Antigenic Variation at the *vlsE* Locus of the Lyme Disease Spirochete, *Borrelia burgdorferi*

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ABSTRACT

Leanna Kei Nosbisch: Antigenic Variation at the *vlsE* Locus of the Lyme Disease Spirochete, *Borrelia burgdorferi*

(Under the direction of Aravinda de Silva)

Lyme disease is caused by the tick-borne spirochete, *Borrelia burgdorferi*. The spirochete produces over 100 membrane anchored lipoproteins. One lipoprotein, designated VlsE, changes its antigenic properties through DNA recombination, most likely to evade adaptive immune responses in the host. Here we demonstrate that VlsE is on the surface of the spirochete and exposed to the outside. Our hypothesis is that *vlsE* recombination is active in the host but not in the tick, which does not have an adaptive immune system. We characterized the number of *vlsE* alleles present in culture and at different stages of the Borrelia life cycle in the vector and host. The locus was stable in culture and in ticks, unlike in mice where many novel recombinants were readily observed. These results support the hypothesis that *vlsE* recombination is more frequent in the host than in ticks.

DEDICATION AND ACKNOWLEDGEMENTS

I would like to dedicate my thesis to my family and friends for all of their love and support during this wonderful period of my life. I would especially like to thank my Mom and my Pop-Pop for giving me perseverance: “You can do anything in life if you want it bad enough.” I thank Aravinda de Silva for his patience, guidance, and support as my mentor. Thanks to all previous and current members of the de Silva lab for their assistance, constant advice, and friendship. Finally, I would like to thank Ben Kendall for his amazing encouragement, strength, and love.

PREFACE

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LIST OF ABBREVIATIONS

bp	base pair
bgp	glycosaminoglycan-binding protein
BSK	Barbour-Stoenner Kelly Broth
cp	circular plasmid
CRASP	Complement regulator-acquiring surface protein
dbpA/B	decorin binding protein A and B
Erp	OspE related protein
IFA	Immunofluorescence assay
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	kilobase
kDa	kilodalton
lp	linear plasmid
Mb	megabase
Msp2	Major surface protein 2, *Anaplasma marginale*
Osp	outer surface protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PMSF	Phenylmethylsulphonylfluoride
RFLP	restriction fragment length polymorphism
TBST	Tris buffered saline + 0.5% Tween-20
TrospA	*Ixodes scapularis* tick receptor for OspA
rVlsE	recombinant VlsE
VESA1	Variant erythrocyte surface antigen 1, *Babesia bovis*
VlsE	Vmp-like sequence expressions site, *Borrelia burgdorferi*
Vmp	Variable membrane protein, relapsing fever *Borrelia*
Vlp	Variable large protein, relapsing fever *Borrelia*
Vsg	Variant-specific glycoprotein, African trypanosomes
Vsp	Variable small protein, relapsing fever *Borrelia*

Chapter 1: Background and Significance

1.1 Introduction to Lyme Disease

Lyme disease is the most common vector-borne disease in the United States and Europe and is also found in Asia (43). The disease is caused by spirochetes of the *Borrelia burgdorferi sensu lato* genospecies complex (95) and is transmitted by bites of *Ixodes* species ticks. The vector on the east coast of the United States is *I. scapularis*, on the west coast is *I. pacificus*, in Europe is *I. ricinus*, and in Asia is *I. persulcatus* (134). Lyme disease is a multi-system disease that starts off in people with mild flu-like symptoms such as malaise, fatigue, headaches, and fever. The appearance of a characteristic localized "bulls eye" rash, *erythema migrans*, at the site of the tick bite occurs in 70-80% of cases (131, 137). During disease progression, the bacteria migrate from the site of infection throughout the body, leading to a disseminated infection that affects the joints, heart, and nervous system (134). Antibiotic treatment is effective if the patient is treated within the early stages of infection (147).

Lyme disease was first recognized in 1976 in Lyme, Connecticut when a group of children were thought to have juvenile rheumatoid arthritis (136). In 1981, Burgdorfer and colleagues identified the spirochete, *Borrelia burgdorferi*, as the etiologic agent of Lyme disease (24, 80, 133, 135). The spirochete was found in ticks and also cultured from patients and recognized by convalescent patient serum, conclusively linking *B. burgdorferi* as the agent of Lyme disease (17, 135). Since this original description, the genus *Borrelia* has been divided into multiple species that can cause infection. In the United States, *B. burgdorferi sensu stricto* is the only cause of Lyme disease, while in Europe and Asia, *B. garinii*, and *B. afzelii* are responsible for illness (6, 28, 135). Relapsing fever spirochetes such as *B. recurrentis*, and *B. turicatae*, are also included in this genus, although they are transmitted by different tick species and have a different progression of disease pathogenesis.

The spirochetes live in nature in enzootic cycles involving ticks and a range of animal hosts including mice, rabbits, and birds. The white-footed mouse, *Peromyscus leucopus*, is the main reservoir in the U.S. (95). *Ixodes* ticks have 4 life stages: egg, larval, nymphal, and adult stages that require a blood meal at each of the latter 3 stages. Ixodid ticks pierce the skin of an animal with their mouthparts, secrete a cement substance to aid in attachment to the skin, and feed for 3-5 days (132). During this feeding period, there are alternating periods of ingesting blood and salivating into the host during which pathogen transmission occurs. The spirochetes are acquired from infected mice by feeding larvae, and are transmitted to uninfected mammals, including humans, during the nymphal stage. Humans are an incidental host, and are not part of the enzootic life cycle. Adult *I. scapularis* ticks feed on large mammals such as deer in order to mate and produce an egg mass, hence the common name, the deer tick.

The reasons for the emergence of Lyme disease in the late 1970s are unclear; however, increases within the deer population may allow for more adult ticks to feed and lay eggs. In addition, movement of families from cities to the suburbs raises the chance to encounter an infected tick, thus increasing the number of cases. Spirochetal DNA has been found in museum specimens of white-footed mice and ticks collected in the late 19th and early 20th centuries (89, 107, 108), indicating the infection has been present in North America for decades (43); however, human disease did not occur frequently enough to be recognized.

1.2 Tick-Borne *Borrelia*

In order for a pathogen to be successfully transmitted by an arthropod vector, it must be acquired by the vector either transovarially (adult female to egg) or when the vector feeds

on an infected host, it must be able to colonize and persist within the vector, it must be transmitted to a new host and persist until it can be acquired by another feeding vector. Borrelia cause two important tick-borne syndromes: Lyme disease and relapsing fever. While Lyme disease spirochetes are transmitted via an Ixodid tick vector, relapsing fever spirochetes are transmitted by Argasid ticks, or soft ticks. Both types of spirochetes grow extracellularly but are able to infect every tick organ except the ovaries, and are therefore not transovarially transmitted (132). The structure of all Borrelia species consists of a protoplasmic cylinder surrounded by periplasm containing flagella that contribute to the corkscrew shape, and an outer membrane (9). These bacteria are considered diderms for their two membranes; however, they are neither Gram positive nor Gram negative. Borreliae are microaerophilic, 20-30 μm in length, and 0.2-0.5 μm in width.

1.3 Lyme Disease Spirochetes

When a human or other mammalian host encounters a tick, it is important to remove the tick from the skin as quickly as possible, as *B. burgdorferi* is not transmitted until at least 48 hours into the bloodmeal (98). Once*B. burgdorferi* have been injected by a feeding tick into a naïve vertebrate host, the infection can persist if not treated by antibiotics. Spirochetes disseminate into organs in infected mammals and are rarely found in the blood. The 1.5 Mb genome of *B. burgdorferi* strain B31 has been sequenced (53) and includes a linear chromosome of 950 kb as well as 12 linear and 9 circular plasmids. The 853 genes located on the chromosome are responsible for most of the basic cellular functions such as growth and metabolism (53), with homologues in other bacteria. The large number of plasmids in *B. burgdorferi* is the most known for any bacterium, and encode another 535 proteins unique to

Borrelia (53). Over 150 of these genes are responsible for the production of putative lipoproteins and are thought to assist the bacterium in adaptation to different environments and different hosts. *B. burgdorferi* is a fastidious organism as it has few proteins with biosynthetic activity and depends on the host for most of its nutritional requirements or a rich medium supplemented with mammalian serum for growth *in vitro* (53). Barbour-Stoenner-Kelly (BSK) II and a variation, BSK-H, are two rich medias that allow researchers to study *Borrelia* gene and protein expression within the laboratory (7, 111). One unique feature of *B. burgdorferi* is it does not require iron during *in vitro* growth (112). The genome contains no homologues for specialized secretion systems, lipopolysaccharide, or toxins, and the only known virulence factors allow for attachment, migration, dissemination through mammalian tissues, and evasion of immune clearance.

Until recently, methods for manipulating genes within *B. burgdorferi* was limited, so the functions of many genes are unknown (20, 48, 141). We have only begun understanding gene function in *B. burgdorferi* through transposon mutagenesis (94, 140), transformation, gene inactivation (20, 47, 103, 121, 129), and generation of conditional lethal mutants (45). Plasmid loss with all of these methods is still a problem, as strains can become noninfectious.

Both innate and acquired immunity are important for controlling infection. *Borreliae* encode several proteins that can prevent complement-mediated lysis, one of the host's first defense mechanisms. Phagocytic cells such as macrophages and dendritic cells can clear bacteria before they disseminate from the site of inoculation. *B. burgdorferi* lipoproteins can signal through toll-like receptors and the adaptor molecule MyD88 on host cell surfaces (2, 16, 18, 19, 26, 54, 65, 87, 122), as well as stimulate adaptive T cell-independent B cell

responses (50, 92, 96). Additionally, antibody-mediated responses are crucial for spirochetal killing and resolution of disease (1, 44, 51, 62).

1.4 *B. burgdorferi* Gene Expression in Ticks and Mammals

In order to be maintained in the environment, *B. burgdorferi* adapts to the different environments of the tick vector and the mammalian host by changing the expression profile of several genes. Differential gene expression in *B. burgdorferi* is linked to cell density, temperature and pH of the surrounding environment (29, 30, 117, 139, 148). During tick feeding, the environment of the tick gut changes from 22 ºC to 37 ºC and from pH 7.4 to pH 6.8 (148). These conditions can be mimicked during *in vitro* cultivation to examine gene functions and gene expression patterns.

Bacteria that are acquired by feeding larvae remain in a dormant state within the tick midgut during the fall, winter, and early spring when the ticks are also dormant. An outer surface protein (Osp) encoded on lp 54, OspA, is primarily expressed during the dormant phase, during blood feeding, and through the molt from larvae to unfed nymphs (126). Recently, Pal and colleagues identified a tick receptor for OspA, TrospA, which allows the spirochetes to attach to the midgut wall (102). Host antibody responses to OspA prevent tick midgut colonization, therefore blocking transmission (40, 41).

When the nymphal ticks feed in late spring or early summer, the population of Borrelia within the tick midgut increases dramatically in the first 60 hours, and several genes are differentially expressed (38, 39, 66). OspA is downregulated, and OspC, encoded on cp26, is upregulated (38, 39). OspC is thought to be involved in migration of the spirochetes from the midgut into the tick hemolymph, into the salivary glands, and for establishing

infection in the mammalian host (30, 98, 103, 126). Other "OspC-like genes" that are upregulated upon infection include decorin binding proteins A and B (dbpA and dbpB) (52, 57), fibronectin binding protein (BBK32) (113), and a glycosaminoglycan-binding protein (Bgp) (83, 105). The proteins encoded on these genes allow for *B. burgdorferi* dissemination and adherence to integrins, proteoglycans, and glycoproteins on host cells or tissue matrices. The reciprocal gene expression in ticks is demonstrated *in vitro* where OspA is expressed at low cell density, high pH (7.4) and low temperature (22 ºC), while OspC and OspC-like genes are expressed at high cell density, low pH (6.8) and high temperature (37 ºC).

B. burgdorferi also encode complement regulator-acquiring surface protein (CRASPs), also known as Erps, or OspE and OspE-related proteins, that bind complement factor H and factor H-like protein 1 (22, 75). These proteins inactivate C3b, preventing further activation of the complement cascade, thus protecting the organism from complement-mediated killing (3, 75, 76, 91, 138). Specific genes are required for mammalian infection including a nicotinamidase encoded on lp25 (115) and a linear plasmid, lp28-1 (78). *B. burgdorferi* genes that are known to be important during tick colonization or mammalian infection are listed in **Table 1.1**.

1.5 Antigenic Variation as a Biological Process

Different strains of a pathogen can vary the immunodominant antigens on their surface differently, resulting in strain specific immune responses in a vertebrate host. Diverse sequences between proteins as well as different immune responses can influence selection of the pathogen (10). Diversity of an immunodominant antigen between strains is called antigenic variation. The strict definition of antigenic variation "involves the loss, gain,

or change in a particular antigenic group, usually by loss, gain, or change in one of the polypeptide or polysaccharide antigens…(13)." The adaptive immune system of a host mounts an immune response against the original infecting serotype, but this specific immune response may be ineffective against new emerging variants. Antigenic variation may also be used by a pathogen during adaptation to different niches within an infected host, or in order to colonize different host species (86, 130).

There are three main mechanisms that pathogens are known to use to change surface antigens: phase variation, slipped-strand mispairing, and gene rearrangement. Phase variation, or on/off switching of a gene, is employed by *Salmonella typhimurium* to switch flagellin genes and fimbriae of *Escherichia coli*species, where promoter inversions can activate transcription of two different gene loci (144). Slipped-strand mispairing within long nucleotide repeats in promoter regions of genes can also lead to changes in the reading frame of a gene and on/off phase variation in bacteria, such as the *opa* genes in *Neisseria* species (61). Gene rearrangement is a mechanism involving gene conversion during DNA recombination that leads to replacement of an expressed recipient gene with variable DNA segments from silent copies located in different parts of the genome. Recombination is uni-directional and non-reciprocal, ensuring that the DNA sequence of the donor locus remains unaltered (61). As we are focused on gene conversion methods for varying surface antigens, phase variation and slipped-strand mispairing will not be discussed further.

1.6 Overview of Antigenic Variation Systems and Organisms

Organisms within *Borrelia* spp., *Anaplasma*, African trypanosomes, *Plasmodium* spp., and *Babesia* spp. are all vector-borne pathogens that use antigenic variation to evade the

host immune system (**Table 1.2**). The exact mechanism between each organism differs; however, a switch between at least three variable antigens rather than two, is common to all. Variation is achieved through switching one of several gene alleles expressed at any one time, rather than accumulating mutations in a single expressed gene. Antigenic variation can happen through gene conversion mechanisms and multiple point mutations (42). Gene conversion is the most widespread method for replacing expression of one gene with another (10). The change in the protein occurs when a central hypervariable region of a protein is replaced through crossovers in highly homologous flanking regions. Examples of this mechanism include major surface protein 2 (msp2) of *Anaplasma marginale,* variant-specific glycoprotein (VSG) of African trypanosomes, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), variant erythrocyte surface antigen 1 (VESA1) of *Babesia bovis, vmp* genes in relapsing fever *Borrelia*, and VlsE in *B. burgdorferi* (10). The second mechanism, multiple point mutations, usually occurs in a gene that is already activated through gene conversion.

The molecular mechanisms for many of the examples listed above are still not well understood; therefore, we will discuss antigenic variation through gene conversion using the well-studied type IV pilin genes of *Neisseria gonorrhoeae*. Type IV pili are present on all clinical isolates of *N. gonorrhoeae* and are essential for colonization of the urogenital tract during infection (34). There is one pilin expression locus in the genome, *pilE*, and one to six loci containing silent cassettes, termed *pilS* (144). All of the *pilS* sequences lack the promoter, ribosomal binding site, and 5' 150 bp found at *pilE* (59, 60, 127). The pilin proteins are conserved for two-thirds of the N terminus, but vary at the remaining C terminus (reviewed in (144)). Variation at the C terminus occurs when a sequence from one of the silent *pilS* loci transfers into *pilE*. Recombination only requires 2 bp of conserved sequence,

occurs at a high frequency (>10^{-3}), and is dependent on the recombinases RecA and RecF (68, 74, 93, 152).

1.7 Antigenic Variation in *Borreliae*

Relapsing fever borreliae are capable of antigenic variation in an outer membrane protein called Vmp, or variable membrane protein (10). Vmps within relapsing fever spirochetes can occur in two classes: the variable large proteins (vlps) of approximately 36 kDa, and the variable small proteins (vsps) of approximately 20 kDa (11, 25, 118). Up to 30 antigenic variants can arise within a single cell of *B. hermsii*, and each can confer a specific serotype (10, 142). Each acute phase of febrile illness and spirochetemia contains a population of spirochetes composed of one serotype, and relapses occur with a population of a differing serotype (142). In *B. hermsii*, gene conversion between linear plasmids containing at least 40 silent *vsp* and *vlp* gene cassettes results in the replacement of one variable antigen gene with another downstream from a promoter of a single expression site in the genome (8, 71, 109). Kitten et al. demonstrated that chimeric *vlp* genes can be created from partial gene conversion events (72). The rate of change in *B. hermsii* has been calculated to be 10^{-4} to 10^{-3} per cell per generation (33). No function has been assigned to the relapsing fever Vmps, other than antigenic variation.

1.8 Antigenic Variation in *Borrelia burgdorferi*

Borrelia strains missing linear plasmid 28-1 show decreased infectivity to mice but not to ticks, indicating an important role for this plasmid within the vertebrate host (70). Lp28-1 carries 32 open reading frames (53), and one gene locus on lp28-1 that likely

contributes to *B. burgdorferi* survival in a mammalian host is the variable major protein like sequence (*vls*) gene locus (4, 149). The expression site (*vlsE*) encodes a 34 kDa lipoprotein that is expressed during *in vitro* cultivation (98, 149, 151), at low levels within ticks (70, 99), and highly expressed in the vertebrate host (149, 151). However, *vlsE* mRNA was not differentially transcribed in response to temperature or pH changes *in vitro* by microarray analysis (100, 119). When spirochetes harvested from mouse chamber implants were compared to spirochetes harvested from culture, *vlsE* message increased threefold in the mammal, indicating that VlsE may only be activated in the host (119). The *vlsE* locus has also been shown to be expressed *in vitro* in response to endothelial cell membranes, further supporting its role in the host (69).

The *vlsE* locus consists of two constant domains at the amino and carboxy terminii of the protein and a central variable domain enclosed by 17 bp direct repeats (4, 149) (**Figure 1.1**). Within the central variable region of *vlsE*, there are six variable regions interspersed between 6 invariant regions (149). Upstream of the expression site are 15 partial gene copies, or cassettes, that are promoterless, have no ribosome binding sites, and are therefore "silent" (149, 150). Mammalian infection with *B. burgdorferi* induces sequence changes through partial, non-reciprocal gene conversion events between cassettes with the central variable domain to generate alleles that are a mosaic of the silent copies (149, 150). In theory, this mechanism could create 10^{30} different alleles at the expression site (143), thus altering the antigenic properties of VlsE (149). Indeed, when the crystal structure of VlsE was solved, the most distal outer part of the protein exposed to the immune system consists primarily of the central variable region (46), and the variable regions could mask the invariant regions of the protein.

1.9 Potential and Proven Functions of VlsE

VlsE is highly immunogenic, as Lyme disease patients mount a strong antibody response to VlsE (81, 88), especially to the IR6 invariant region of the protein (5, 64, 79, 84, 85, 104, 106, 123). Recombinants can be detected within the mammalian host as early as 4 days after infection and continue throughout the course of infection (151). Although VlsE is expressed in culture, novel recombinants have not been observed among spirochetes grown in culture (98), indicating that the recombination mechanism may be inactive, or the rate of recombination in culture is too low to be detected (99). Furthermore, recombinants were not detected within mouse chamber implants (Steven Norris, personal communication). This indicates that*vlsE* recombination is initiated by either a host specific mechanism (extracellular matrix or cell contact dependent), or an antibody specific mechanism.

Recombination could help spirochetes escape antibody-mediated defense against VlsE variants that arise during infection, and variants are antigenically distinct from one another (4, 90, 143, 149). The fact that *B. burgdorferi* is able to persist in the presence of an active anti-VlsE antibody response suggests that changes to the surface exposed epitopes in the variable region protect the spirochetes from host recognition. The mechanism for how *vlsE* recombination in *B. burgdorferi* happens is unknown; however, the *cis* gene arrangement of *vlsE* and the upstream silent cassettes may be important (82). The *vls* locus of two other Lyme *Borrelia* strains was recently characterized by Wang and colleagues (146). *B. garinii* lp90 and *B. afzelii* ACAI were found to have 11 and 14 silent *vls* cassettes, respectively. This indicates that the mechanism of antigenic variation is a common feature of Lyme disease borrelia and is conserved (146).

1.10 Two Models to Explain *vlsE* Recombination

Mutations and natural selection is a way for all organisms to genetically adapt to their environment. Growth under selection allows for gene mutations to happen and allows for reproductive success for organisms that carry those mutations. The major population expressing the dominant allele will be selected for, while any minor populations with mutated alleles will continue to survive. Selection is distinct from mutation, where errors occur during DNA replication and repair. The mutation rate is generally thought to be constant within a population, and is dependent on population size, genome size, and recombination frequency (120), but not environmental stimuli such as antibodies or immune effector cells.

Because recombination at the *B. burgdorferi vlsE* locus can be detected within mice, but not within spirochetes grown in culture, we thought of two models for how *vlsE* recombination may be influenced: an active model, and a passive model. The active model is based on mutation frequency, and whether the frequency changes between *in vitro* and *in vivo* growth. While it has been shown that antibodies can influence Borrelia gene expression (67, 86), it is generally not accepted that a bacterial pathogen can change its rate of mutation in the presence of antibody. The passive model is based on selection and the mutation rate staying constant. In this model, under selective pressure such as a specific antibody, minor *vlsE* variants could be detected.

1.11 Thesis Overview

It is important to understand how a pathogen changes its antigenic profile during infection in order to understand disease pathogenesis. It is well known that pathogens such

as *B. burgdorferi* are able to control gene expression in the many environments they encounter. Sequence changes by recombination at the *vlsE* locus are hypothesized to be important for *B. burgdorferi* to establish a chronic infection within the mammalian host; however, it is unknown what happens to the *vlsE* sequences during acquisition by larval *I. scapularis* ticks, through a blood meal, through the molt into nymphal ticks, and through a second blood meal. As many genes responsible for host colonization are upregulated during transmission from the feeding nymph, we asked the following questions: 1. Can we detect recombination at the *vlsE* locus during tick feeding? 2. Is there a role for VlsE recombination in tick colonization? 3. Is there selection within feeding ticks that allows for only certain alleles to be carried to the next stage? In Chapter 2, we describe a series of experiments using an immersion technique with a clonal population of *B. burgdorferi* to infect larval *I. scapularis*ticks in order to follow the populatio n through the tick life cycle.

In addition to antigenic variation, we were interested in the antibody-mediated immune response to VlsE. The fact that variation at the *vlsE* locus does not occur, or is at a frequency too low to be detected during *in vitro* cultivation led us to the following two questions: What is the rate of recombination at the *vlsE* locus? Does the rate increase as spirochetes are adapting to the host environment (active model)? Or are variants detectable within the mouse due to host immune selection of the minor population (passive model)? These questions led to creation of several tools to study whether antibodies to VlsE are bactericidal, and where in the cell VlsE is located. In Chapter 3, we describe immunofluorescence, electron microscopy, and proteinase K experiments that were performed to understand the location of VlsE within bacterial cells grown *in vitro*. The

results of these techniques were inconclusive, however we discuss several possibilities *B. burgdorferi* may use to avoid host immune recognition.

Table 1.1 Important genes in *B. burgdorferi* enzootic life cycle.

Gene	Location	Expression	Function	Mechanism
OspA	Lp54	Tick	Midgut colonization	Expressed in unfed tick and downregulated during the bloodmeal
OspC	Cp26	Tick/mammal	Migration from midgut to salivary gland, colonization of host	Upregulated during bloodmeal, downregulated during dissemination in host
Erps	Cp32	Mammal	Inactivation of host alternative complement	Expressed in mammalian host
DbpA, DbpB	Lp54	Mammal	Binds decorin	Host colonization
Bbk32	Lp36	Mammal	Binds fibronectin	Host colonization
P66	Chromosome	Mammal	Binds integrin	Host colonization
vlsE	Lp28-1	Mammal	Immune evasion	Recombination leads to antigenic variation
Bgp	Chromosome	Mammal	Binds glycosaminoglycans	Host colonization
CRASP	Lp54 and cp32	Mammal	Binds factor H, blocks host complement activation	Upregulated in mammalian host
pncA	Lp25	Tick/Mammal	Nicotinamidase	Host colonization

Table 1.2 Vector-borne infections with antigenic variation (adapted from (10)).

Disease	Pathogen(s)	Vector	Variable antigen*
Relapsing Fever	Several *Borrelia* species	Soft (argasid) ticks and body lice	Vlp and Vsp
Anaplasmosis	*Anaplasma marginale*	Hard (ixodid) ticks	MSP2
African trypanosomiasis	African *Trypanosoma* spp., e.g. *T. brucei*	Tsetse fly	VSG
Malaria	*Plasmodium falciparum*	Mosquito	PfEMP1
Babesiosis	*Babesia bovis*	Hard (ixodid) ticks	VESA1

* Vlp = variable large protein; Vsp = variable small protein; MSP2 = major surface protein 2; VSG = variant-specific glycoprotein; PfEMP1 = *P. falciparum* erythrocyte membrane protein 1; VESA1 = variant erythrocyte surface antigen 1

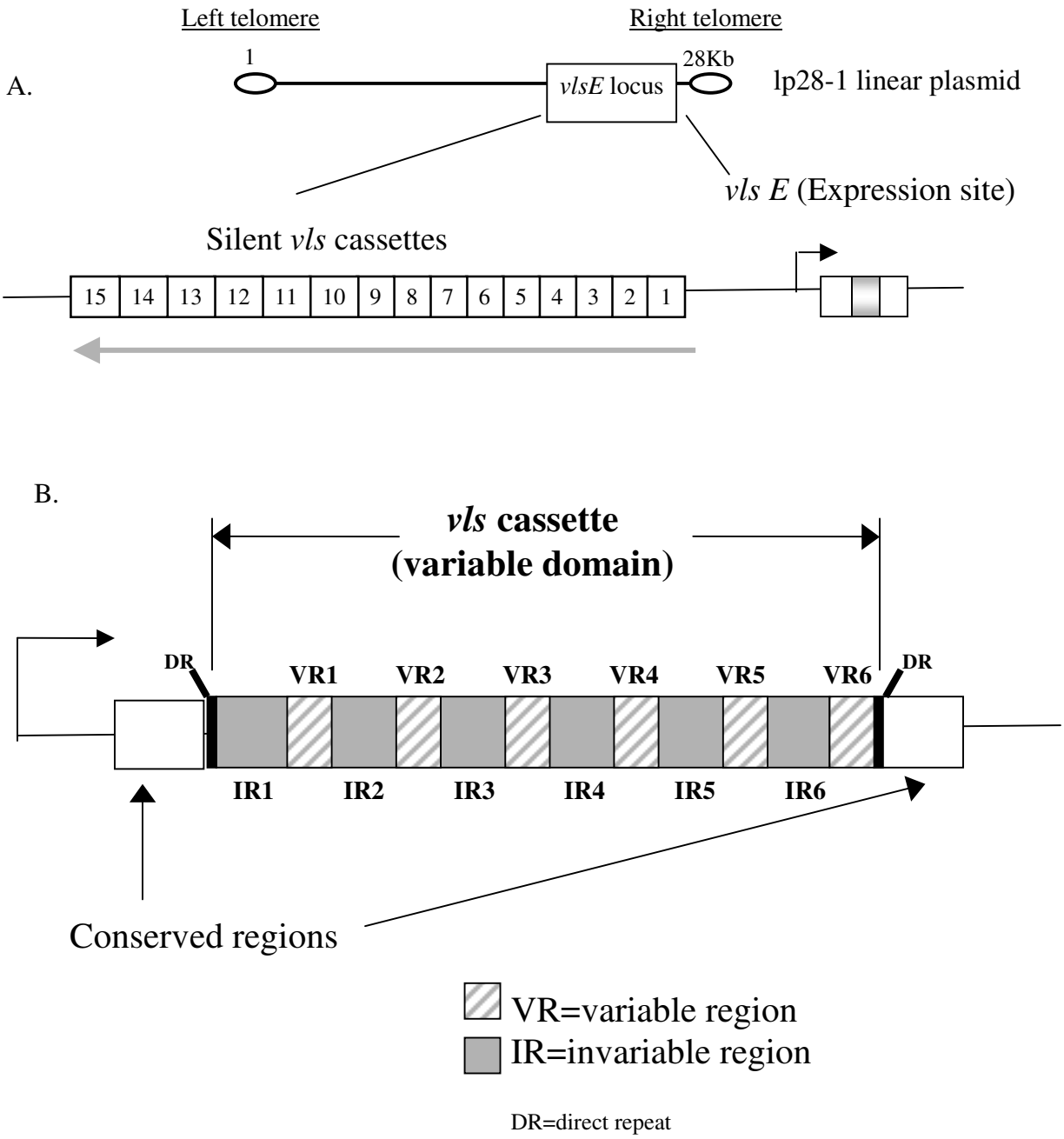

Figure 1.1 Schematic diagram of the *vlsE* locus of *B. burgdorferi.* A. *vlsE* locus on lp28-1. B. Diagram of the central variable domain of *vlsE*

Chapter 2: Lack of Detectable Variation at the *Borrelia burgdorferi vlsE* Locus in Ticks

2.1 Abstract

VlsE is a surface exposed lipoprotein of the Lyme disease spirochete, *Borrelia burgdorferi*. Novel antigenic variants at this site are readily observed in the infected vertebrate host. We followed a clonal population of spirochetes through a tick transmission cycle and report that unlike in vertebrates, the *vlsE* locus is stable in ticks.

2.2 Journal of Bacteriology Note

The spirochete *Borrelia burgdorferi*, the causative agent of Lyme Disease, is able to maintain a persistent infection of mammals in spite of an active immune response (125, 134). One mechanism that contributes to persistence is recombination at the plasmid encoded variable-major-protein (Vmp)-like sequence, or *vls* on linear plasmid lp28-1 (149). This locus has been well characterized in *B. burgdorferi* sensu stricto strain B31 (149-151), and consists of an expression site encoding a lipoprotein, *vlsE*, and 15 silent cassettes located upstream (149). A nonreciprocal gene conversion mechanism recombines portions of the silent cassettes within a central variable domain of the expression site, allowing for novel alleles to be expressed and variation of the antigenic properties of VlsE (149-151). Recombination is detectable within days after infection of the mammalian host, but is not detectable in spirochetes cultured *in vitro* (98, 99, 151).

As Lyme disease is spread through the tick vector *Ixodes scapularis*, we were interested in studying whether recombination can be detected in ticks. Indest et al. observed the *vlsE* locus in infected nymphal ticks and reported the absence of recombination in ticks(70, 98, 99); however they studied a clonal population of spirochetes in nymphs introduced by capillary feeding (70). Our group has previously studied ticks fed on mice

inoculated with a clonal population of *B. burgdorferi* to follow populations of *vlsE* alleles in ticks (98, 99); however, these studies are confounded by the fact that ticks feeding on an infected animal acquire many different *vlsE* alleles and it is not possible to separate recombinants that might arise in the vector from recombinants acquired from the host. In this study we used a recently described larval immersion method (110) to introduce a clonal population into larval ticks and then followed the *vlsE* population by sequencing clones at different stages in order to determine whether the locus is stable through a larval blood feeding, through the molt into nymphs, and during a second nymphal blood feeding. This method is faster than previous methods of analysis (99).

The study design is outlined in Figure 2.1. Low passage *B. burgdorferi* strain B31 (CDC, Fort Collins, CO) was grown on solid Barbour-Stoenner-Kelly II (BSKII) medium (7, 77, 110) and a single clone designated B31-C1 was used in this study. *B. burgdorferi* B31-C1 was inoculated into liquid BSK-H medium(111) and grown to 1 x 10^8 cells per ml. *B. burgdorferi* B31-C1 was introduced into larval *I. scapularis* ticks (J. Bowman, Oklahoma State University) using the previously described larval immersion method (110). Briefly, larval ticks were transferred to a 1.5 ml screw cap microcentrifuge tube (Starstedt, Newton, NC), and immersed in 1 ml of *B. burgdorferi* B31-C1 in BSK H media. Tubes were gently vortexed to suspend larvae, and incubated for 2 hours at 34ºC on a rocker (110). After incubation, tubes were centrifuged at 200 x *g* for 30s, and supernatant medium was removed. The larval ticks were washed twice with phosphate-buffered saline (PBS) and excess moisture was wicked with strips of Whatman #1 filter paper (Whatman, Maidstone, England). The larvae were immediately placed on a naïve 4-6 week old female C3H-HeN mouse (NIH) and allowed to feed until repletion.

Engorged larvae were held in a humidity controlled incubator at 21°C for 1 week and then a portion were separated for the fed larval group. The rest of the ticks were held approximately 7 weeks postrepletion through the molt into nymphal ticks. A second group was separated for the unfed nymph group, and the rest of the ticks were placed on a second naïve mouse. This group of nymphs fed until repletion and were designated the fed nymph group. To culture spirochetes from ticks, the ticks were surface sterilized by immersion in 3% hydrogen peroxide for 10 min, followed by a 10 min. immersion in 70% ethanol and 2 rinses with PBS. Ticks were then crushed with a sterile molecular grinding pestle (Kontes, Vineland, NJ) in a microfuge tube in 50 µl PBS. 20 µl of the tick homogenate was used to inoculate 1.5 ml BSK-H media with Borrelia antibiotic mix containing fosfomycin, rifampin, and amphotericin B (Sigma Chemical Co. St. Louis, MO). Cultures were incubated until they reached a density of 1 x 10^6 cells per ml. DNA was harvested using the DNeasy kit (Qiagen, Valencia, CA). In order to verify our sequencing method could detect *vlsE* allele differences, we sacrificed mice 3 weeks post feeding and cultured spirochetes from mouse tissue. Cultures were treated as described above.

The *vlsE* variable region was amplified from DNA purified from spirochetes harvested from each tick group and mouse culture using the primers vlsE-F and vlsE-R (98). PCR products were visualized by 0.8% agarose gel electrophoresis, and then cloned into a TOPO PCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) and electroporated into *Escherichia coli* strain Top10 using the manufacturer's instructions. White *E. coli* colonies carrying *vlsE* variable region sequences from each original tick or mouse culture were randomly selected and analyzed by either restriction fragment length polymorphism (RFLP) (99) (data not shown) or were submitted for sequencing. The sequences were aligned using

Vector NTI Software (Invitrogen) against the parental B31-C1 sequence, which is identical to the sequence in the Genbank database (U76405) (149).

The *vlsE* variable regions from 876 tick samples were sequenced or analyzed by RFLP and all had the same sequence or RFLP pattern as the parental strain, *B. burgdorferi* B31-C1, with no insertions, deletions, or point mutations. This indicated that no recombination events could be detected and there is no role for *vlsE* recombination in the tick. A complete summary of the results is presented in Table 2.1. Our results are consistent with the findings of Indest et al. (70) with our analysis of more sequences and a method for infecting larval ticks that is closer to the natural cycle of tick infection. In contrast to ticks, we detected several sequence changes in mice using both RFLP and sequencing. Our method was able to detect sequence changes within variable regions of mouse cultures (Table 2.1), consistent with previous groups being able to detect recombinants *in vivo*.

There are two possible explanations for why we were unable to detect recombinants in feeding ticks. One may be the recombination mechanism is specifically turned on within mice in order to change surface antigenic properties, or detection of recombinants is easier in mouse samples due to immune selection (149-151). We interpret our results as novel alleles of *vlsE* are not generated or not detectable by our methods throughout acquisition by larvae, through the larval feeding, through the molt into nymphs, and through a second nymphal feeding. We conclude that there is no role for *vlsE* recombination in ticks and selection on *vlsE* is not occurring within the tick during the tick transmission cycle. This indicates that the *vlsE* allele present in a population is stable for several months through various population changes and morphological changes in ticks.

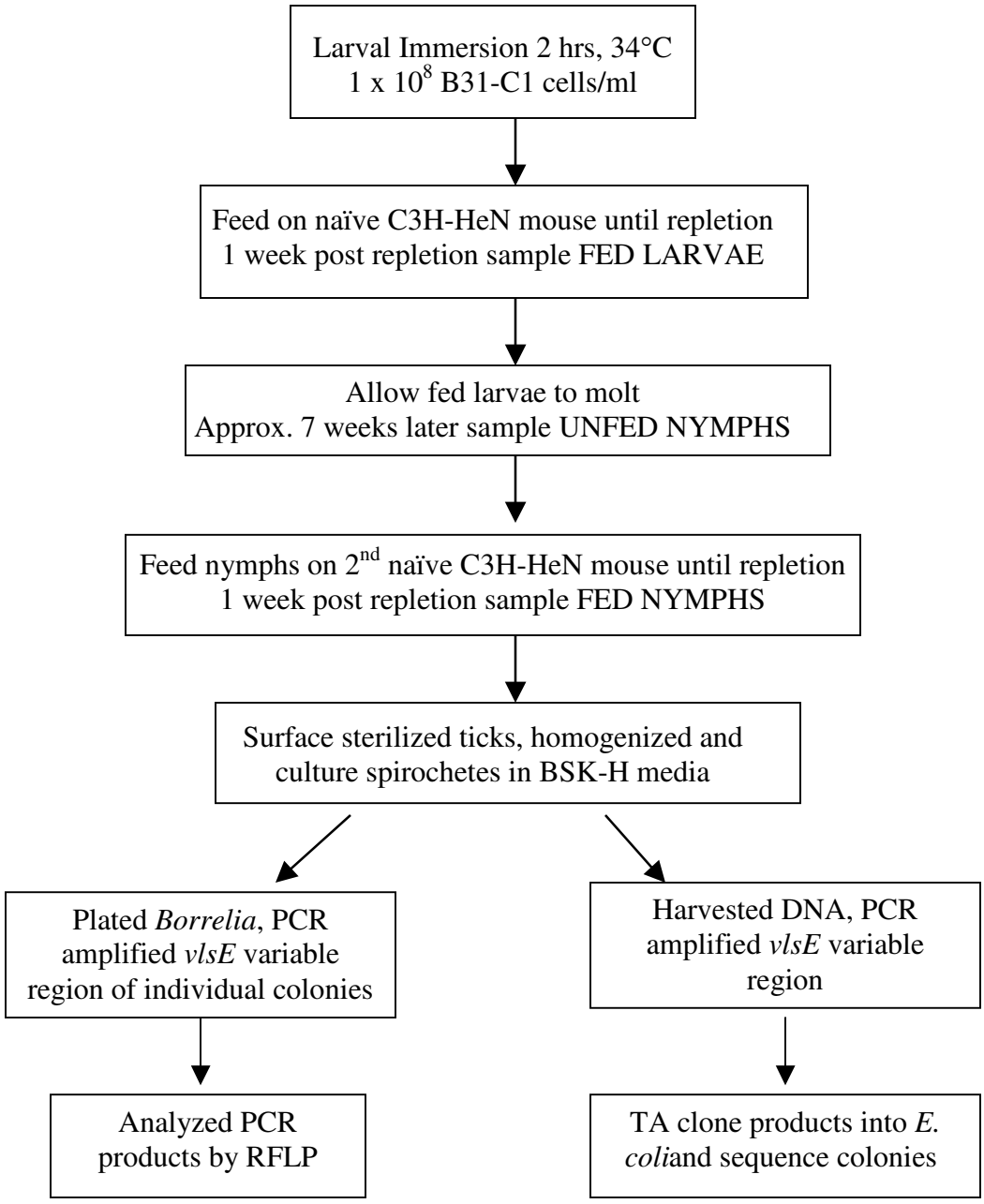

Figure 2.1 Experimental design. Experimental design for monitoring *Borrelia burgdorferi* B31-C1 population through a tick feeding cycle.

Table 2.1 Summary of all RFLP and sequencing data from each tick group.

Sample	# ticks	# clones RFLP	# clones sequenced	Analysis
Fed larvae	32	88	168	B31-C1
Unfed nymphs	32	123	157	B31-C1
Fed nymphs	44	123	217	B31-C1
Mouse	NA	28	12	Sequence changes in variable regions
Totals	108	334	554	

10-12 Clones were picked per tick to analyze by RFLP digestion with *AluI* and *MboI* or cloned into *E. coli* and sequenced. All *E. coli* clones sequenced or *B. burgdorferi* clones analyzed by RFLP had the same sequence as the parental strain, B31-C1. NA=not applicable

Chapter 3: Do antibodies directed against VlsE kill *Borrelia burgdorferi*?

3.1 Introduction

The genome of *B. burgdorferi* encodes at least 105 outer membrane lipoproteins that represent 8% of the coding sequences (53). The function of many of the lipoproteins is unknown; however, it is well established that many genes are differentially regulated in response to temperature, pH, and cell density (29, 30, 116, 117, 148). Several lipoproteins are important for colonization of the tick vector, transmission from the tick vector, and colonization of the mammalian host. Borrelia lipoproteins are surface exposed, and anchored to the outer membrane lipid bilayer by an N-terminal triacyl-modified cysteine (15, 21).

The exact mechanism for secretion and anchoring of the lipoproteins to the membrane is not well understood; however *B. burgdorferi* has homologues of all the essential genes involved in the general secretion pathway of *E. coli* (*secA/D/E/F/Y*, only the non-essential *secB* is missing) to secrete proteins across the inner membrane (53, 56). Detailed studies on lipoprotein secretion to the outer membrane in other bacteria have been limited to a few model proteins, which are transported by a type II apparatus or through an autotransporter secretion pathway (36, 114, 145). The Borrelia genome does not contain any homologues of type II secretion machinery, or other sec-dependent or independent secretion pathways (31, 53, 58), and *Borrelia* lipoproteins do not contain autotransporter translocation domains (101). It was recently shown that Borrelia lipoproteins are secreted to the surface by default, not a active mechanism, although there are sequences that retain lipoproteins in the periplasm, rather than allowing surface exposure (124).

One *B. burgdorferi* lipoprotein, VlsE, undergoes antigenic variation within the mammalian host through non-reciprocal DNA conversion events (149). This feature of VlsE may be driven by host immune response or cell contact dependent mechanisms during host

colonization and dissemination (see **Chapter 1**). Several assumptions must be made in order for VlsE antigenic variation to be driven by host antibodies: 1. VlsE must be surface exposed, 2. antibodies to VlsE must be bactericidal in order to select against variants expressing a specific allele, and 3. the subsequent antibody response must be specific for variants that arise during antigenic variation of VlsE. In this chapter we describe studies to investigate the surface exposure of VlsE and the ability of anti-VlsE antiserum to kill spirochetes.

3.2 Materials and Methods

Cloning B31-C1 VlsE

In order to express *B. burgdorferi* VlsE in *E. coli*, we first designed primers with restriction enzyme sequences at the 5' and 3' ends to facilitate cloning. The *vlsE* full open reading frame (1200 bp) was amplified by vls5A3F (CGGCATATGAGCCAAGTTGCTGATAAGGACGACCC) with a Nde1 restriction site at the 5' end, and vlse5A3R (CGGCTCGAGCAATCATGAGGGCATAGTCGTGTCCATACA) with a Xho1 restriction site at the 3' end, based on a previous strategy (149). Primers were diluted to a working stock concentration of 10 μM. Total DNA was harvested from *B. burgdorferi* strain B31-C1 using a DNeasy kit (Qiagen). PCR reactions were set up using high fidelity Vent Taq DNA polymerase in a 50 μl reaction in buffer concentrations recommended by the manufacturer, including negative controls for DNA contamination. Reaction mixtures were subjected to 5 min. at 95ºC, 35 reactions of 40 sec. denaturation at 95ºC, 40 sec. annealing at 60ºC, 2 min extension at 72ºC, and a final extension for 10 min at 72ºC. PCR products were visualized

using a 0.8% agarose gel electrophoresis.

Once a product of the correct size was amplified, it was cloned and transformed into *E. coli* cells using the TOPO 2.1 TA cloning vector according to the manufacturer's instructions (Invitrogen). DNA from a single white colony was harvested using a miniprep kit (Qiagen), and correct insertion of PCR products was confirmed by restriction digest analysis with NdeI and XhoI restriction digest enzymes (NEB Biolabs). Digested VlsE was cloned into the pET28 vector (Novagen) and transformed into Novablue*E. coli* cells (Novagen). The resulting plasmid was named pLN6, and contained the pET28 vector backbone, *vlsE* inserted in frame under a lactose inducible promoter, and DNA sequence for an N-terminal six histidine tag for purification. The final plasmid was confirmed by sequencing and restriction digest analysis.

Production of recombinant B31-C1VlsE

A single colony of *E. coli* pLN6 was used to inoculate a 25 ml LB + 50 µg/ml kanamycin culture, which was grown overnight and then used to inoculate 1 L LB + kanamycin broth. Cells were grown to an OD_{600} of 0.6, and then protein expression was induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 1 hour. Cell pellets were harvested by centrifugation and resuspended in lysis buffer (Phosphate buffered saline with 0.1% Triton-X 100, 0.5 mM Phenylmethylsulphonylfluoride (PMSF), and protease inhibitor cocktail (Sigma)). Cells were sonicated, centrifuged to remove cell debris, and supernatants containing recombinant protein were bound to Ni-NTA slurry (Invitrogen) for protein purification. The slurry mixture was loaded onto a column, the column was washed several times, and then recombinant VlsE protein was eluted from the column using

250 mM imidazole. Protein concentrations were determined using a Bradford protein assay.

Production of variant B31 VlsE

To obtain recombinant VlsE proteins with different sequences within the variable region, a heterogeneous culture was grown from a *B. burgdorferi* infected mouse ear biopsy. The culture was plated on Barbour-Stoenner-Kelly H (BSK-H) *borrelia* agar plates and several colonies were submitted for sequencing at the UNC-CH genome analysis facility. The primers VlsE-F (GTAGTACGACGGGGAAACCAGATAGTAC) and VlsE-R (CCTAAAACTTTGCGAACTGCAGACTCAGC), which amplify the central 682 bp variable region of *vlsE*, were used for sequencing. Two clones with diverse sequence from the parental B31-C1 *B. burgdorferi* sequence were chosen and recombinant VlsE was cloned into pET28, expressed in *E. coli,* and purified as described above. The resulting plasmids were called pLN2 and pLN8.

Production of rabbit polyclonal antisera

To obtain a polyclonal anti-VlsE antiserum, we sent rVlsE (1mg/ml) to Strategic Biosolutions, Newark, DE. They immunized 2 pathogen-free rabbits 4 times with 200 μg rVlsE each time using complete Freund's adjuvant the first time, and incomplete Freund's adjuvant each subsequent time. Approximately 75 mls of polyclonal rabbit anti-VlsE sera was collected, and was subsequently used in immunofluorescence assays, transmission electron microscopy, and western blotting experiments.

Immunofluorescence assay

Low passage *B. burgdorferi* B31-C1 was grown in BSK-H media to mid-exponential

phase and washed several times with PBS to remove media. Depending on the experiment, cells were either spotted on silylated slides (approximately 10^5 cells/spot) or fixed in a microfuge tube with 4% paraformaldehyde (PFA) for 30 min. Slides were fixed with acetone for 5 minutes. Both PFA fixed spirochetes and slide spirochetes were blocked with 5% FBS-PBS for 30 min., and then detected with rabbit polyclonal anti-VlsE at a 1:500 dilution. Some samples were treated with 0.05% Triton-X to permeabilize the cell. PFA-fixed spirochetes were spotted on slides, allowed to air dry, and both aceteone fixed and PFA fixed bacteria were detected with a secondary goat anti-rabbit alexa 594 antibody and a goat anti *Borrelia* FITC-conjugated antibody for 30 min at room temperature. Slides were mounted with slowfade/antifade (Molecular Probes) and observed by epifluorescence microscopy (Eclipse E 600, Nikon). Images were captured with a digital camera (SPOT II, Diagnostic Instruments, Sterling Heights, MI) using SPOT software version 2.2.

Transmission Electron Microscopy

Low passage *B. burgdorferi* B31-C1 weregrown in BSK -H media to mid-exponential phase, and washed several times with PBS to remove media. Cells were fixed in a microfuge tube with 4% paraformaldehyde (PFA) for 30 min and sent to the Microscopy Services Laboratory (Department of Pathology and Laboratory Medicine, UNC-CH). The cell pellet was washed, stained en bloc with 2% uranyl acetate, and dehydrated in an increasing concentration of ethanol series (75%, 90%, 100%, 100%). The pellet was infiltrated overnight in L.R. White resin (hard grade, Ted Pella, Inc,. Redding, CA) at 4ºC. The embedded cells were sectioned using a diamond knife and a Leica Ultracut UCT ultramicrotome (Leica, Inc., Deerfield, IL) to 80 nm thickness. The sections were mounted

on 300 mesh nickel grids, immunostained, and post-stained with 4% aqueous uranyl acetate and Reynolds' lead citrate. Grids were observed and photographed using a LEO EM910 transmission electron microscope operating at 80 kV (LEO Electron Microscopy, Thornwood, NY).

Western blot

B. burgdorferi B31-C1 was grown in BSK H media at 34 ºC to a density of 1-2 x 10^8cells/ml. Cells were pelleted by centrifugation, washed in PBS, resuspended in SDS-PAGE running buffer and 0.4 M DTT. In Western blots with rVlsE, protein was resuspended in SDS-PAGE running buffer and 0.4 M DTT as above, and 5 µg was electrophoresed on the gel. Samples were boiled for 5 minutes at 90 ºC, and electrophoresed in a single prep well on a 12% SDS-PAGE running gel, 5% SDS-PAGE stacking gel. Gels were transferred to nitrocellulose, and blocked with 2-5% milk in Tris-buffered saline-1% Tween 20 (TBST). Rabbit polyclonal anti-VlsE antibody or rabbit anti-C6 antibody was added at a 1:500 dilution in Milk-TBST for 1 hour, washed, and detected with goat anti-rabbit alkaline phosphatase antibody for 30 min. Blots were developed using BCPIP/NBT alkaline phosphatase substrate (KPL, Gaithursburg, MA).

Proteinase K Accessibility Experiments

Low passage *B. burgdorferi* B31-C1 was grown in BSK-H media to mid-exponential phase, washed several times, and resuspended in PBS. Aliquots of washed cells were removed and digested for 45 min. at room temperature with 200 µg proteinase K (Sigma), and stopped with the addition of 1.6 mg/ml phenylmethyl-sulfonyl fluoride (PMSF) (Sigma).

Some samples received 0.05% Triton-X 100 to permeabilize the outer membrane. SDS-PAGE sample buffer and DTT were added to samples before they were boiled at 90 ºC for 5 min and run on 12% SDS PAGE resolving, 5% stacking gels. Polyacrylamide gels were transferred to nitrocellulose at 380 mAmps for 75 mins, and blocked with 2% Milk-TBS for 30 min. at room temperature. Blots were probed with rabbit polyclonal anti-VlsE serum, mouse anti-FlaB or mouse anti-OspA sera, and detected with goat anti-rabbit or goat anti-mouse conjugated to alkaline phosphatase. Finally, blots were developed with BCPIP/NBT alkaline phosphatase substrate (KPL, Gaithursburg, MA).

Borreliacidal Assays

Borreliacidal assays were adapted from existing protocols (35, 128). *B. burgdorferi* B31-C1 were grown to mid-exponential phase in BSK-H media, centrifuged, and resuspended to 1 x 10^7 cells/ml. Cells were plated at 2 x 10^6 bacteria per well of a sterile 96 well plate in duplicate. Serum was heat inactivated for 45 min at 56 ºC, and added to the wells at various dilutions. Antibodies were allowed to bind for 20 minutes, and then normal rabbit serum was added as a source of complement. Plates were allowed to incubate for 2-4 days and then spirochetes were visualized by dark field microscopy for motility indicating viability. Normal rabbit serum or BSK H media alone served as controls for no killing.

3.3 Results

Creation of recombinant VlsE and polyclonal serum

If antigenic variation at the *vlsE* locus is driven by the host immune system, the protein must be surface exposed and antibodies directed against the protein must be

bactericidal. Here we describe the results of studies that were conducted to test if VlsE is surface exposed and if antibodies against the protein are bactericidal.

In order to produce immune sera against VlsE, we cloned, expressed and purified the VlsE allele from *B. burgdorferi* B31-C1. The recombinant protein (**Figure 3.1 A**) was recognized by polyclonal rabbit sera specific for constant region 6 within the variable region by Western blot (**Figure 3.1 B**). As only 1 band is detected in lanes 3,4,7, and 8, we determined that there was no cross reactivity of the antibody with the *E. coli* lysate and that protein bands underneath main protein bands are rVlsE degradation products. We repeated this same strategy with 2 heterologous *vlsE* alleles from two *B. burgdorferi* clones isolated from a mouse (**Figure 3.2**) in order to create variant recombinant proteins.

The first goal of this project was to produce and characterize a polyclonal rabbit anti-serum using recombinant VlsE protein to localize the protein on the spirochete's envelope. Rabbits were immunized with recombinant VlsE. The immune rabbit serum recognized the VlsE protein in Western Blots.

Bactericidal activity of VlsE antiserum

In order to test our hypothesis that VlsE is involved in immune evasion *in vivo*, we wanted to see if our B31-C1 VlsE rabbit polyclonal antiserum was bactericidal using *in vitro* assays with cultured spirochetes. We used heat inactivated polyclonal rabbit antiserum at various dilutions as our test serum, and normal rabbit serum or no rabbit serum (BSK media only) as controls. In addition, we used various concentrations of fresh rabbit serum as our complement source, to see if the addition of complement would increase antibody-mediated killing. The results of one representative experiment are shown in **Figure 3.3**. As the

doubling time of *B. burgdorferi in vitro* is approximately 12 hours, we allowed the spirochetes to be in the presence of specific antibody and complement for several days. The spirochetes continued to grow and all wells reached the same density under all conditions tested. There was no membrane blebbing or loss of motility to indicate loss of membrane integrity or cell lysis.

VlsE cellular location experiments

Several other groups have demonstrated VlsE expression by IFA in culture, however they always used acetone fixation methods, which do not distinguish between surface exposed and intracellular proteins (70, 99). We tried to answer where VlsE was located within cells using IFA, TEM, and proteinase K experiments. For IFA, we used two different fixation methods: acetone and paraformaldehyde (PFA). Acetone fixation will create spaces within the lipid bilayers, allowing antibodies to freely diffuse to their antigenic targets, whether on the surface or inside the cell. PFA fixation will fix the whole bacterium but will not create spaces for antibodies to penetrate beyond the outer surface of the spirochete. Addition of Triton-X 100 to PFA fixed cells will disrupt the lipid bilayer and create spaces for antibodies to enter the periplasm and cytoplasm.

Using the rabbit polyclonal anti-VlsE serum, we were able to detect VlsE in acetone fixed spirochetes and paraformaldehyde plus 0.5% Triton-X 100 samples the majority of the time (95.4% and 89%, respectively); however, we only detected VlsE on paraformaldehyde fixed spirochetes without Triton-X 100 36% of the time (**Figure 3.4**). We also performed immuno EM using this rabbit serum (Department of Pathology and Laboratory Medicine, UNC-CH). **Figure 3.5** shows VlsE immunogold labeling mostly on the inner membrane of

in vitro grown *B. burgdorferi* B31-C1. Some outer membrane labeling was observed by TEM, which is consistent with the small proportion of PFA only cells (36%) that were positively stained with the polyclonal rabbit anti-VlsE serum.

To confirm the location of VlsE, we performed proteinase K experiments. Proteinase K is a nonspecific serine protease that cleaves proteins off the surface of bacteria. We used FlaB, a component of the periplasmic flagella, as a control for outer membrane integrity. We also included OspA, an outer membrane lipoprotein, as a control for extracellular protein digestion (**Figure 3.6**). Triton-X 100, a nonionic detergent, was included to disrupt the lipid bilayer (37) and allow for total protein digestion. OspA does not completely go away with the addition of Triton-X 100, which could be from incomplete lysis of all of the cells. Although the FlaB signal is slightly diminished with the addition of proteinase K, the majority of it is protected, and the VlsE signal is gone (**Figure 3.6B**). These results indicate that VlsE is surface exposed, as FlaB is protected and OspA is digested (**Figure 3.6C**).

3.4 Discussion

We are interested in the mechanism that drives antigenic variation at the *B. burgdorferi vlsE* locus because recombinants can be detected in mice as early as 4 days after inoculation, but not in culture-grown spirochetes. We thought of two possible scenarios influencing recombination: an active increase in the frequency of recombination within the mouse, and a passive selection model, where specific anti-VlsE antibodies kill the major population expressing that allele, allowing for minor variants within the population to be detected. In order to test these two models, we first cloned and expressed recombinant proteins from B31-C1 and variant *vlsE* alleles in *E. coli* Although we are unsure if the

recombinant protein is properly folded, it is the same size as protein from culture lysate (data not shown) and is recognized by immune mouse serum.

Recombinant VlsEwas highly immunogenic, and we were successful in c reation of a polyclonal rabbit anti-serum. The serum recognized variant VlsE alleles as well as the parental allele, and we think this is due to the serum recognizing the many conserved domains of VlsE, not just the variable domain. Because *vlsE* variants can be detected within the mouse, it is surmised to be involved in evasion of the host's immune system; however, the exact function of VlsE is unknown. The next goal was to investigate whether our polyclonal antiserum was bactericidal against B31-C1 *B. burgdorferi* cells expressing the *vlsE* allele that was used to immunize rabbits. The sera did not kill*B. burgdorferi* B31-C1 bacteria in the presence or absence of rabbit complement (**Figure 3.3**). These results indicate that the antibody present in each individual well did not have borrelia cidal activity with or without a complement source. It is possible that VlsE is not surface exposed *in vitro*, while it may be abundant on the surface *in vivo*. In addition, it is possible that the bacteria expressing the B31-C1 allele on the surface underwent antigenic variation in the presence of antibodies and complement source in order to escape selection; however, we did not test the spirochetes for recombination after incubation with antibodies.

Antibodies against several *B. burgdorferi* antigens are borreliacidal, and efficiency of antibody-mediated killing goes up in the presence of a complement source (14, 27, 32, 49, 51, 55, 73, 97). Because our serum did not kill spirochetes in our *in vitro* assay, we used several techniques to understand where in the cell VlsE was expressed. Using two different fixation methods and IFA, we determined that VlsE was intracellular, either within the cell or on the inner membrane, but not surface exposed. This was confirmed by TEM where we

observed immunogold labeling on the inner membrane, but rarely on the outer membrane.

We tried to confirm the microscopy results by protease digestion with proteinase K and we saw a different result. VlsE was surface exposed and had the same digestion pattern as OspA, while FlaB protein within the periplasm was protected. There are several interpretations for this: 1. Another protein (unknown protein X) could be masking VlsE on the surface, or 2. VlsE is expressed at low levels *in vitro*. The first possibility should be considered, and it is possible that protein X is OspA as it is one of the most abundant lipoproteins expressed on the surface of *B. burgdorferi* during *in vitro* cultivation (12, 148). There is a precedence for Osp proteins masking or shielding other antigens on the surface of *B. burgdorferi* (23). It is also possible that another protein would hide surface exposed VlsE from antibody recognition due to steric hindrance, but not proteinase K digestion. This masking protein (protein X or OspA) could be downregulated within the feeding tick or during host colonization, thus allowing for VlsE to be exposed to the host immune system.

Other groups have argued that surface lipoproteins may have limited surface exposure or low expression *in vitro* (37, 63), and VlsE may fall into this category. Surface lipoproteins could be tethered to the inner leaflet of the outer membrane (37, 63), or exist within an intracellular pool. The mechanism for regulating surface exposure is unknown. We favor a masking protein model to explain our data. Our IFA, transmission EM, and proteinase K digestion experiments were conflicting, and we were unable to resolve which mechanism may be occurring.

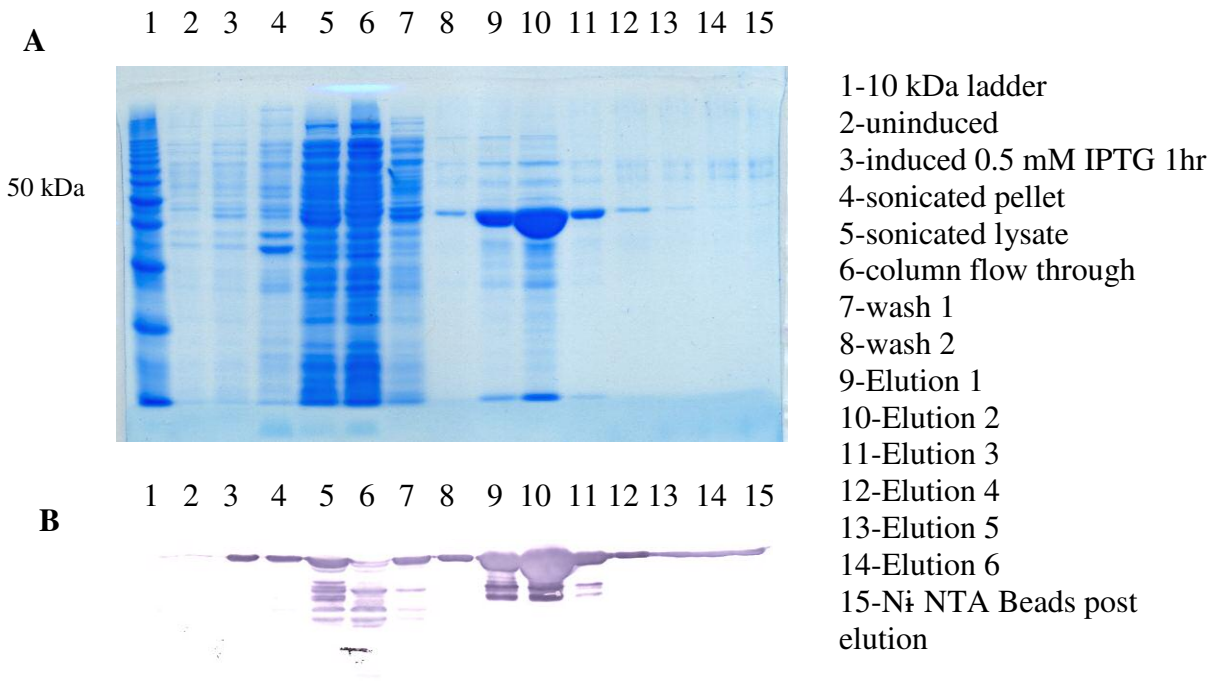

Figure 3.1 Creation of recombinant VlsE protein in *E. coli*. A. Simply blue stained 12% resolving SDS-PAGE gel of rVlsE. B. Western blot using rabbit anti-C6 VlsE antibody. As only 1 band is detected in lanes 3, 4, 7, and 8, we determined that there was no cross reactivity of the antibody with the *E. coli* lysate and that protein bands underneath main protein band are rVlsE degradation products. Lanes indicated on the right are same for both A and B, rVlsE is approximately 48 kDa.

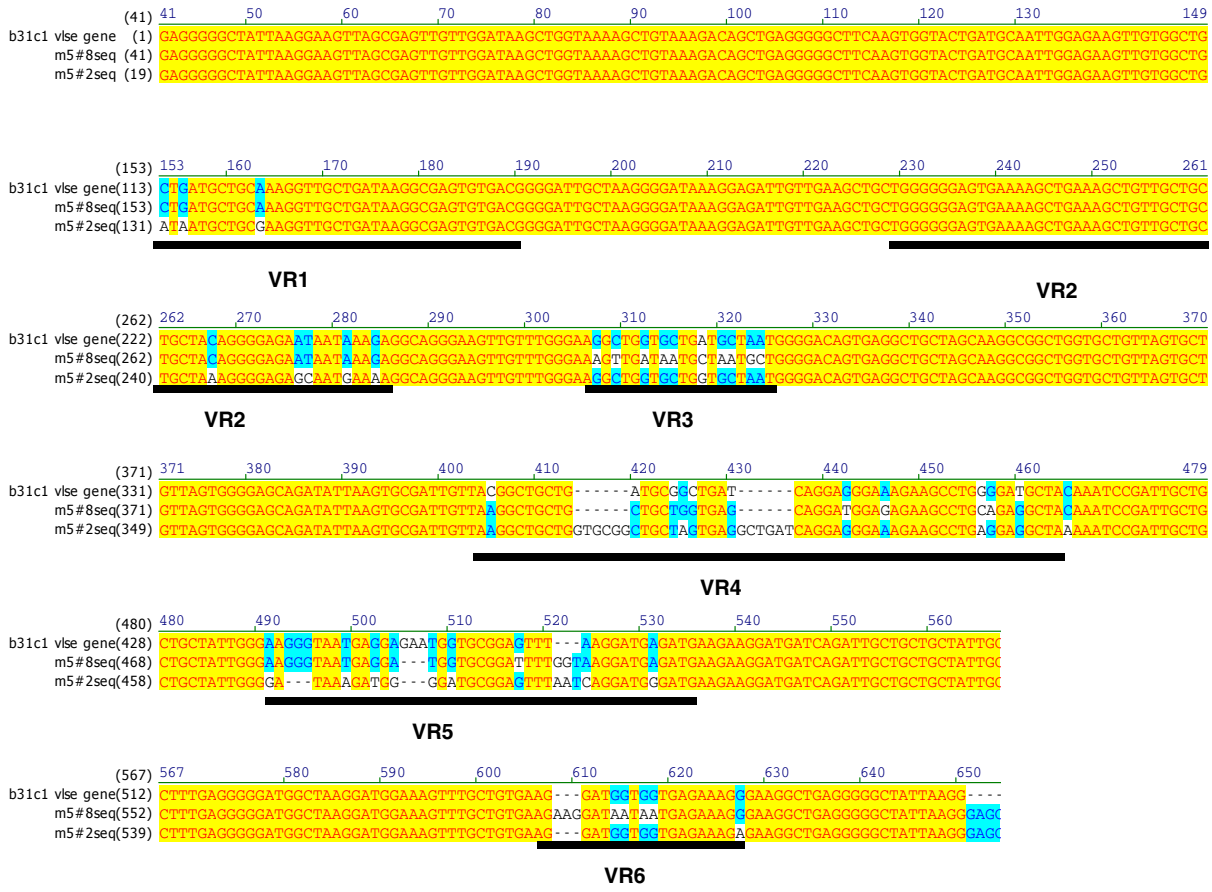

Figure 3.2 Alignment of variant *vlsE* alleles. Two *B. burgdorferi* clones isolated from an infected mouse are shown aligned with the parental strain, B31-C1. Variable regions are underlined.

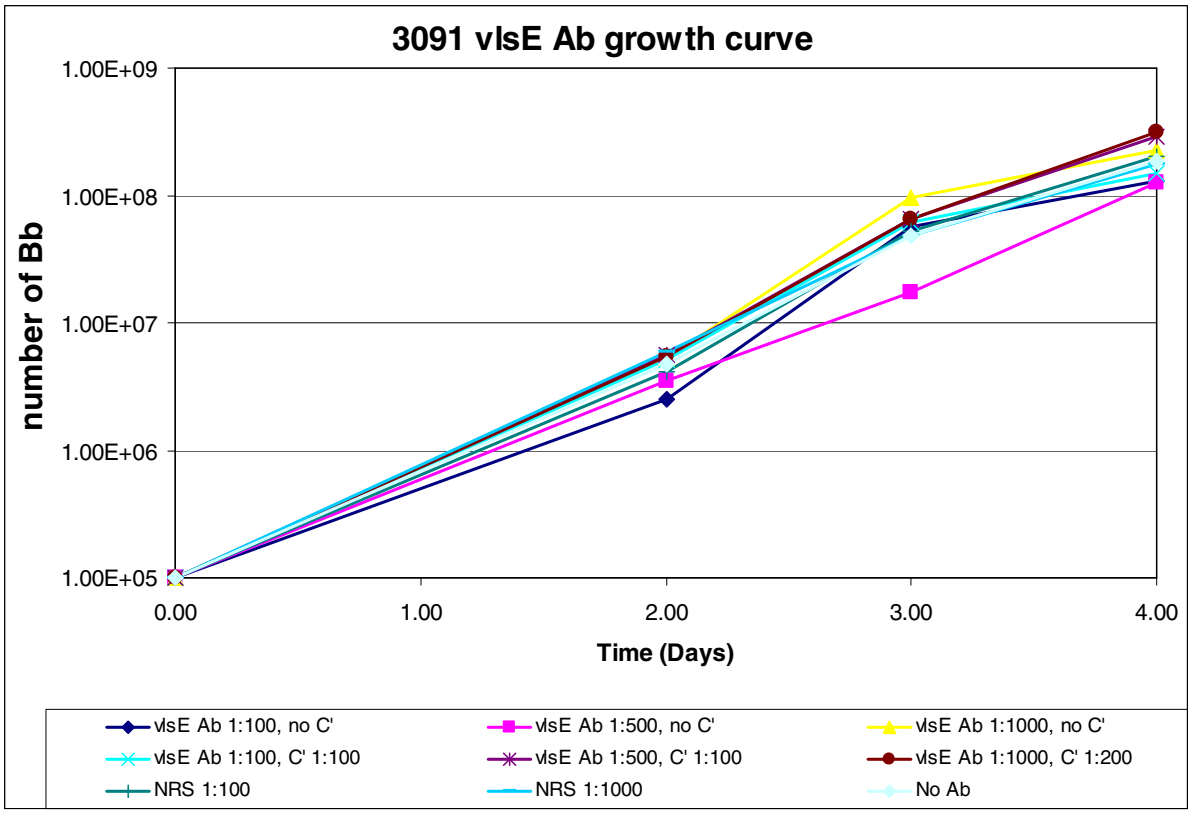

Figure 3.3 Borreliacidal assays with rabbit polyclonal anti B31-C1 VlsE serum. Various dilutions of polyclonal rabbit anti-B31-C1 rVlsE serum were mixed with 1 x 10^5 B31-C1 *B. burgdorferi* with various dilutions of rabbit complement in BSK-H media. Cultures were followed for 4 days post-incubation with the antibody.

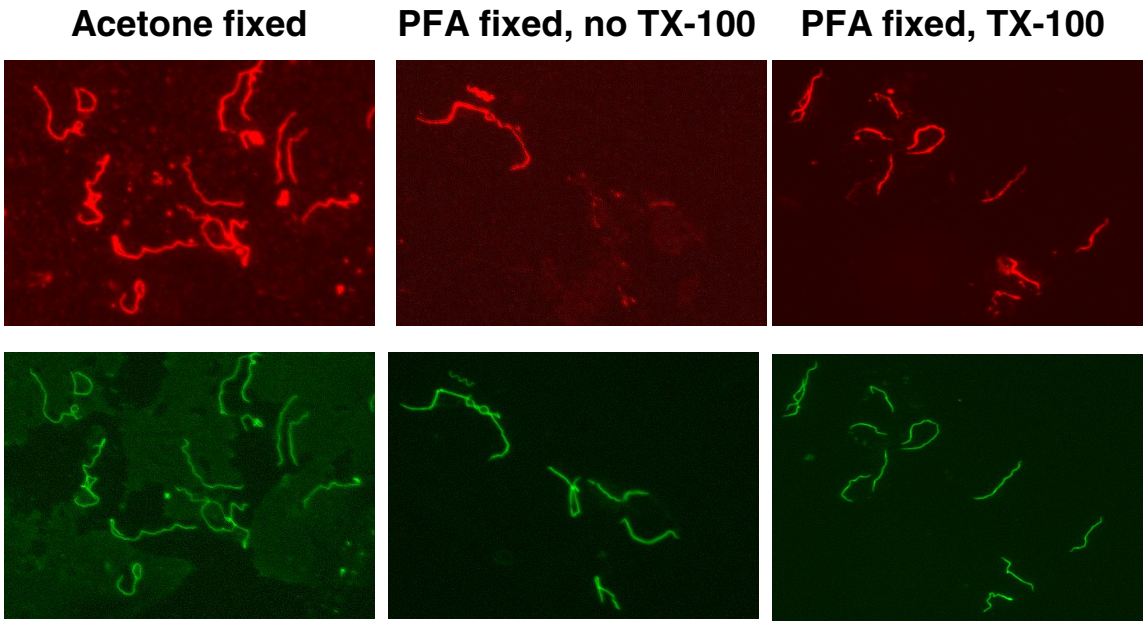

Figure 3.4 Acetone vs. PFA fixation IFA. *B. burgdorferi* B31-C1 were grown to mid-exponential phase in BSK-H media and stained with rabbit polyclonal anti-VlsE sera. The top row of panels is VlsE expression, detected with goat anti-rabbit alexa 594 antibody. The bottom row is goat anti-Borrelia FITC antibody. The number underneath the figure represents the percentage VlsE positive cells in each condition.

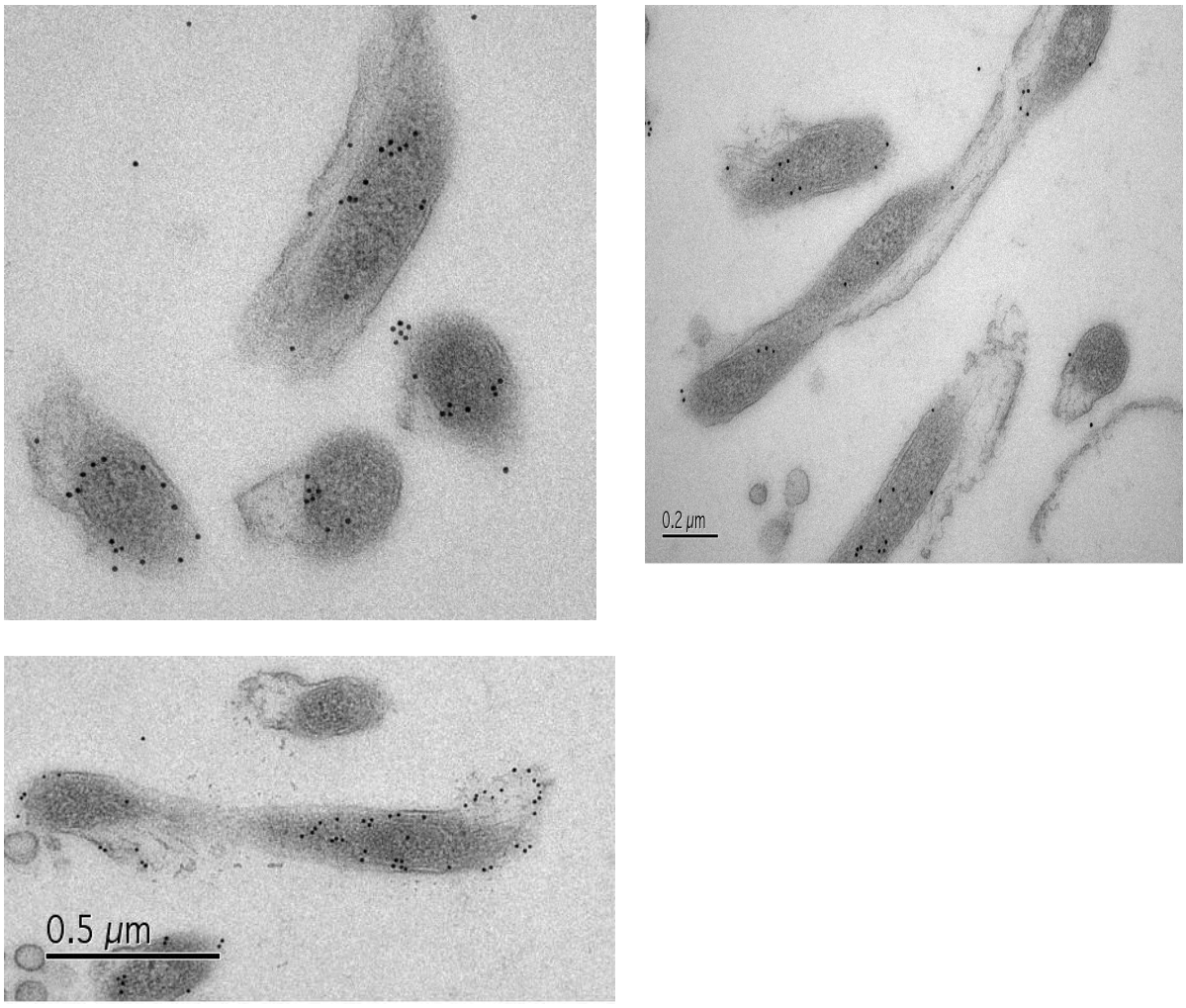

Figure 3.5 Transmission electron micrographs of VlsE. All 3 pictures represent VlsE cellular localization detected by rabbit polyclonal anti-VlsE serum and anti-rabbit secondary antibody conjugated to colloidal gold. Notice inner and outer membrane staining, and location of periplasmic flagella.

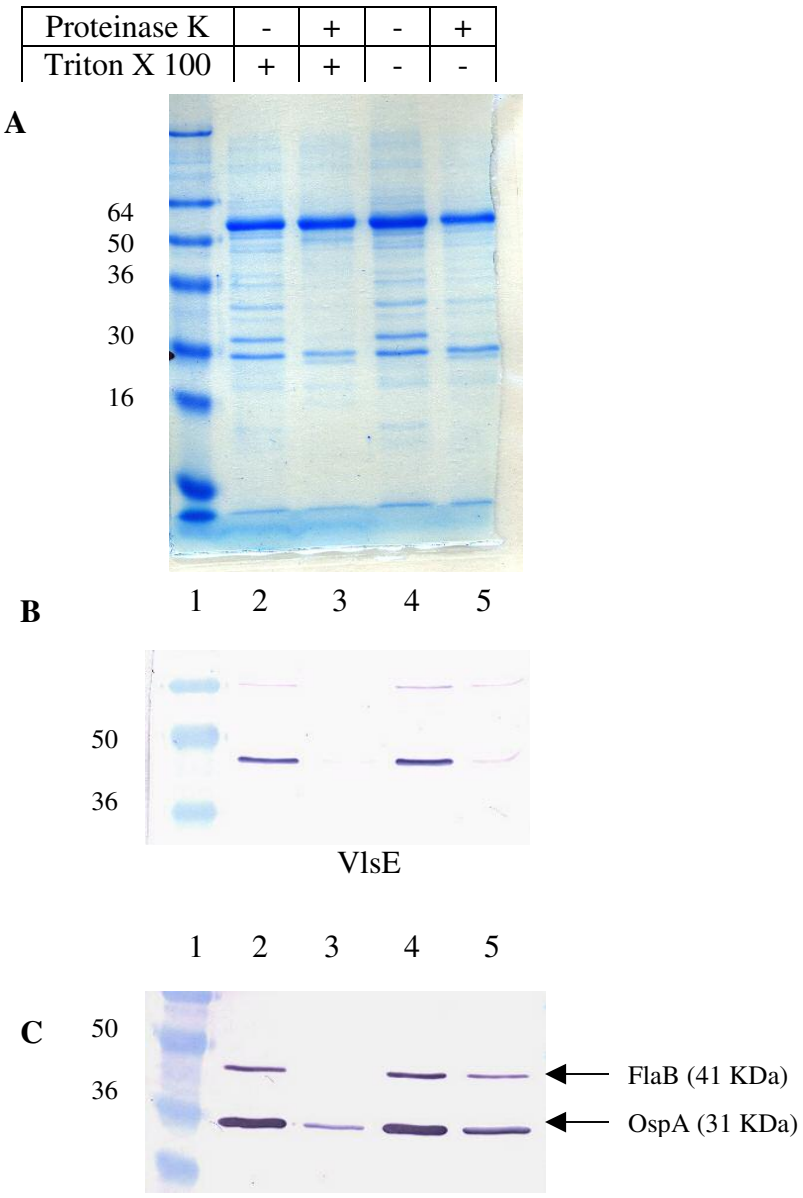

Figure 3.6 VlsE Proteinase K experiments. A. Simply Blue stained 10% SDS-PAGE gel. Lane 1 is See Blue molecular weight standards in KDa on left. Band above 64 KDa is carry-over bovine serum albumin from BSK-H. B. Western blot with rabbit anti-VlsE antibody. C. Western blot with mouse anti-FlaB (upper band) and mouse anti-OspA (lower band) antibodies. Lanes are same for A, B, and C, and indicated above.

Chapter 4: Discussion and Perspectives

Lyme disease is a good example of an emerging disease where not much is understood about how the bacterium causes disease. In addition, as it is the most prevalent vector-borne disease in the United States, it is important to understand how the bacterium, *B. burgdorferi* is able to colonize both the vector and the host. Changes in gene expression between the vector and host lead to changes in the antigenic profile that is displayed to the host's immune system. Many vector-borne pathogens, including *B. burgdorferi*, are able to undergo antigenic variation at a specific locus through non-reciprocal gene conversion events with silent gene cassettes. The experiments described in this thesis were aimed at understanding recombination in the tick vector and surface localization of a specific lipoprotein, VlsE.

Variant *vlsE* alleles can be rapidly detected within the mammalian host shortly after infection, but not in cultured spirochetes. One of our goals was to determine whether recombinants could be detected within a tick transmission cycle. Our initial hypothesis was that we would be able to detect *vlsE* recombinants within feeding nymphs, the tick stage responsible for transmission of*B. burgdorferi* to a naïve host; however, we found that this is not what happens. We used the larval immersion technique to infect larval *I. scapularis* ticks with a clonal population of *B. burgdorferi.* We were unable to detect changes within the variable region of *vlsE* through a larval tick feeding, through the morphological changes during the tick molt, and through the subsequent nymphal feeding (**Chapter 2**). Recombinants could readily be detected in the infected mouse. This indicates that the *vlsE* locus is stable in feeding ticks. This also would indicate that there is not a role for recombination within feeding ticks. The fact that VlsE protein is expressed at low levels in the tick and high levels within the host supports the role for VlsE in host immune evasion.

We were also interested in trying to understand the mechanism of recombination interacting with the host immune response. We presented two models: an active model, and a passive model. The active model asserts that recombination at the *vlsE* locus is actively being turned on specifically in response to mouse colonization. The passive model asserts that recombination frequency does not change during mouse colonization, only that selection is occurring in the presence of specific antibodies. While we were successful in creating the necessary reagents needed to try and test these two models, we were unable to figure out a frequency of recombination. Teasing out the differences between the two models is also not as simple as we have stated. There are many variables involved within a host that are difficult to control and that confound easy interpretation of data from experiments. Our polyclonal antiserum was not bactericidal for Borrelia grown in culture (**Chapter 3**), and therefore we were unable to look for minor allele variants that may exist within the population.

Because our serum was not bactericidal, wequestioned the cellular location of VlsE within the bacterial cell. While our immuno-microscopy experiments indicated that VlsE was intracellular, protease digestion experiments indicated that VlsE was indeed on the surface (**Chapter 3**). These conflicting results raised several questions about possible masking proteins, limited surface exposure of VlsE, or low levels of expression *in vitro*. It is difficult to look at bacteria within host tissues, and unfortunately we have to use a culture amplification step to be able to get substantial spirochetes to do experiments.

The next step to understand VlsE surface localization would be to use high resolution microscopy such as transmission electron microscopy using the same polyclonal sera with protease digested spirochetes, to see if there is a masking protein. Another idea that is in

progress is to develop monoclonal antibodies to *B. burgdorferi* B31-C1 VlsE in hopes of finding one that is bactericidal and specific to the variable region of the protein. This reagent could be used in experiments to try and determine a recombination frequency, or used in mouse immunization studies. Finally, it is possible that VlsE may be differentially expressed in response to pH and temperature, and both parameters are easily manipulated during lab culture conditions. Therefore, surface exposure and antibody accessibility may be dependent on pH and temperature and still needs to be tested.

Several questions in regard to VlsE function in the host still remain, such as is VlsE involved in colonization of specific niches (i.e. joints, heart, attachment to host extracellular matrix proteins)? Because *B. burgdorferi* colonizes the tissues and does not stay in the blood like relapsing fever *Borreliae*, it is possible that changes in the variable region provide multiple functions for the bacteria. Finally, is immunization to one allele of VlsE protective against that allele? Is it possible to design a multi-valent vaccine composed of several diverse VlsE sequences that protects against subsequent challenge with spirochetes? Basic mechanism questions also still exist, such as is recombination RecA dependent as it is in *Neisseria*? Or does another recombinase exist specifically for DNA conversion events at the *vlsE* locus? Why do the upstream cassettes have to be in the *cis* orientation in *B. burgdorferi*, while they are scattered throughout the genome in relapsing fever spirochetes? As the ability to manipulate genes within Borrelia is rapidly progressing, some of the answers to these questions will soon be addressed.

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