme disease was first recognized in a group of children displaying arthritic symptoms in Lyme, Connecticut in 1975 by Allen Steere (Steere et al., 1977). Since then, Lyme disease is the most commonly reported vector-borne illness in the United States and is commonly found in Europe (Figure 1) (CDC, 2012). The disease does not occur nationwide and is mostly concentrated in the northeast and upper Midwest (Figure 2). In 2011, 96% of Lyme disease cases were reported from 13 states: Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Vermont, Virginia, and Wisconsin. The disease is most commonly diagnosed in the summer months, June and July (Figure 3). For humans, initial diagnosis involves considering the history of a person being in an area endemic for Lyme disease and direct observation of symptoms most notably a bulls-eye rash known as erythema migrans (EM) (CDC, 2013c) (Figure 4). This rash occurs in approximately 70-80% of infected people and begins at the site of a tick bite, radiating outwards. Other symptoms include a feeling of general malaise. A blood test may also be administered considering other symptoms, but this usually is only effective two to four weeks after a tick bite. Without the EM rash, diagnosis is difficult due to the familiar symptoms universally representing many other common ailments. If allowed to persist, the disease is characterized by arthritis, Bell's palsy, severe headaches and neck stiffness due to meningitis, heart palpitations and dizziness due to changes in heartbeat. When Lyme

disease is diagnosed early in infection (3-30 days post tick bite), treatment consists of a regiment of antibiotics such as doxycycline, amoxicillin, or cefuroxime axetil for two to four weeks (CDC, 2013a). In 10 to 20% of cases treated for Lyme disease, patients will experience lingering symptoms of pain and joint swelling (CDC, 2013b). This is termed "chronic Lyme disease" or "Post-treatment Lyme Disease Syndrome" (PTLDS). Unfortunately, for these cases, studies have shown patients who received prolonged courses of antibiotics do not fare better than patients receiving a placebo (Krupp et al., 2003). Additionally, continued antibiotic use resulted in adverse effects in patients and no improvement of cognitive function (Fallon et al., 2008).

A vaccine for Lyme disease was removed from the market due to insufficient consumer demand in 2002 (CDC, 2011). Precautionary measures should be heeded. These involve avoiding environments where ticks would be found, such as wooded and bushy areas, use repellents and wear appropriate clothing, bathe or shower after coming indoors and check the body for ticks. Gear and pets should be investigated as well, as ticks can be transported into a home and later attach to a person.

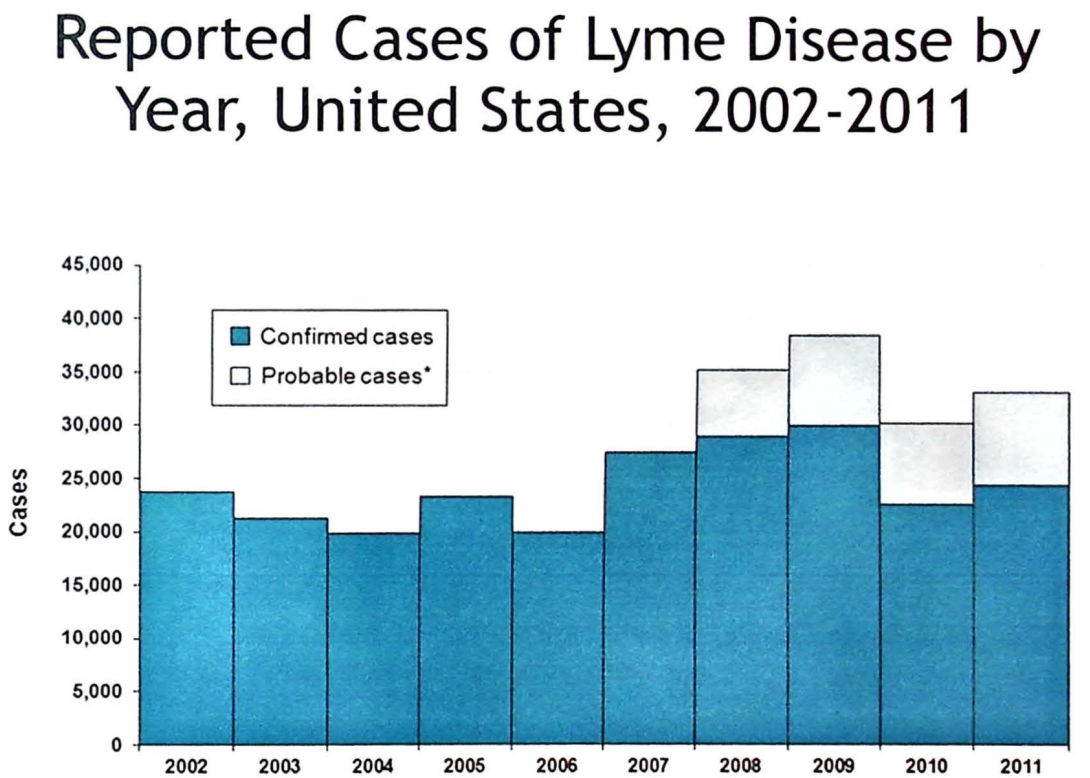

Figure 1. The graph displays the number of reported cases of Lyme disease from 2002 through 2011. The number of confirmed cases ranged from a low of 19,804 in 2004 to high of 29,959 in 2009 (CDC, 2012).

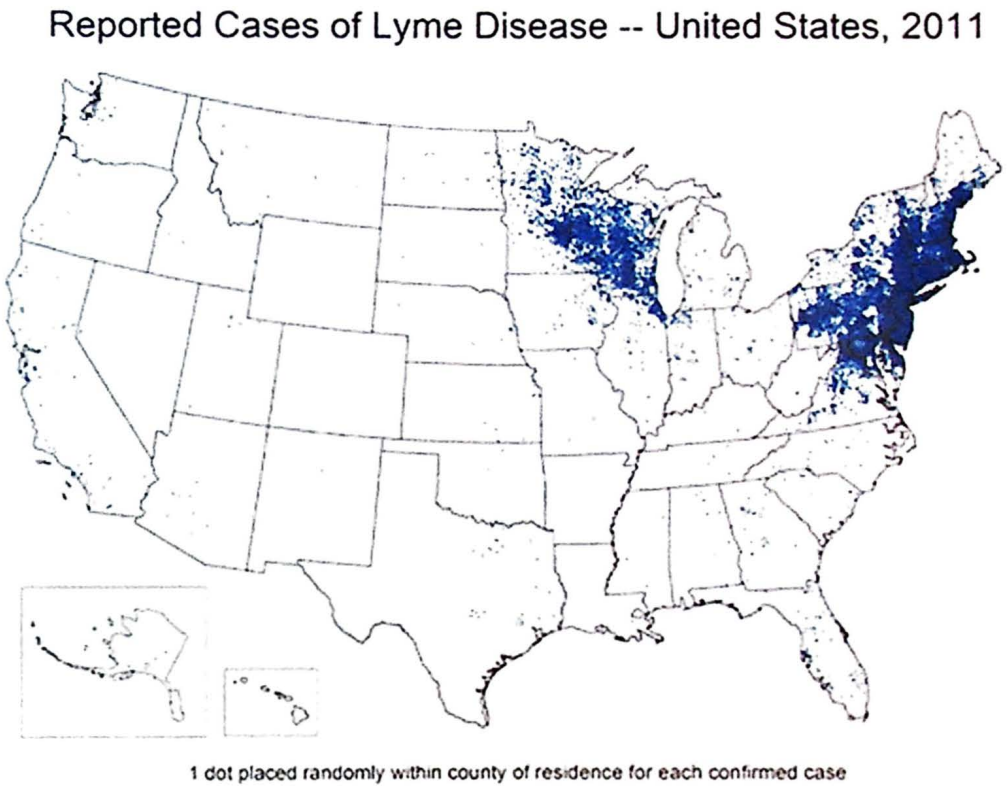

Figure 2. Geographic location of diagnosed Lyme disease cases concentrated in northeast and upper Midwest regions (CDC, 2012).

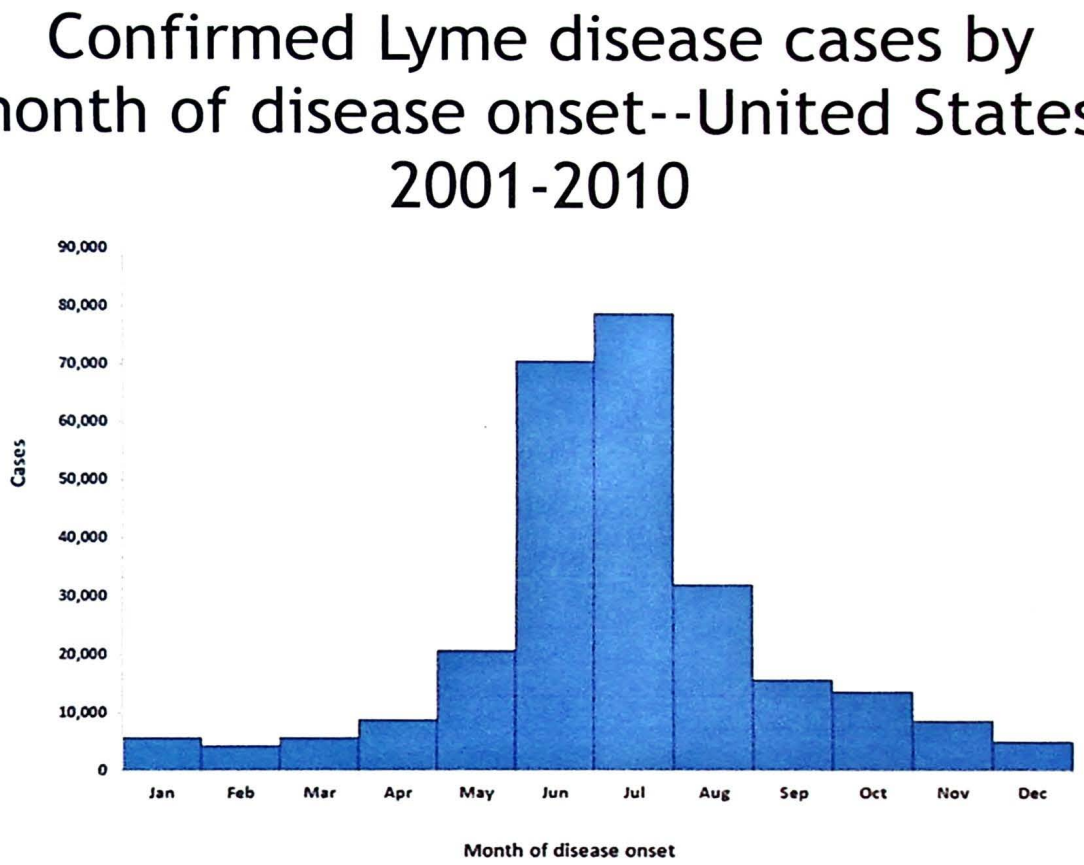

Figure 3. Lyme disease patients are most likely to have illness onset in June, July, or August and less likely to have illness onset from December through March (CDC, 2012).

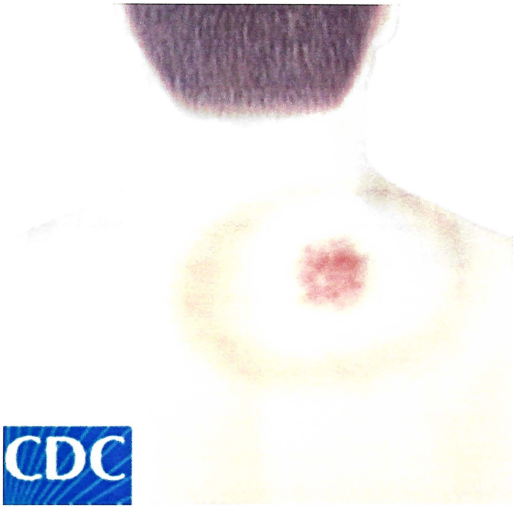

Figure 4. Erythema migrans (EM) rash; Characteristic symptom associated with Lyme disease tick bite and early infection of *B. burgdorferi* (CDC, 2013c).

Lyme disease is caused by a bacterium known as *Borrelia burgdorferi* (Figure 5). *Borrelia burgdorferi* is a pathogenic spirochete and shares ancient ancestry, similar morphology, as well as a protean strategy of infection, with another pathogen, *Treponema pallidum*, a spirochete known to cause syphilis (Porcella & Schwan, 2001). *Borrelia burgdorferi* leads an interesting enzootic life cycle (Figure 6), circulating between ticks of the species *Ixodes* and mammal hosts (Caimano, Hu, Radolf, & Stevenson, 2012). The tick larva hatch naïve of the pathogen, as there is no transovarial transmission, and acquire the spirochete during their first blood meal from an infected reservoir, which may include mice, squirrels, birds, and/or reptiles. After molting to the nymphal stage, the ticks transmit the pathogen to another animal serving as the next blood meal. It is believed at this stage humans are most likely to acquire the pathogen. The nymphs are very small and go easily unnoticed. The tick will go through one more molt into the adult stage where the pathogen is further disseminated to a range of vertebrate hosts. As an adult, ticks will mate on animal hosts, the eggs will drop to the ground, hatching, and perpetuating the life cycle of the *Ixodes* tick species.

The vagrant life cycle of *B. burgdorferi* is challenged to some degree by the environment it encounters. Each reservoir host *B. burgdorferi* inhabits contains a unique defense mechanism, known as the complement cascade, designed to contend with and eliminate foreign entities. The successful establishment of infection requires *B. burgdorferi* to circumvent the immune system long enough to reach a sustainable number of spirochetes within the host. The ability for *B. burgdorferi* to effectively infect a variety of hosts has been attributed to its elegant genome.

Figure 5. Scanning electron micrograph of *B. burgdorferi* spirochetes in the midgut of a nymphal *Ixodes* tick. (Steere et al., 2004).

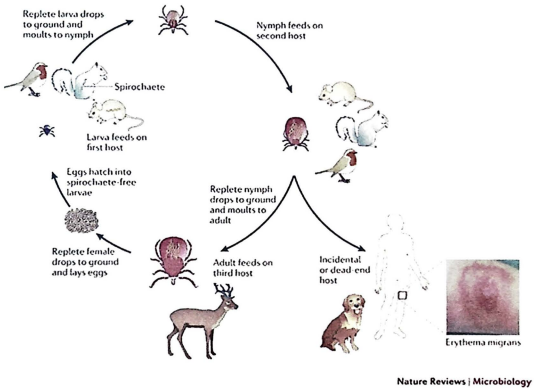

Figure 6. Enzootic life cycle of *B. burgdorferi* and *Ixodes* tick species (Caimano, Hu, Radolf, & Stevenson, 2012).

The elaborate genome of Borrelia burgdorferi

The genome of *B. burgdorferi* was first sequenced entirely in 1997 and showed the bacterium contained a linear chromosome of 910,725 base pairs and at least 17 to 20 linear and circular plasmids with a combined size of more than 533,000 base pairs (Fraser et al., 1997). The plasmids and chromosome have a precise one to one relationship with one another. Of the 11 plasmids analyzed during this study, Fraser et al. found 58% of plasmid DNA of *B. burgdorferi* did not match any sequence within their database software (Table 1). Much of the identified genes coded for membrane proteins, recombination cassettes, and purine ribonucleotide biosynthetic enzymes. It has been observed different plasmids have regions of homologous DNA (Casjens, van Vugt, Tilly, Rosa, & Stevenson, 1997; Zückert & Meyer, 1996). Fraser et al. found 47 paralogous gene families which accounts for 39% of the plasmid-encoded genes with no known biological role.

In the past, it was shown plasmids are lost over long periods of culture (Norris, Howell, Garza, Ferdows, & Barbour, 1995; Schwan, Burgdorfer, & Garon, 1988; Xu, Kodner, Coleman, & Johnson, 1996). This loss results in different protein expression profiles and a loss in the ability to infect laboratory animals, suggesting plasmids encode important genes necessary for the survival of *B. burgdorferi* and establishment of infection. This is known as the species-specific hypothesis – each plasmid or set of plasmids encoding unique genes is required to infect a certain reservoir host.

Table 1. Genome features in *B. burgdorferi* (Fraser, et al., 1997).

Chromosome	910,725 bp (28.6% G+C)
Coding sequences (93%)	
RNAs (0.7%)	
Intergenic sequence (6.3%)	
853 coding sequences	
500 (59%) with identified database match	
104 (12%) match hypothetical proteins	
249 (29%) with no database match	
Plasmids	
cp9	9,386 bp (23.6% GC)
cp26	26,497 bp (26.3% GC)
lp17	16,828 bp (23.1% GC)
lp25	24,182 bp (23.3% GC)
lp28-1	26,926 bp (32.3% GC)
lp28-2	29,771 bp (31.5% GC)
lp28-3	28,605 bp (25.1% GC)
lp28-4	27,329 bp (24.4% GC)
lp36	36,834 bp (26.8% GC)
lp38	38,853 bp (26.1% GC)
lp54	53,590 bp (28.1% GC)
Coding sequences (71%)	
Intergenic sequence (29%)	
430 coding sequences	
70 (16%) with identified database match	
110 (26%) match hypothetical proteins	
250 (58%) with no database match	
Ribosomal RNA	**Chromosome coordinates**
16S	444581-446118
23S	438590-441508
5S	438446-438557
23S	435334-438267
5S	435201-435312
Stable RNA	
tmRNA	46973-47335
mpB	750816-751175
Transfer RNA	
34 species (8 clusters, 14 single genes)	

Of particular interest, are *B. burgdorferi* genes encoding proteins which bind to host complement regulatory proteins, specifically factor H (FH) and factor H-like 1 (FHL-1) protein. Factor H and FHL-1 protein are cofactors for factor-I mediated cleavage of C3b (Alitalo et al., 2001). Cleavage of C3b at the cell surface prevents the further assembly of complement proteins leading to the formation of the membrane attack complex and ultimately opsonization of cells. Figure 7 diagrams the classical and alternative pathways of the immune system. Using reverse affinity ligand binding immunoblot assays (ALBI), Hovis et al. showed the capacity for *B. burgdorferi* outer surface protein E (OspE) to bind to serum proteins, presumably FH, from a subset of animals, supporting the species-specific hypothesis (Figure 8). This study showed OspE to bind to human, monkey, dog, pig, minipig, and rabbit serum proteins. Rogers and Marconi concentrated on a gene with five known paralogues. Complement regulator-acquiring surface protein 2 (also known as: BbH06, CspZ, CRASP-2) was shown to bind specifically to FH of varying animal subjects as well (Figure 9). In this study, Rogers showed CspZ was capable of binding to serum from 9 out of 15 animals tested. These included: human, monkey, cow, pig, minipig, rabbit, guinea pig, mouse, and duck. These are similar to the results shown by Hovis et al. except CspZ bound to animal FH more times than the OspE protein. This is an indication, in certain animals, CspZ may be key in allowing for the survival of *B. burgdorferi* instead of OspE. Brooks et al. examined the role of complement regulator-acquiring surface protein 1 (also known as: BbA68, CspA, or CRASP-1) *in vitro*. Figure 10 shows the capacity for this *B. burgdorferi* protein to bind to FH in humans, thus permitting the persistence of *B. burgdorferi* infectivity. In the same study, Brooks et al. developed a strain lacking the CRASP-1 gene and demonstrated

the mutant to be susceptible to human complement pathway. Another study produced somewhat conflicting results, concluding CRASP-1 is not essential for *B. burgdorferi* to evade the human complement cascade (McDowell et al., 2006). Their research included serum from mice and humans. All results showed CRASP-1 did not bind to FH from serum isolated from infected mice or any other animals tested as in Hovis et al. Their results also showed CRASP-1 did not bind to FH, and therefore is not expressed, from serum isolated from patients diagnosed with Lyme disease. These results support a species-specific interaction showing CRASP-1 may not be necessary for *B. burgdorferi* to successfully infect a mouse host, or even humans, the opposite of which was suggested by Brooks et al.

In one figure, McDowell et al showed the ability for CRASP-1 to bind only to human FH (mimicking the experiment conducted by Hovis et al.) but this experiment is glossed over in the discussion, making mention how Brooks et al. showed CRASP-1 necessity *in vitro* rather than *in vivo*. Obviously, more experiments will need to be conducted to clarify ambiguity. Based on the evidence, ruling out CRASP-1 involvement in human Lyme disease infection seems premature.

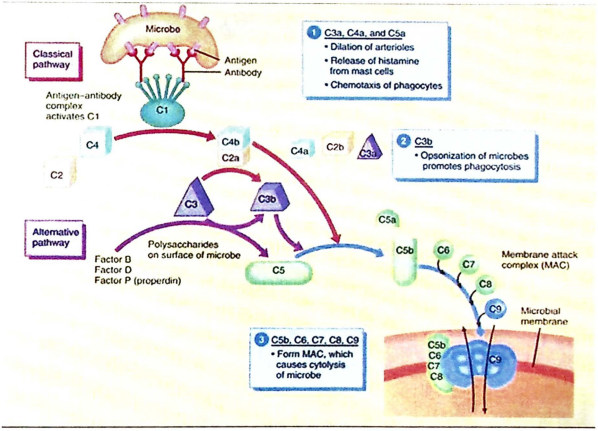

Figure 7. Classical and alternative pathways of immune system. C3b activation promotes the formation of membrane attack complex ending in death of cells.

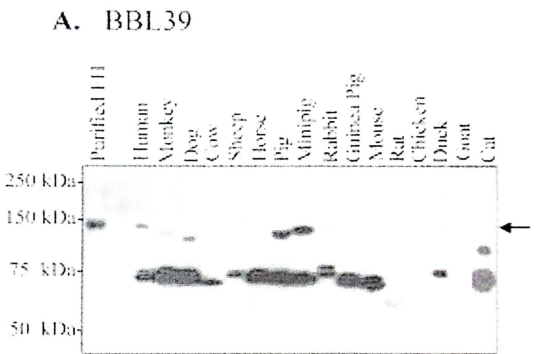

Figure 8. Reverse ALBI analysis showing recombinant OspE binding to serum proteins from different animals. Purified FH (150kDa) was included as a control. Results show *B. burgdorferi* OspE to bind to 6 out of 16 animal FH (Hovis et al.,2006).

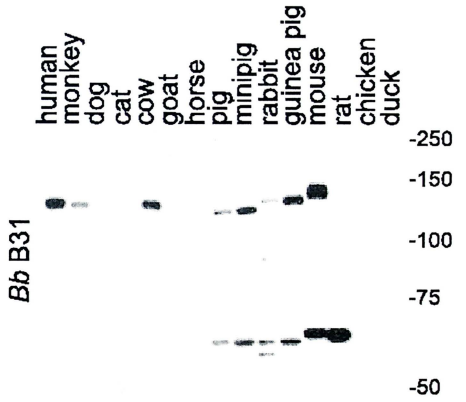

Figure 9. Reverse ALBI analysis showing recombinant CspZ binding to serum FH (150kDa) from different animals. Results show CspZ binds to 9 out of 15 animal FH (Rogers and Marconi, 2007).

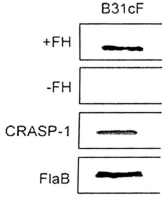

Figure 10. Results showing CRASP-1 binding to purified FH from human serum (Brooks et al., 2005).

In Brooks lab at Austin Peay State University, Jillian Kay, a former graduate student, conducted an experiment to determine the plasmid profile of *B. burgdorferi* exposed to different animal sera (Kay, 2009). Kay passed cultures of *B. burgdorferi* which mentioned previously, lost plasmids over time. Once a heterogeneous population of spirochetes was achieved, she exposed the cultures to horse, rat, and dog sera. She then isolated plasmid DNA from each of the cultures and conducted quantitative real-time polymerase chain reaction. She found spirochetes equipped with certain plasmids made up distinct populations in the different immunological environments. In her study concerning the exposure to dog sera, she found spirochetes harboring plasmids lp17, lp28-3, and lp28-4 made up 100% of the spirochete population in the dog sera (Figure 11). This is strong evidence suggesting a gene or set of genes encoded on these plasmids plays a role in the survival of *B. burgdorferi* in a canine host. Interestingly, one of the CRASPs ,CRASP-2 or CspZ, is encoded on lp28-3.

Another former graduate student, Elisa Takalo Lund, constructed a mutant strain of *B. burgdorferi* B31cF containing the *cspZ* gene under the control of a constitutively expressed promoter *flgB* (Lund, 2011). In a microscopic study, she showed B31cF containing the *cspZ* gene survived dog sera, whereas B31cF, which does not natively express the *cspZ* gene, does not survive at all when exposed to dog serum (Figure 12).

The goal of this study is to characterize the role of the CspZ protein by showing it is surface localized and expressed during infection in a canine host. Ultimately, the aim is to show this protein would be a reasonable target for vaccine production for dogs against Lyme disease.

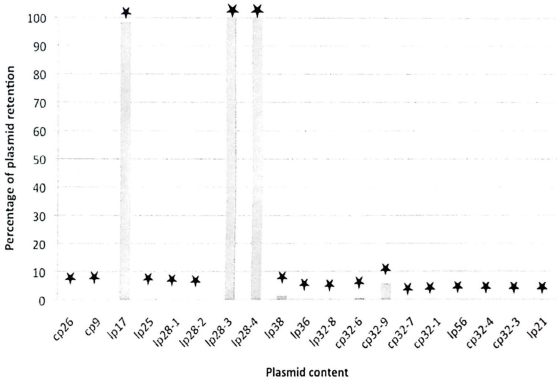

Figure 11. Jillian Kay experiment showing plasmid retention in spirochetes exposed to dog sera. Spirochetes with plasmids lp17, lp28-3, and lp28-4 are preferentially selected for when exposed to dog sera (Kay, 2009).

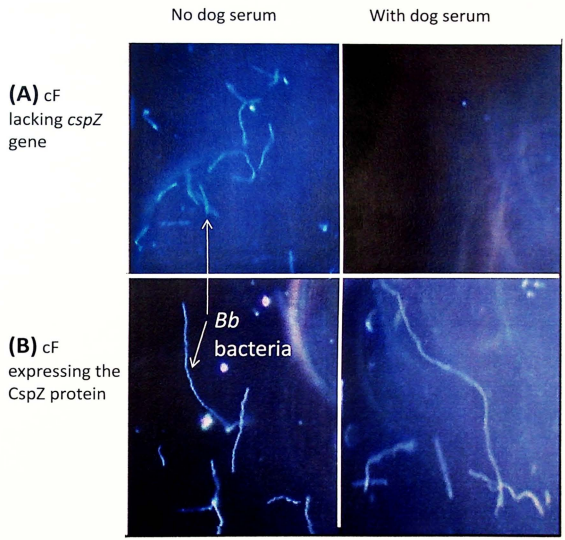

Figure 12. Elisa Takalo Lund microscopic observations during dog sera challenge to mutant strain B31cF expressing *cspZ* versus native B31cF (Lund, 2011). Results show spirochetes without *cspZ* die.

CHAPTER II

Methods

Bacterial strains and growth conditions

Strains of *B. burgdorferi* used in this study include B31MI, B31cF, and B31cF(flgB::cspZ). All strains were maintained at 34°C in complete BSK-II medium (complete BSK-II media is composed of BSK-II medium supplemented with 6% rabbit serum heat inactivated for 1h at 55°C) (Sigma).

Strains were counted at 400X total magnification daily using dark-field microscopy. Cultures were enumerated by placing 10µl of sample culture on to a clean glass slide and covered with a glass coverslip. The number of spirochetes per ten fields of view was counted and the number averaged. This averaged number was multiplied by a constant, 2.5 X 10^5. Strains were passed once cultures reached mid-logarithmic phase (1 X 10^7 organisms/ml). New cultures were seeded at a concentration of 10000 spirochetes/ml in a new 14ml conical vial.

Confirmation of Elisa Takalo-Lund mutant strain pBSV2(flgB::cspZ)

Escherichia coli strain DH5α used to maintain the plasmid construct pBSV2(flgB::cspZ) was sequenced to confirm the accuracy of the insert, subjected to polymerase chain reaction (PCR), and digested with a restriction enzyme.

Initially, *E. coli* DH5α harboring pBSV2(flgB::cspZ) was retrieved from a -80°C frozen stock culture and inoculated onto a nutrient agar plate supplemented with 100µg of Kanamycin (Sigma). Deoxyribonucleic acid (DNA) from successfully growing

colonies was isolated using a PureYield Plasmid Miniprep Kit (Promega) following manufacturer protocol. The concentration and purity of the DNA was enumerated using a NanoDrop ND-1000 Spectrophotometer.

A portion of this isolated DNA (100ng) was sent to Vanderbilt University DNA Sequencing Facility for Sanger sequencing using M13F and M13R primers (Table 2). Sequence analysis was performed using Basic Local Alignment Search Tool (BLAST).

Polymerase chain reaction was carried out using 10µl 2X GoTaq Green Master Mix (Promega), 0.5µM each of M13F and M13R primers (Table 2), 2µl of plasmid DNA (approximately 5ng – 100ng), and volume adjusted with molecular grade water (Cellgro) for a 20µl total volume reaction. Positive and negative controls were included to ensure size of PCR products and confirm reactions were free of contaminants. Polymerase chain reaction was conducted using an AB Verti Gradient Thermal Cycler. After an initial denaturation of 95°C for 5 minute, the samples were run for 40 cycles at 95°C for 30sec, varying annealing temperatures for 45sec, and 72°C for 1:10min. All PCR products were analyzed using a 0.8% - 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer.

Restriction enzyme digestion was also used to confirm presence of *cspZ* insert. HindIII cuts *cspZ* 216 base pairs (bp) from the beginning of the *cspZ* gene (711 bp). Twelve microliters of purified pBSV2(flgB::cspZ) DNA (approximately 500ng) was added to 5µl of Buffer 3 (Promega) and 32µl of molecular grade water. Next, 1µl of restriction enzyme HindIII (Promega) was added to the mixture. This sample was incubated at 37°C for 1 hour and then held at 4°C in a Techne TechGene Thermal Cycler. Plasmid pBSV2 lacking any insert in the multiple cloning site was also digested with

HindIII for comparison. Samples were analyzed using a 1% agarose gel in 1X TAE buffer.

Another experiment was run using another mutant from Brooks et al. which has CRASP-1 knocked out and then also rescued with a plasmid known as pKFSS-1. Primers from Kay were used to detect the presence of select plasmids and determine if amplifying regions of DNA from transformed plasmids was possible (Table 2).

Table 2. Primers used during this study for traditional polymerase chain reaction. The bolded sequences denote restriction sites incorporated into designed primers.

Designation	Sequence	Annealing Temperature (°C)
M13F	GTAAAACGACGGCCAGT	50 - 54
M13R	CAGGAAACAGCTATGAC	
Kan-F	TGAGGGAGGTTTCCATATGAGCCA	57
Kan-R	TGCTCTGCCAGTGTTACAACCAAT	
aadA-F	CATATGAGGGAAGCGGTGATC	54
aadA-R	GACGTCATTATTTGCCGACTACC	
FlaB (456) F	AGAGCTTGGAATGCAGCCT	52
Flab (993) R	GGGAACTTGATTAGCCTGCG	
flgBp(BamH1)F	GCG**GGATCC**TACCCGAGCTTCAAGG	58
flgBp(Not1)R	GCGG**CGGCCG**CATGGAAACCTCCCT	
cspZ(outer)F	GTAGCAATATACTTGTGCTAGAGG	50
cspZ(outer)R	TCTCTTTTGATAAATTGGCTTAAGC	
CspZ(trunc)F	GCG**GGATCC**AACGAATGTACAGG	58
cspZ(XhoI)R	GCGCG**CTCGAG**CTATAATAAAGTTTG	
pGEX(MCS)F	GGGCTGGCAAGCCACGTTTGGTG	58 - 60
pGEX(MCS)R	CCGGGAGCTGCATGTGTCAGAGG	
cspZ2581	CATGTCTGGCATTAGACACATCATT	55
lp54F	CCCAAAAGCCCACGATACA	50
lp54R	CAAGTGCAACTTCAAATCCTTGTT	
lp28-3F	TGGACCTGCTAATAGATGGGATAAA	50
lp28-3R	TGCTGAATGTTCTGCCTTATACTTTG	
lp17F	CAACGAATAGAATGTTGCTAAATCTAAGC	50
lp17R	CACCCCATTCTCATTCTCAATAAAAG	
lp25F	ACGCCGATCCACAAGCAT	50
lp25R	AACATCTACCCCTTGTTCTTCCAA	

(not cleaned), and adjusted volume to 50µl with molecular grade water. This PCR reaction was carried out using an AB Verti Gradient Thermal Cycler, following the same protocol used for the nested PCR. These PCR products were cleaned using a Wizard SV Gel and PCR Clean-Up System (Promega) and DNA was isolated from a nutrient broth bacterial culture of pGEX-4T-3 using a PureYield Plasmid Miniprep Kit following manufacturer protocol. The cleaned PCR products previously described and the pGEX-4T-3 plasmid DNA were then subjected to restriction enzyme digestion in separate vessels using BamHI (New England BioLabs) and XhoI (New England BioLabs). The reaction consisted of 5µl of Buffer 3, 0.5µl bovine serum albumin (BSA), 1µg of pGEX-4T-3 plasmid DNA or truncated *cspZ* PCR product, adjusted volume to 48µl with molecular grade water. One microliter of restriction enzyme XhoI was added first to each reaction mixture and incubated at 37°C for 1 hour using a Techne TechGene Thermal Cycler. Next, 1µl of restriction enzyme BamHI was added to each reaction mixture and incubated again at 37°C for 1 hour Techne TechGene Thermal Cycler. Both the restriction enzyme digested PCR product and plasmid DNA were cleaned using a Wizard SV Gel and PCR Clean-Up System and quantified using a NanoDrop ND-1000 Spectrophotometer. The restriction enzyme digested pieces were ligated together using T4 DNA Ligase (New England BioLabs) enzyme. This reaction consisted of 5µl ligase buffer, 350ng restriction enzyme digested pGEX-4T-3 plasmid DNA, 152ng restriction enzyme digested truncated *cspZ* PCR product, adjusted volume to 49µl with molecular grade water, and 1µl of T4 DNA ligase enzyme. This reaction was incubated at 25°C for 3 hours using a Techne TechGene Thermal Cycler (Figure 13). The ligation reaction was transformed into *E. coli* JM109 (Promega) cells for plasmid maintenance following

manufacturer protocol. The transformants, pGEX(tcspZ), were screened using PCR and M13F and M13R primers.

One hundred nanograms of DNA from successful transformants, including M13F primer, were sent to Vanderbilt University DNA Sequencing Facility to confirm sequence integrity. Sequence analysis was performed using BLAST.

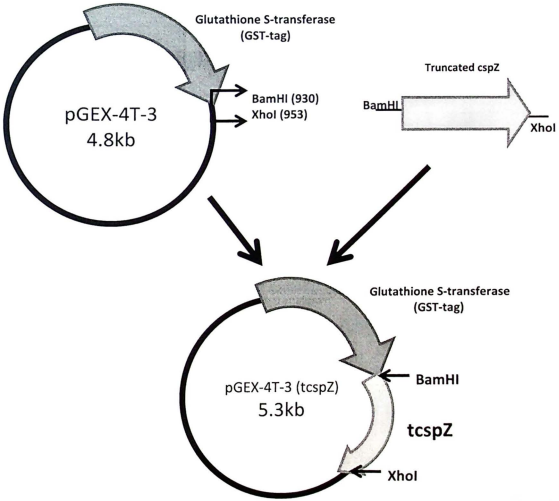

Figure 13. Schematic of plasmid construction for protein expression purposes. Induction will produce a GST-tCspZ fusion protein which can be purified using an affinity column.

Optimizing protein expression of truncated cspZ

Protein expression was performed using a variety of methods to try and optimize conditions. *Escherichia coli* JM109 harboring plasmid construct, pGEX(tcspZ), was cultured in Luria Broth (LB) (10g tryptone, 5g yeast extract, 10NaCl per 1 liter of deionized water) supplemented with 100μg/ml Ampicillin salt (Sigma). Growth of JM109 cells harboring either pGEX-4T-3 or pGEX(tcspZ) was monitored for an 8 hour period.

To induce fusion protein production, isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fischer), an allolactose analog, was added to JM109 cells at differing concentrations. Contentrations of IPTG included 0.2mM, 0.3mM, 0.4mM, 0.5mM, and 0.6mM were added to bacterial cultures once the cell density reach mid-logarithmic phase and incubated overnight at 37°C shaking (110rpm).

Another optimization experiment included bacterial cultures incubated at 37°C or 30°C (shaking at 110rpm), induced with 1mM IPTG, for 3 hours.

Protein from induced cells was isolated by taking a 1ml sample from bacterial cultures and centrifuging sample at max speed (14000rpm) for 5min to pellet bacteria. The supernatant was removed and the pellet re-suspended in 200μl of phosphate buffered saline (PBS) (HyClone). Next 200μl of 2X sodium dodecyl sulfate (SDS) loading buffer was added and the samples were boiled for 10min. Samples were then run on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) in 1X SDS running buffer (25mM Tris, 192mM glycine, 0.1% SDS).

For protein purification, bacterial cultures were induced at mid-logarithmic phase and grown overnight. Then using Glutathione Spin Columns (Pierce Thermo Scientific), protein was purified following manufacturer protocols. Purified protein was quantitated using the NanoDrop ND-1000 Spectrophotometer and/or run on a 10% SDS-PAGE in 1X SDS running buffer.

Quantitative real-time polymerase chain reaction

For experiments involving quantitative real-time polymerase chain reaction (qRT-PCR), strain B31MI was cultivated in complete BSK-II media at 34°C. They were enumerated using dark-field microscopy and once the culture had reached a concentration of at least 10^8 spirochetes/ml, two samples containing 10^8 spirochetes were gently centrifuged at 4,000 X g for 4 minutes. The supernatant was removed and the pellets gently washed with a volume of BSK-II media. The samples were centrifuged again, supernatant removed, and one sample re-suspended in 100µl of BSK-II media and 100µl of heat-inactivated dog sera (56°C for 30 min) and the other sample re-suspended in 200µl of BSK-II media. The two samples were incubated at 34°C for 16 hours. Isolation of total RNA was conducted using TRIzol (Invitrogen) reagent following manufacturer protocol. Isolated total RNA was DNase (Invitrogen) treated following manufacturer protocol. The concentration and purity of total RNA was quantified using an NanoDrop ND-1000 Spectrophotometer. Isolated RNA was stored at -80°C until further use. First strand cDNA synthesis was carried out utilizing SuperScript II RT (Invitrogen), 250ng random primers, and 100ng of DNase treated total RNA. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using SYBR Select Master Mix (Applied Biosystems). Reactions run in triplicate loaded into a 96-well plate consisted of

10µl SYBR Select Master Mix, 0.5µM of each forward and reverse primers (Table 3), 5ng of cDNA, and adjusted volume with molecular grade water to 20µl. The RT-PCR reaction was carried out using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). After an initial heating at 50°C for 2min, the AmpliTaq Gold ® Polymerase was activated by heating the mixture to 95°C for 10min, the samples were run through 40 cycles of 95°C denaturation for 15sec and annealing/extending at 55°C for 1min.

Statistical analysis included a paired t-test employing statistical software JMP 9.

Table 3. Primers used for quantitative real-time polymerase chain reaction.

Designation	Sequence	Annealing Temperature
CspZ(qRT)Fwd	TGCCAGACATGTTGCTGATT	55
CspZ(qRT)Rev	CCCCCTCAAGTTCTACAGCA	
OspC(qRT)Fwd	CTGATGCAAAAGAAGCCATTTTAA	55
OspC(qRT)Rev	TGCTTTTGACAAGACCTCTACTGATT	
FlaB(qRT)Fwd	ATGTTAGCAGCCTTGACGAGAAA	55
FlaB(qRT)Rev	GATCGTACTTGCCGTCTTTGTTTT	

B31cF challenge to dog sera

For experiments involving the challenge of B31cF to dog sera, spirochetes were cultivated in complete BSK-II media at 34°C. They were enumerated and once the culture had reached a concentration of at least 2.5 X 10^6 spirochetes/ml, samples containing 2.5 X 10^6 were centrifuged at 4,000 X g for 4 minutes. The supernatant was removed and the pellets were gently washed three times with a volume of BSK-II media. The samples were centrifuged again and re-suspended in 100µl of BSK-II media and 100µl of dog sera. The samples were incubated at 34°C for the duration of the experiment. The samples were then enumerated using dark-field microscopy at 400X total magnification every hour post exposure.

Proteinase K treatment of B31MI

B31MI spirochetes (1 X 10^7) were gently washed three times with BSK-II media and split into two aliquots. The experimental (E) aliquot was subsequently induced with heat-inactivated (56 °C for 30 min) dog sera and the control (C) received no induction. Both aliquots were incubated at 34 °C for 1 hour; the exposure to dog sera complement should elicit *Bb* transcriptional response. Spirochetes were then treated with proteinase K (PK) and incubated for 1 hour at RT to digest all outer surface proteins. Samples were washed three times with PBS and re-suspended in BSK-II media. A sample was taken and exposed to dog sera (not heat-inactivated) and then counted (40X total magnification) using dark-field microscopy. Spirochetes were repeatedly counted every hour until no further change was detected. Samples were also taken continually from the original

aliquot, exposed to dog sera, and counted (40X total magnification) until no further change was detected. B31cF was used as a control.

CHAPTER III

Results

Experiments aiming to confirm the findings from Lund were successful. The sequence analysis from the pBSV2(flgB::cspZ) construct showed 97% identity match compared to the native *cspZ* gene sequence from *B. burgdorferi* B31 (Figure 14). Restriction enzyme digestion with HindIII of the pBSV2(flgB::cspZ) plasmid also revealed the insertion of additional nucleotides compared to pBSV2 plasmid (Figure 15). It is difficult however to determine if 1000 extra base pairs are included (the expected approximate size of the MCS from pBSV2(flgB::cspZ)). The most curious part concerning the confirmation of *cspZ* in the plasmid was the attempted PCR from pBSV2(flgB::cspZ) (Figure 16) and B31cF transformed with pBSV2(flgB::cspZ) (Figure 17). While this experiment amplified the multiple cloning site (MCS) with the *cspZ* gene, which appears to be approximately the right size, trying to amplify across the MCS using primers specific for *cspZ* or *flgB*, the sizes of the resulting bands were too small and was not consistent with expectations.

When trying to amplify *cspZ* from B31cF transformed with pBSV2(flgB::cspZ), there was no evidence at any point in time of a detectable PCR product by agarose gel electrophoresis showing the amplification of neither the *cspZ* gene, nor the kanamycin cassette from thisB31cF mutant (Figure 17). Curiously, this mutant survives in BSK-II media supplemented with the antibiotic kanamycin, while B31cF lacking additional genetic elements does not.

Another experiment was run for comparison which included running PCR from another mutant strain of B31cF with CRASP-1 knocked out from Brooks et al. The results of this experiment show the possibility of PCR amplification of nucleotides located on an extra-chromosomal plasmid (Figure 18). This experiment used primers specific for genes located on each plasmid. Kanamycin was included as a control. The first five lanes are consistent with expectations. The strain with CRASP-1 knocked out shows no cross-reactivity with streptomycin or M13 primers and amplified lp54 (encodes CRASP-1) and *flaB*. The next five lanes show the knockout strain but with the inclusion of pKFSS-1 (nothing inserted into the MCS). A very faint band showing the amplification of the streptomycin cassette is present, confirming the presence of the transformed plasmid, pKFSS-1. However, the M13 primers were not successful in amplifying the MCS region of this plasmid. The last five lanes show the knockout strain rescued with pKFSS1::crasp-1 (encoding the CRASP-1 gene). There is clearly amplification of the streptomycin cassette and a faint band confirming lp54 is still present, but the M13 primers amplified a very small fragment, which would be consistent with a MCS with no insert.

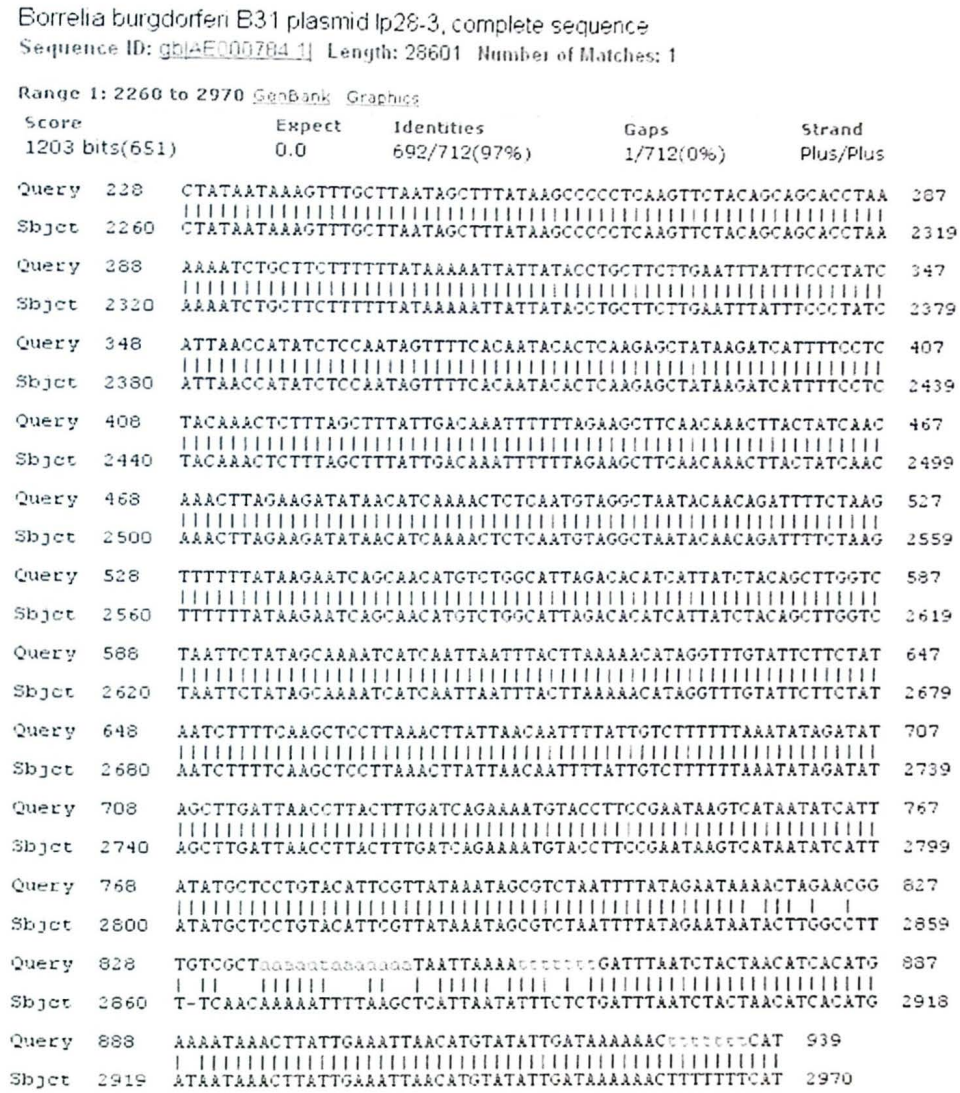

Figure 14. BLAST sequence analysis of Lund construct pBSV2(flgB::cspZ) MCS. Sequencing was conducted at Vanderbilt DNA Sequencing Facility using M13F primer. The "Sbjct" line shows the sequence from the native *cspZ* gene and the "Query" line shows the sequencing data from the construct. The results show a 97% match comparing the native and experimental sequence and one missing nucleotide.

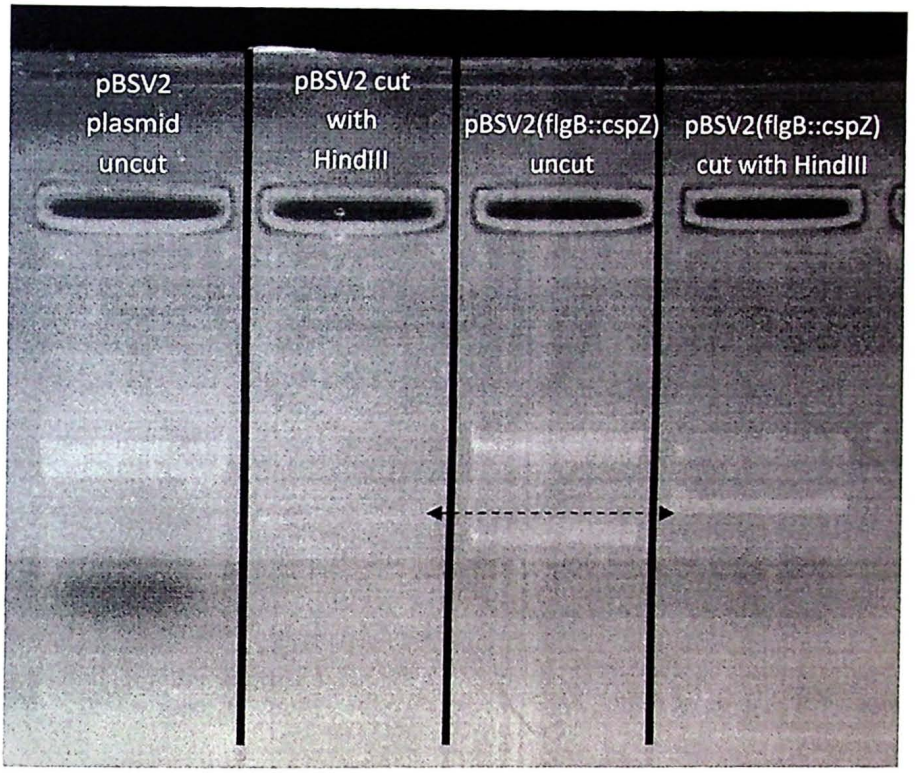

Figure 15. Restriction enzyme digestion of pBSV2(flgB::cspZ) (7.3 kb) with HindIII. The results show the pBSV2(flgB::cspZ) construct ran slightly slower than the pBSV2 plasmid (6.2kb) suggesting the construct is larger and includes more nucleotides (arrow). However, it is difficult to say whether the extra nucleotides constituted an extra 1000 bp.

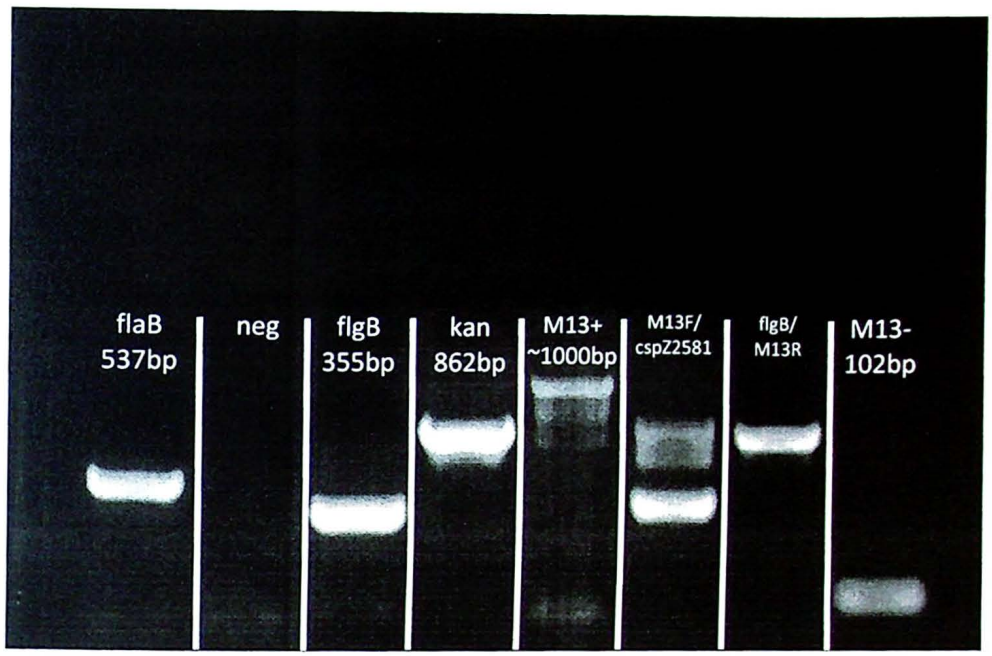

Figure 16. Polymerase chain reaction showing amplification of pBSV2(flgB::cspZ) MCS. flaB (537bp) (DNA from *B. burgdorferi*), flgB (355bp) (DNA from pBSV2), kan (862bp) (DNA from pBSV2), and M13- (102bp) (DNA from pBSV2) were included as controls. M13+ shows the amplification of the MCS from pBSV2(flgB::cspZ) using the M13F and M13R primers. The M13F/cspZ2581 lane amplified DNA from pBSV2(flgB::cspZ) using M13F forward primer and reverse primer cspZ2581. The expected size was approximately 1000bp. The flgB/M13R lane amplified DNA from pBSV2(flgB::cspZ) using the flgB forward primer and the M13R reverse primer. The expected size was approximately 1000 bp.

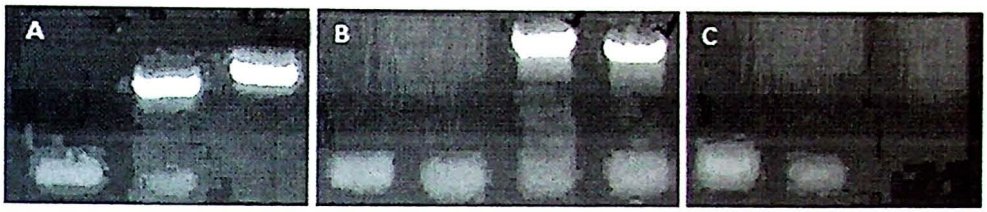

Figure 17. Amplification of *cspZ* and *kan* from B31cF transformed with pBSV2(flgB::cspZ). Panel A shows DNA amplified from B31MI, used as controls. The first lane should have shown a band from primers amplifying a region on lp28-3. The second lane shows a band from primers amplifying a region on lp25. The last lane shows a band from primers amplifying *flaB*. Panel B shows DNA amplified from B31cF. The first lane should not have shown a band from primers amplifying a region on lp28-3 (B31cF does not contain lp28-3). The second lane should not have shown a band from primers amplifying a region on lp25 (B31cF does not contain lp25). The third lane shows a band from primers amplifying a region on lp17. The last lane shows a band from primers amplifying lp54. Panel C shows DNA amplified from B31cF transformed with pBSV2(flgB::cspZ). The first lane did not show a band indicating the amplification of *cspZ* and the second lane did not show a band indicating the amplification of *kan*. The last lane in panel C is a negative control.

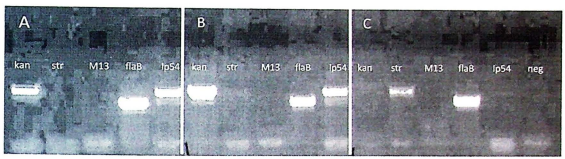

Figure 18. Amplification of regions of DNA from mutant supplied by Brooks et al. lanes labeled kan (862 bp) were used as controls. Panel A shows results from DNA from CRASP-1 KO cF. The results are consistent with expectations. Bands were not present when using primers to amplify a region of the streptomycin (str) gene. The M13 primers also do not bind to native DNA as no band is present. Panel B shows results from DNA from CRASP-1 KO cF transformed with the pKFSS-1 plasmid. The results show a faint band in the lane labeled "str" indicating primers amplified a region of the streptomycin gene located on the pKFSS-1 plasmid. The M13 primers did not appear to amplify a region of DNA. Panel C shows results from DNA from CRASP-1 KO cF transformed with pKFSS-1::CRASP-1. The M13 primers amplified a small region of DNA not consistent with expectations (approximately 1000 bp).

Construction of a plasmid for protein purification was successful. The resulting transformants were screened using PCR with M13F and M13R primers (Figure 19). The colony designated with an arrow was selected for sequence analysis and further protein expression and purification. The *cspZ* gene was included as a control with an expected size of 711bp. The multiple cloning site of pGEX-4T-3 alone was included to detect transformants which did not have the insert. Of 17 colonies screened, only two did not have the truncated *cspZ* gene ligated into the MCS.

The sequence analysis was conducted using BLAST software. The analysis revealed only two nucleotide mismatches (Figure 20). When the protein sequence from the construct was compared to the protein sequence from the native *cspZ* gene, the protein sequence also showed 99% identity with two amino acids differing from the native sequence (Figure 21).

The growth of the JM109 cells with pGEX-4T-3 and with pGEX-4T-3(tcspZ) showed mid-logarithmic phase was approximately 8 hours after the culture was seeded, with an optical density of 0.500 at 600nm (Figure 22).

Protein expression and purification was never successful. Despite all efforts for optimization, there was never a substantial amount of protein expressed or purified from pGEX(tcspZ) or pGEX-4T-3, which was used as control. The SDS-PAGE gels all appeared to show a typical protein profile with no "protein puff" typically seen when a specific protein is overexpressed (Figure 23).

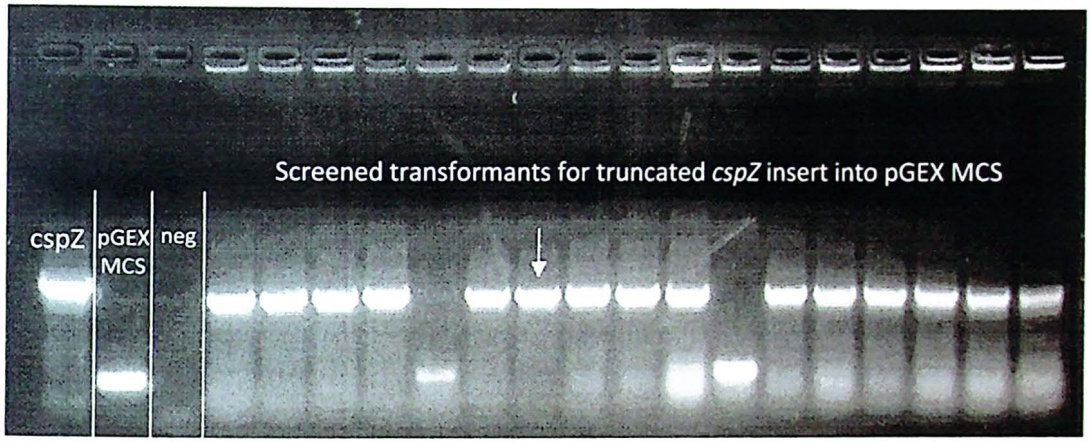

Figure 19. Polymerase chain reaction of transformants from pGEX-4T-3(tcspZ) transformation in JM109 cells. cspZ (711bp), pGEX MCS (102bp), and neg were included as controls. DNA from successfully transformed colonies is expected to show a band to be approximately 561bp.

Borrelia burgdorferi B31 plasmid lp28-3, complete sequence
Sequence ID: gb|AE000784.1| Length: 28601 Number of Matches: 1

Range 1: 2260 to 2820 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1026 bits(555)	0.0	559/561(99%)	0/561(0%)	Plus/Minus

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Figure 20. BLAST analysis of truncated *cspZ* showing 99% identity. The sequence compared to the native sequence shows two mismatches.

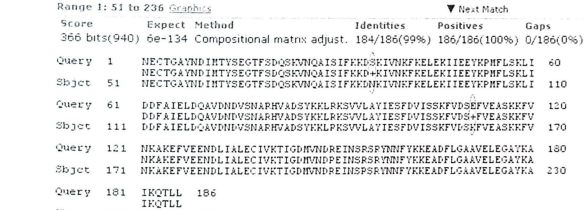

Figure 21. Protein sequence from pGEX(tcspZ). Comparing this sequence to the native sequence, the results show there is a 99% match. The first substitution is a serine for an asparagine. The second substitution is a glutamic acid for a lysine.

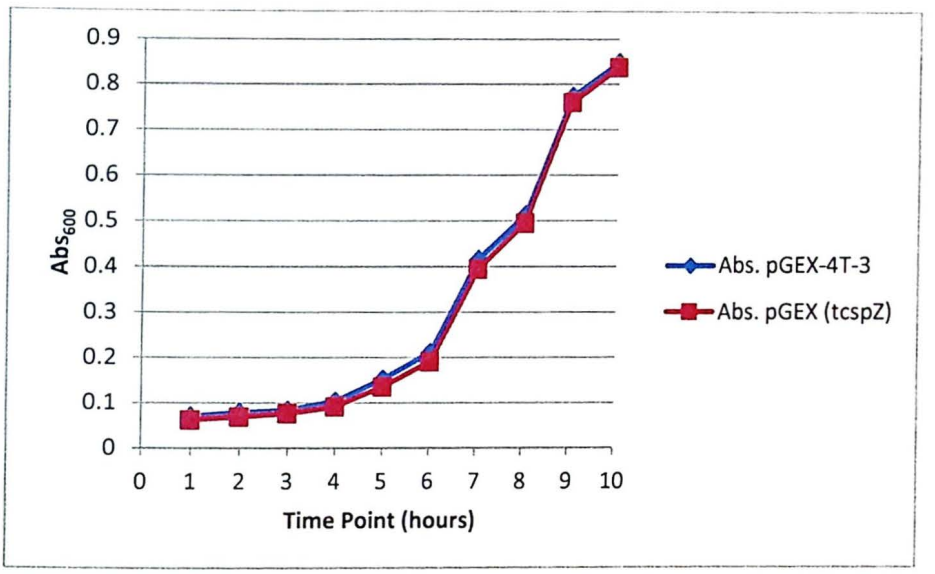

Figure 22. Growth curve of JM109 cells harboring either pGEX-4T-3 or pGEX(tcspZ). Mid-logarithmic phase was determined to be approximately 8 hours after seeding of culture with bacteria. The optical density at mid-logarithmic phase was determined to be 0.500 at 600nm.

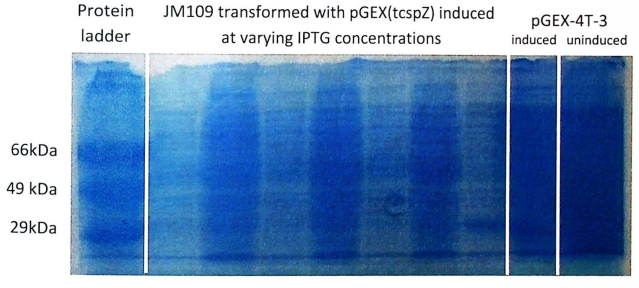

Figure 23. Typical SDS-PAGE gel showing protein profile of JM109 cells. A large "puff" is expected at approximately 25kDa. This was never seen even in .

Quantitative real-time polymerase chain reaction did not reveal the presence of *cspZ* gene upregulation, as the levels of *cspZ* were undetectable. The expression levels of *flaB* and o*spC* were however detectable. When B31MI is exposed to heat-inactivated dog sera, *flaB* gene regulation did not differ significantly depending on treatment with dog sera, whereas, *ospC*was significantly down-regulated 1.42 folds (p-value 0.039, α=0.05) (Figure 24).

Curiously, B31MI was screened for plasmids present, as the passage number of the B31MI sample used was greater than passage 1. The PCR revealed the plasmid, lp28-3, encoding the *cspZ* gene, was not detectable (Figure 17); while other plasmids, included as controls were shown to be present.

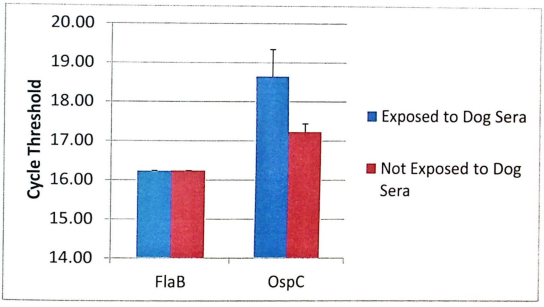

Figure 24. Quantitative real-time polymerase chain reaction results showing both *flaB* and *ospC* from B31MI exposed to dog sera and not exposed to dog sera. Both genes appear to be slightly down-regulated. Conducting a paired t-test using statistical software JMP 9, *flaB* gene regulation did not differ significantly depending on treatment with dog sera. *ospC* gene regulation did differ significantly with a p-value of 0.039 (α=0.05). *ospC* was significantly down-regulated 1.42 folds.

Experiments concerning the proteinase K treatment revealed B31MI can survive in dog sera even when all outer surface proteins were eliminated. Strain B31MI levels were comparable to control levels (B31MI receiving no induction to heat-inactivated dog sera) throughout both experiments (Figure 25 and Figure 26). As expected B31cF, lacking the genetics to produce the *cspZ* gene, dies when exposed to dog sera.

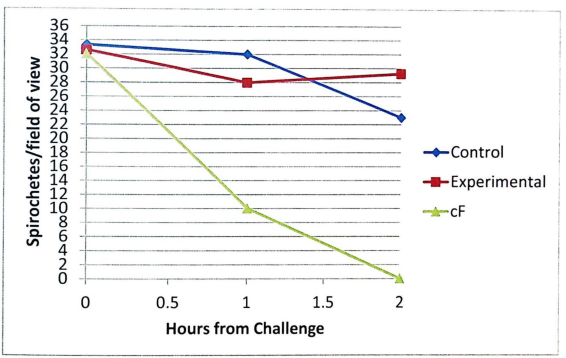

Figure 25. Graph showing results from PK treated spirochetes and exposure to dog sera over a course of two hours

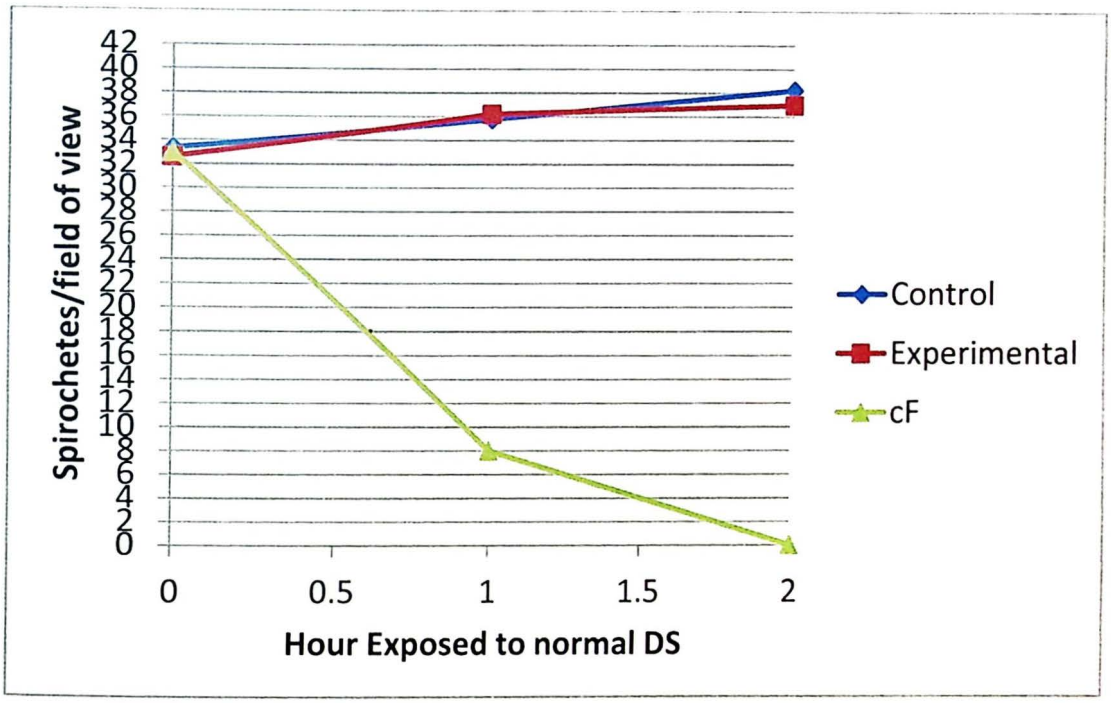

Figure 26. Results from time-dependent exposure to dog sera after PK treatment

CHAPTER IV

Discussion

Confirming the presence of *cspZ* inserted in the MCS of pBSV2 was very difficult and puzzling. The sequence analysis revealed the beginning and end of the gene to be intact and should not have posed a problem for the binding of appropriate primers. However, using these primers with PCR and analyzing PCR fragments using agarose gel electrophoresis never showed a successful PCR product. However, using M13 primers showed the amplification of a large product consistent with size expectations (1000 bp). Restriction enzyme digestion also showed a linearized vector for both pBSV2 and pBSV2(flgB::cspZ). There is a HindIII restriction site at the very end of the MCS of pBSV2. With this in mind, there should have been an approximately 300bp band visible as well as the rest of the vector. In hindsight, this may have just been a loading issue. One hundred nanograms of DNA is typically the lowest threshold for visualizing DNA on an agarose gel. It is interesting B31cF transformed with pBSV2(flgB::cspZ) survives in BSK-II media supplemented with kanamycin. Also interesting was the PCR ran to ensure amplifying a product from a transformed plasmid. According to the results, the MCS in the strain rescued with pKFSS-1 encoding the CRASP-1 gene is very small. Taking this into consideration, it has been observed, *B. burgdorferi* to spontaneously become resistant to kanamycin (Criswell, Tobiason, Lodmell, & Samuels, 2006) or the spirochete may have gone through recombination events leading to the elimination of the transformed gene.

The construction and transformation of pGEX-4T-3 with a truncated version of *cspZ* went very well. The mutations in the protein sequence (a serine for an asparagine and glutamic acid for lysine) would still allow for hydrogen bonding within the tertiary structure. However, it is difficult to predict the exact folding.

The overexpression of the pGEX-4T-3(cspZ) plasmid was very discouraging. The growth curve showed mid-logarithmic phase to be when the bacterial culture reached an absorbance of 0.500 at 600nm. This was interesting considering most protein expression protocols suggest inducing protein production when the bacterial cultures reach "mid-logarithmic phase", which was an absorbance reading of 0.600-0.800 at 600nm. Inducing protein expression during mid-logarithmic phase maximizes the chance for protein production. When protein expression seemed to never be successful, the sequence was checked again to make sure it was in frame with the GST moiety included in pGEX-4T-3. It was confirmed the sequence was in frame and translation of the mRNA should have proceeded until the expected stop codon was reached. The next thought was perhaps it was an induction problem as pGEX-4T-3 with nothing in the MCS would not overexpress the GST protein. More experiments optimizing the growing conditions may need to be performed for protein expression. One idea was to actually add lactose to the broth bacteria are growing in thereby possibly removing the lac repressor more effectively than IPTG. Although, IPTG is a very common reagent used during protein expression and there has been many successful protein expression experiments. Another idea was to switch vectors.

The qRT-PCR was supposed to be a pinnacle in the experiment showing the up-regulation of the *cspZ* gene. Unfortunately, the gene was not detected by the real-time

system and upon later investigation with traditional PCR, the plasmid encoding the *cspZ* gene was not detected either. The data was interesting however as *flaB* and *ospC* were both detectable. The data revealed the down-regulation of *ospC* while *flaB* levels remained not significantly different. It was interesting to see the *ospC* gene be down-regulated as *ospC* is known to be up-regulated when spirochetes are being transmitted from a tick environment into an animal host. Although, the experiment was conducted using *in vitro* conditions, this may have influenced the experiment. Also, traditional PCR conducted to check for the presence of lp28-3 did not show any amplified PCR fragment from B31MI or cF. This was unexpected as B31MI encodes *cspZ*, this may be an indication the primers used may be contaminated.

The proteinase K treatment of spirochetes yielded interesting data showing B31MI was capable of surviving dog sera even when all outer surface proteins were removed. This may have been because there was no ligand for the C3b to bind. The next few experiments involving fluorescent microscopy tracking protein localization would add to our understanding of how *B. burgdorferi* populates the outer surface landscape allowing for immune evasion in a canine host.

CHAPTER V

Conclusion

In conclusion, the pBSV2(flgB::cspZ) construct sequence analysis is 97% correct. B31cF transformed with pBSV2(flgB::cspZ) allows this strain to survive in dog sera, while B31cF without *cspZ* does not survive. Troubleshooting protein expression and purification will continue, optimizing growing conditions and/or switching vectors. The plasmid vector constructed for this work retains the integrity of the *cspZ* gene and is in frame with the GST moiety. Once the CspZ protein has been purified, antibodies can be generated using rats. These antibodies can then be harvested and used for downstream reactions showing surface localization of the CspZ protein. Quantitative real-time polymerase chain reaction will need to be redone to confirm *cspZ* is up-regulated when exposed to dog sera.

Overall, evidence from Kay and Lund still support the hypothesis CspZ is a key player in allowing *B. burgdorferi* to survive in a canine host.

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VITAE

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By 2011, she was ready to begin a Masters Degree of Science in Biology studying the genetics of B. burgdorferi. While completing a Masters Degree she also taught three semesters of microbiology for pre-nursing students mainly. She was also a peer mentor during this time, guiding incoming freshmen. She attended many seminars and conferences including winning first place at the Tennessee Academy of Sciences, Tennessee Mosquito and Vector Control Association, and the Graduate Student Research and Creative Activity Extravaganza.

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