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Analysis of the role of outer surface protein C (OspC) in Borrelia burgdorferi pathogenesis

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ANALYSIS OF THE ROLE OF OUTER SURFACE PROTEIN C (OSPC) IN *BORRELIA BURGDORFERI* PATHOGENESIS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
B. afzelii	*Borrelia afzelii*
B. burgdorferi	*Borrelia burgdorferi*
B. garinii	*Borrelia garinii*
B. hermsii	*Borrelia hermsii*
B. recurrentis	*Borrelia recurrentis*
BCA	bicinchoninic acid assay
BN-PAGE	blue native polyacrylamide gel electrophoresis
BSA	bovine serum albumin
BSK	Barbour-Stoenner-Kelly
C130	cysteine residue at position 130
cm	centimeter
C terminus	carboxyl terminus
cp9	9 kilobase circular plasmid
cp26	26 kilobase circular plasmid
dPBS	Dulbecco's phosphate buffered saline
DMC	dialysis membrane chamber
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
FlaB	flagellin B
IFA	immunofluorescence assay
IgG	immunoglobulin G
IgM	immunoglobulin M
IPTG	isopropylthio-β-D-galactoside
GTG	genetic technology grade
kbp	kilobase pair
kDa	kilodalton
kg	kilogram
LB	lysogeny broth
LIC	ligase-independent cloning
lp5	5 kilobase linear plasmid
lp21	21 kilobase linear plasmid
mg	milligram
mL	milliliter
mM	milimolar
mmol	milimole
N terminus	amino terminus
ng	nanogram
OD	optical density

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Tween 20
PCR	polymerase chain reaction
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
UV	ultraviolet
V	volts
µg	microgram
µM	micromolar
µL	microliter
°C	degree Celcius

Abstract

ANALYSIS OF THE ROLE OF OUTER SURFACE PROTEIN C (OSPC) IN *BORRELIA BURGDORFERI* PATHOGENESIS

By DeLacy V. L. Rhodes, PhD

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: Dr. Richard T. Marconi
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Lyme disease is an emerging infection that is caused by the *Borrelia burgdorferi* sensu lato complex. These bacteria exist in nature through an enzootic life cycle involving *Ixodes* ticks and various reservoir hosts. One way that this bacterium adapts to the different hosts in the enzootic cycle is through the expression of outer surface protein C (OspC). OspC is a surface exposed lipoprotein encoded on circular plasmid 26 that forms homodimers on the bacterial surface and has distinct conserved and variable portions of sequence. When *ospC* is deleted, the spirochetes are unable to cause mammalian infection although the mechanism of this is unknown. Additionally, OspC is thought to be involved in reservoir host specificity/association and in tissue

dissemination. In order to better understand the functional domains of OspC, the different conserved and variable portions of this protein were investigated.

Three conserved portions of OspC were investigated: (1) the conserved cysteine residue at position 130 (C130), (2) the last ten C-terminal amino acids (C10), and (3) ligand binding domain 1 (LBD1). The C130 residue was mutated and this substitution disrupted OspC oligomerization in vitro and in vivo. A *B. burgdorferi strain* lacking the C10 retained full infectivity and plasminogen binding. The mutation of a single residue within LBD1 rendered *B. burgdorferi* noninfectious, indicating the importance of this domain in infection establishment.

The variable portion of OspC was investigated by: (1) altering the surface charge of ligand binding domain 2 (LBD2), (2) inserting different OspC types into a constant genetic background, and (3) creating OspC hybrids. Alteration of the surface charge of LBD2 by site directed mutagenesis resulted in a lack of persistence in mice. By inserting an OspC type known to be noninfectious in mice into an infectious strain, infectivity was abolished. Strains expressing OspC hybrids indicated that multiple domains of OspC are involved in species specificity.

Together these analyses demonstrated that OspC is as important protein that plays multiple roles in pathogenesis. The work presented here helps to increase the understanding of this crucial protein and the strains described can be used to decipher the full function of OspC.

Chapter 1: Introduction

1.1 Lyme disease

1.1.1 History

Lyme disease, an emerging zoonotic infection, was recognized as a clinical disease in North America in 1977 (Steere *et al.*, 1977a; Steere *et al.*, 1977b; Steere *et al.*, 1978). The unusually large occurrence of cases of arthritis in children in Lyme, Old Lyme, and East Haddam, Connecticut during this time led to a large epidemiological study to identify the cause of this disease. In 1982, a tick-borne spirochete was discovered by Willy Burgdorfer, Allen Steere and Jorge Benach. Dr. Burgdorfer was able to isolate these previously undescribed spirochetes from hard-body *Ixodes* ticks from Shelter Island, New York while Drs. Steere and Benach were able to isolate the spirochetes from patients exhibiting symptoms of Lyme disease, implicating the spirochetes as the causative agent (Burgdorfer *et al.*, 1982; Steere *et al.*, 1983). The newly discovered spirochete was able to be cultured in media that had been developed for other spirochete species and was found to belong to the genus *Borrelia*. This spirochete was eventually designated *Borrelia burgdorferi* (Johnson *et al.*, 1984). Polymerase chain reaction (PCR) was used retrospectively on preserved tissue samples and showed that *B. burgdorferi* was a distinct clinical entity that could be

detected in samples dating as far back as 1894 (Marshall *et al.*, 1994; Persing *et al.*, 1990). Similar symptoms of Lyme disease had been noted during the 20th century in Europe. Shortly after the discovery of *B. burgdorferi* in North America these spirochetes were also recognized as a cause of Lyme disease in Europe followed by the discovery of the other Lyme spirochete species *B.afzelii* and *B.garinii*.

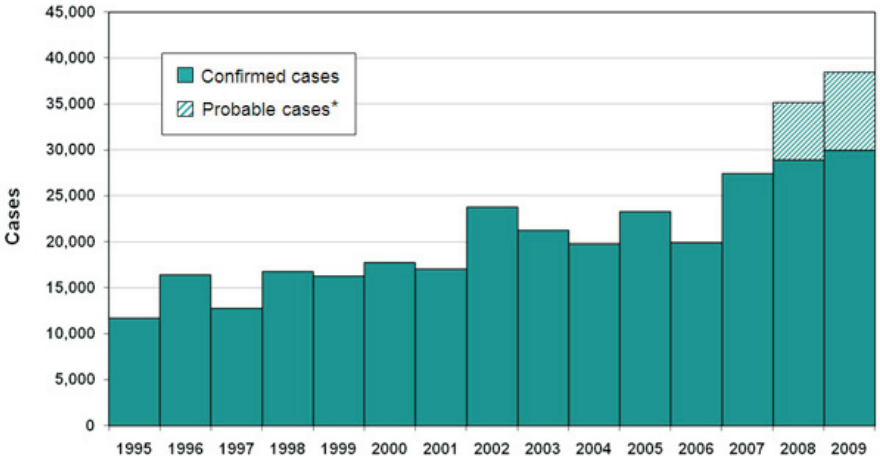
1.1.2 Epidemiology

Lyme disease is the most commonly reported vector-borne disease in North America and Europe. According to the Centers for Disease Control and Prevention, approximately 30,000 cases of Lyme disease were reported in 2009, although the actual number of cases is thought to be much higher due to lack of reporting and correct diagnosis (Yound, 1998) (Figure 1A). Since the identification of the causative agent of Lyme disease in 1982, the number of cases has steadily risen each year. Cases of Lyme disease in the United States are concentrated to the northeast with the states of Delaware, Connecticut, New Jersey, Massachusetts, Pennsylvania, and New York having the highest per capita incidences (McNabb *et al.*, 2007). In addition to the northeastern states, the midwestern states of Wisconsin and Minnesota also report a high number of Lyme disease cases (Figure 1B). Lyme disease exhibits a bimodal age distribution with the peak ages being 5-9 and 55-59 years old. There is no gender predilection of Lyme disease cases. Cases of this disease usually occur between June and August when the nymphal ticks feed and people are most often to engage in outdoor activities (Bacon *et al.*, 2008).

1.1.3 Clinical disease

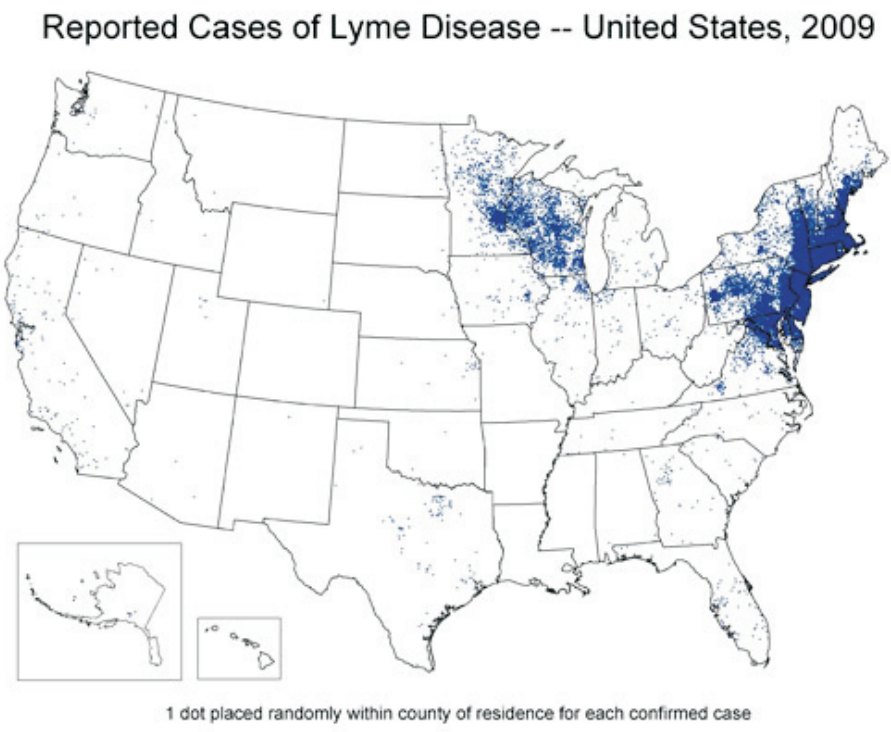
Figure 1. Incidence and number of Lyme disease cases in the United States. (A) Lyme disease case numbers from 1995-2009. Shown are the number of cases reported to the Centers for Disease Control and Prevention. (B) Lyme disease incidence map. Each spot on the map corresponds to one reported case. The source of each figure is given below.

A



http://www.cdc.gov/lyme/stats/chartstables/casesbyyear.html

B



http://www.cdc.gov/lyme/stats/index.html

The bacteria that cause Lyme disease are transmitted primarily by the nymphal stage of infected *Ixodes* ticks. The ticks must feed on their host for approximately 48 hours in order for the spirochetes to migrate into the host tissue (Piesman, 1993). Once the spirochetes enter mammalian skin, they disseminate laterally from the site of the tick bite, resulting in the pathognomonic skin rash, erythema migrans (EM) (Afzelius, 1910). This "bull's eye" rash is called so due to a central zone of clearing that occurs in some patients. The EM commonly occurs at the site of tick bite but in some patients this rash can occur on other parts of the body or not at all. Other symptoms of early Lyme disease include fever, headache, arthralgia, regional lymphadenopathy, and malaise (Nadelman and Wormser, 1995). If left untreated, Lyme disease can present with late stage sequelae such as arthritic, cardiac, neurological, and/or dermatological symptoms. These different late stage symptoms can be debilitating and tend to associate with different species of Lyme *Borrelia*. Severe dermatological symptoms are associated with *B. afzelii* while arthritic conditions occur commonly with *B. burgdorferi* infection and neurological complications occur more frequently with *B. garinii* (Balmelli and Piffaretti, 1995; Busch *et al.*, 1996). According to the Centers for Disease Control and Prevention, Lyme disease is usually diagnosed based on the presence of the erythema migrans. Serological tests and PCR can also be used but the results are unreliable (Murray and Shapiro, 2010). Lyme disease is treated with antibiotics although some controversy exists over the duration of antibiotic treatment and the route of antibiotic administration (Feder *et al.*, 2007; Reid *et al.*, 1998; Stricker, 2007). This disease is typically treated with a 2-4 week course of the antibiotic doxycycline and when treated early, is generally responsive to this treatment. Alternative antibiotics

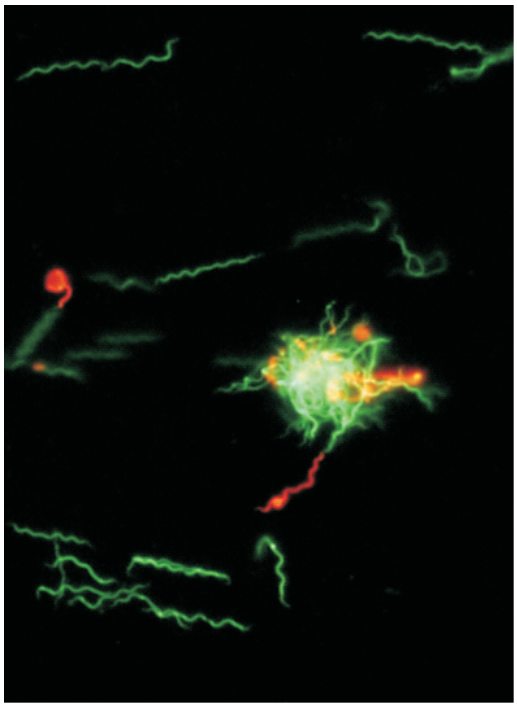
include amoxicillin and ceftriaxone (Wormser *et al.*, 2006). Currently no vaccine for this disease is available for human use and the sole method of prevention is limiting exposure to the ticks that transmit the infection.

1.2 *Borrelia*

1.2.1 General characteristics

The genus *Borrelia* is made up of spirochetes that are helically shaped, motile, and have two sets of periplasmic flagella in between the inner and outer membranes that are attached to each end of the periplasmic cylinder (Barbour and Hayes, 1986) (Figure 2). Although the *Borrelia* are said to be Gram-negative organisms based on the presence of inner and outer membranes, they lack lipopolysaccharide (LPS) on their cell surface. The *Borrelia* are microaerophilic and cultivated in a complex culture medium supplemented with rabbit serum. These spirochetes need a rich medium and are completely host-dependent due to the fact that they lack most de novo amino acid, lipid, nucleotide, and enzyme cofactor synthesis (Fraser *et al.*, 1997). Optimal growth temperatures are between 33° and 37°C. These spirochetes can be easily viewed using dark-field microscopy and typically range from 0.18 to 0.25 μm in diameter and 4 to 30 μm in length (Barbour, 1984; Ruzic-Sabljic *et al.*, 2006). Members of this genus are primarily enzootic and are able to cause several different diseases including Lyme disease, epizootic bovine abortion, relapsing fever, and avian borreliosis, though only Lyme disease and relapsing fever affect humans. There are two different forms of relapsing fever, louse-borne relapsing fever caused by *B. recurrentis* and tick-borne relapsing fever caused by *B. hermsii* and others. Lyme disease in humans is caused by

Figure 2. Fluorescent micrograph of *B. burgdorferi*. The characteristic helical shape of the spirochetes can be seen. Spirochetes were stained using BacLight Live/Dead fluorescent stain. Live spirochetes stain green while dead spirochetes are red.



three *Borrelia* species; *B. burgdorferi*, *B. afzelii*, and *B. garinii*. *B. burgdorferi* was the first species to be associated with Lyme disease and can be found worldwide while *B. afzelii* and *B. garinii* are only in Europe and Asia.

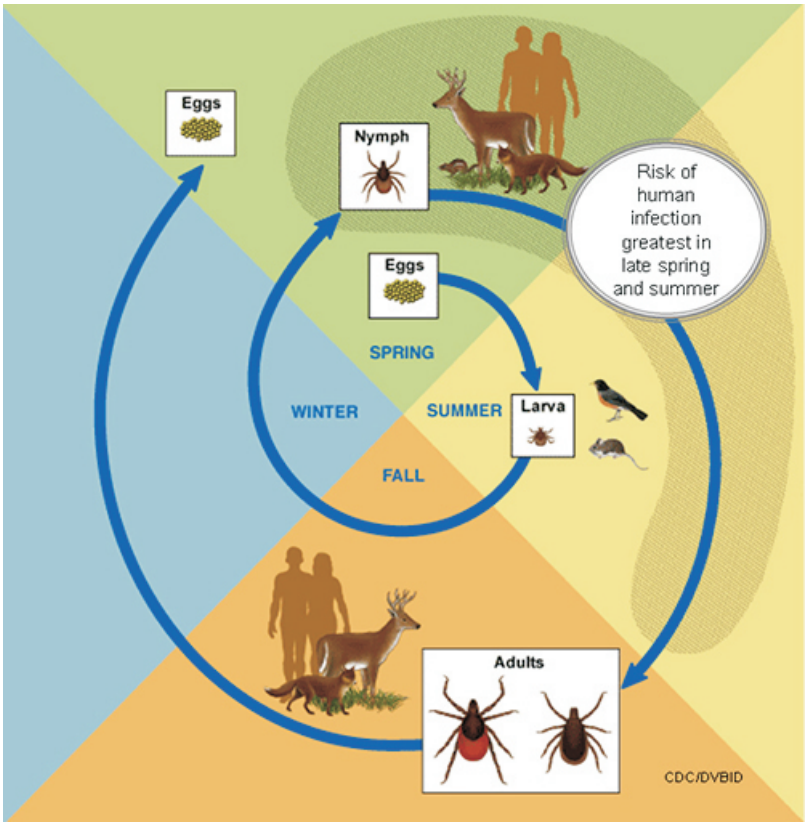
1.2.2 *Borrelia* genetics

The *Borrelia* have a very unique genome. All members possess a large linear chromosome with various circular and linear plasmids. The full genome sequence of the *B. burgdorferi* B31MI strain, a strain commonly used in the laboratory, was one of the first bacterial genomes to be determined (Fraser *et al.*, 1997). It was found that this strain contains 1516 open reading frames encoded on a 910 kbp linear chromosome, 12 linear, and 9 circular plasmids, some of which appear to be prophage (Casjens *et al.*, 2000; Eggers and Samuels, 1999; Eggers *et al.*, 2000; Fraser *et al.*, 1997; Zhang and Marconi, 2005). The plasmid content of different strains of *B. burgdorferi* can vary greatly (Iyer *et al.*, 2003; Terekhova *et al.*, 2006) and can change between in vitro and in vivo cultivation (Barbour, 1988; McDowell *et al.*, 2001; Persing *et al.*, 1994; Schwan *et al.*, 1988). Some plasmids are said to be universal and can be found in all *B. burgdorferi* isolates (e.g., cp26) while others plasmids are required for infectivity but not in vitro cultivation (e.g., lp25) (Byram *et al.*, 2004; Grimm *et al.*, 2004; Labandeira-Rey and Skare, 2001; Labandeira-Rey *et al.*, 2003; Marconi *et al.*, 1993a; Purser and Norris, 2000; Tilly *et al.*, 1997). Several of the genes encoded on these plasmids are house-keeping genes, but the majority of them are unique genes with unknown function (Barbour and Garon, 1987; Barbour and Garon, 1988).

1.2.3 Enzootic life cycle

The *Borrelia* are completely host-dependant and persist in nature through a complex enzootic life cycle. For the Lyme disease spirochetes, this life cycle involves ticks of the *Ixodes ricinus* complex and a wide variety of animals that serve as reservoir hosts (Brown and Lane, 1992; Gern *et al.*, 1998; Keirans *et al.*, 1996). In the northeastern and midwestern portions of North America, *Ixodes scapularis* (previously *I. dammini* (Oliver *et al.*, 1993)) is the primary tick vector while *I. pacificus* vectors the spirochetes in the western United States (Lane *et al.*, 1991). *Ixodes ricinus* is the primary arthropod vector for species of *Borrelia* in Europe while *I. persulcatus* and *I. nipponensis* serve as vectors in Asia (Hengge *et al.*, 2003). Due to the fact that there is no transovarial transmission of the Lyme disease spirochetes (Barbour and Hayes, 1986) animal reservoirs are necessary for maintenance of these bacteria in nature (Figure 3). When larval ticks hatch out of eggs during the spring, they immediately begin questing after a blood meal, which they usually take on a small mammal or bird. It is often during this first blood meal that the ticks acquire a *Borrelia* infection. After feeding to repletion the larvae molt into the nymphal stage and become dormant through the winter. When the infected nymphs emerge in the late spring or early summer of the next year, they will take a blood meal on another animal, possibly transmitting the spirochetes. There is a wide range of competent reservoir hosts including many species of small mammals and birds that can become infected during a blood meal, a major one in North America being the white-footed mouse *Peromyscus leucopus* (Gern *et al.*, 1998; Keirans *et al.*, 1996; Steere *et al.*, 2004). After this blood meal the ticks will molt into adults and feed on larger mammals such as deer. These larger mammals are important in the enzootic cycle due to the fact that they can harbor

Figure 3. Enzootic life cycle of *B. burgdorferi*. Shown is the life cycle of the tick vector, *Ixodes scapularis*. Spirochetes cycle through *Ixodes* tick vectors and animal reservoir hosts.



http://www.cdc.gov/lyme/transmission/blacklegged.html

greater numbers of tick vectors and aid in spreading them over larger areas. Humans often contract the Lyme spirochetes through an encounter with infected nymphal ticks but, due to the fact that they do not aid in the progression of the enzootic cycle, are considered incidental hosts.

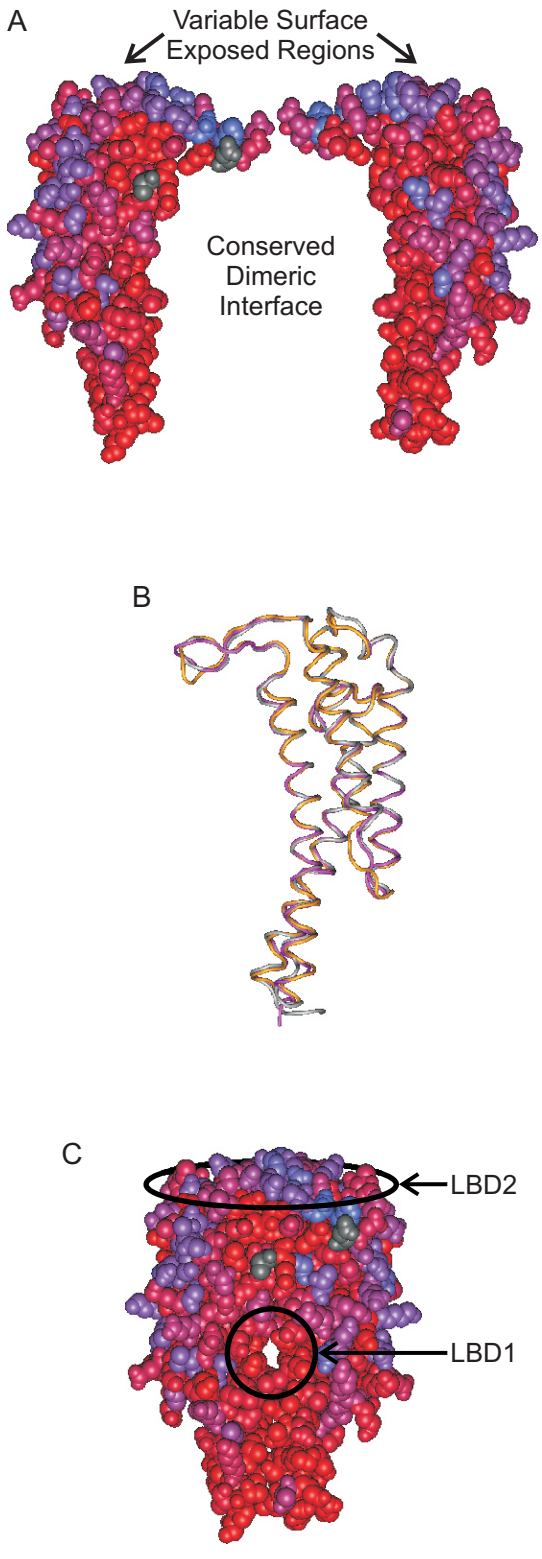
All the different hosts encountered by the Lyme *Borrelia* during their enzootic life cycle present a variety of different environments to which the spirochetes must adapt. One way that these bacteria are able to do this is through differential expression of many outer surface lipoproteins (Boardman *et al.*, 2008; Brooks *et al.*, 2003; Caimano *et al.*, 2007; Revel *et al.*, 2002; Rogers *et al.*, 2009). A well characterized example of this is the reciprocal expression of outer surface protein A (OspA) and outer surface protein C (OspC).

1.3 Outer Surface Protein C (OspC)

1.3.1 General characteristics

Outer surface protein C (OspC) is an approximately 22 kDa surface exposed lipoprotein (Fuchs *et al.*, 1992) that is expressed by all of the Lyme *Borrelia*. This protein is encoded on the universal plasmid cp26, which is a highly stable plasmid present in all strains (Marconi *et al.*, 1993a; Sadziene *et al.*, 1993). The crystal structure of OspC had been determined and shows that this protein is primarily alpha helical in structure, containing five alpha helices that are connected by six loops (Eicken *et al.*, 2001; Kumaran *et al.*, 2001b). Additionally, OspC forms homodimers (Figure 4A) that are tethered to the outer membrane by an N-terminal tripalmitoyl-S-glyceryl-cysteine (Brooks *et al.*, 2006). These homodimers are held together by hydrophobic

Figure 4. Structure of OspC, conservation of residues, and location of putative ligand-binding domains. (A) Two OspC monomers are shown with the conserved to variable residues indicated using a red to blue scale. (B) A VAST alignment is presented, using CN3D 4.1 of type A in purple (1GGQ), type I in yellow (1F1M), and type E in gray (1G5Z). (C) The OspC dimer is shown with the two putative ligand binding domains, LBD1 and LBD2, indicated. Amino acid conservation is shown as in (A).

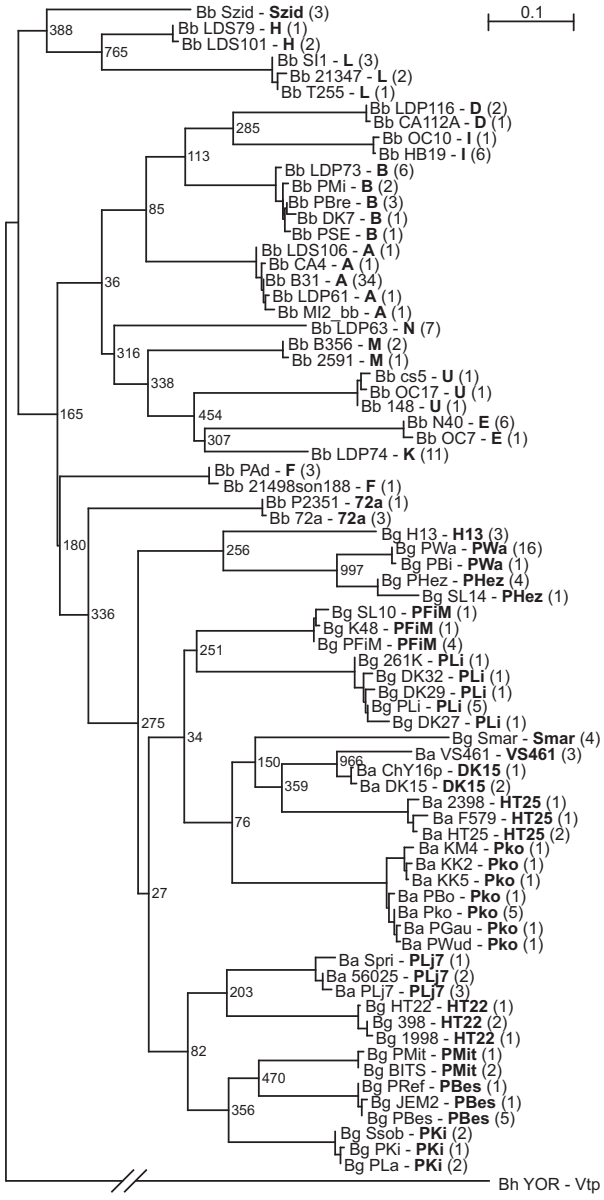


interactions between the amino acids at the dimeric interface and are thought to be the biologically active form of the protein, although higher order oligomers of this protein may occur (Eicken *et al.*, 2001; Zuckert *et al.*, 2001). Expression of OspC is upregulated by spirochetes residing in the midgut of ticks when they take a blood meal. During this time, spirochetes migrate from the midgut to the salivary glands of the ticks and enter the host. OspC remains expressed by spirochetes in the mammal for the first few weeks of infection before it is downregulated (Fingerle *et al.*, 1995; Masuzawa *et al.*, 1994; Schwan and Piesman, 2000). Numerous studies have implicated the expression of OspC with the ability of the spirochetes to cause mammalian infection and it has been shown that mutant strains of the Lyme *Borrelia* lacking OspC are unable to cause infection by either the tick or needle inoculation route (Earnhart *et al.*, 2010; Fingerle *et al.*, 2007; Gilmore and Piesman, 2000; Grimm *et al.*, 2004; Pal *et al.*, 2004; Tilly *et al.*, 2006; Tilly *et al.*, 2007).

1.3.2 Phylogenetics

OspC is highly divergent in sequence. Over 30 different OspC phyletic types have been identified (Earnhart and Marconi, 2007a; Seinost *et al.*, 1999; Wang *et al.*, 1999) (Figure 5). Within an OspC type, amino acid sequences are >95% identical, whereas between OspC types, identity ranges from 55-80% (Attie *et al.*, 2007; Earnhart and Marconi, 2007a; Wang *et al.*, 1999). The sequence divergence that gives rise to the different phyletic types is located in distinct, surface-exposed domains of the protein (Earnhart *et al.*, 2005; Earnhart *et al.*, 2007) (Figure 4A). Even though there is such

Figure 5. Phylogenetic tree of OspC type from the Lyme *Borrelia*. This phylogenetic tree was generated by aligning the *ospC* residues 20 – 200 (lacking the signal sequence) using Clustal X (http://www.clustal.org/) and NJ Plot (http://pbil.univ-lyon1.fr/software/njplot.html). Bootstrap values (1,000 trials) are shown at the nodes for critical differentiation.



sequence diversity between OspC types, these differences do not create structural differences between types (Figure 4B). The domains that make up the variable portion of OspC are also highly antigenic during mammalian infection and elicit a type-specific humoral response. An individual strain only produces one OspC type which remains stable during infection; however it has been demonstrated that multiple OspC types can be found in tick, animal, and human samples from distinct geographic areas (Alghaferi *et al.*, 2005; Anderson and Norris, 2006; Earnhart *et al.*, 2005; Lin *et al.*, 2002; Wang *et al.*, 1999). Particular OspC types, specifically types A, B, I, and K, have been implicated in the development of invasive disease symptoms in humans (Lagal *et al.*, 2003; Seinost *et al.*, 1999) and further studies have suggested additional types which also show this association (Alghaferi *et al.*, 2005; Earnhart *et al.*, 2005). It has also been shown that certain OspC types tend to be commonly associated with different reservoir hosts (Brisson and Dykhuizen, 2004).

1.3.3 Potential structure/function determinants

While it has been shown that expression of OspC is crucial for mammalian infection, the actual function of this protein remains unknown. Although one study has proposed that OspC itself has no actual function other than providing membrane stability (Xu *et al.*, 2008), other studies have proposed that OspC plays multiple roles in the pathogenesis of the Lyme *Borrelia*. OspC may function in three distinct areas of *Borrelia* pathogenesis: establishment of mammalian infection, reservoir host species specificity/association, and tissue dissemination/ability to cause invasive disease (Alghaferi *et al.*, 2005; Brisson and Dykhuizen, 2004; Earnhart *et al.*, 2005; Lagal *et al.*, 2003; Seinost *et al.*, 1999). In the investigation of these different functions of OspC,

several potential ligands have been proposed for OspC, including Salp15 and plasminogen. Salp15, a tick-derived protein with immunomodulatory activity, is able to bind OspC in vitro (Anguita *et al.*, 2002; Das *et al.*, 2001; Hovius *et al.*, 2007). The interaction between these two proteins may serve to protect the spirochetes from antibody-mediated killing as they enter the host tissues (Hovius *et al.*, 2008; Ramamoorthi *et al.*, 2005) and aid in the establishment of mammalian infection; however, the ability of spirochetes to cause infection by the needle inoculation route indicates that Salp15-binding is not the necessary function of OspC required to establish infection. Another proposed OspC ligand is plasminogen. In other bacterial species, plasminogen binding has been shown to be important in tissue penetration and dissemination (Lagal *et al.*, 2006; Lahteenmaki *et al.*, 1998; Lahteenmaki *et al.*, 2001). OspC has been shown to bind plasminogen in vitro but the contribution of this interaction is not known due to the presence of other plasminogen-binding proteins expressed by the *Borrelia* (Brissette *et al.*, 2009; Coleman *et al.*, 1995; Grosskinsky *et al.*, 2009; Hovis *et al.*, 2008; Lagal *et al.*, 2006; Rossmann *et al.*, 2007; Seling *et al.*, 2009). In order to decipher the function(s) of this protein in the absence of a ligand, a novel approach must be employed.

1.4 Research Objectives

Several different portions of OspC have been theorized to play specific roles in this protein's many theoretical functions. The OspC dimer has two solvent-accessible domains that potentially could interact with a ligand(s). These domains have been denoted as ligand binding domain 1 (LBD1) and ligand binding domain 2 (LBD2) (Figure 4C). LBD1 is a pocket formed by the juxtaposition of alpha helix 1 and 1` of the two

opposing OspC monomers. The residues that make up this domain are highly conserved in sequence with some being invariant. LBD2 is located on the membrane distal 'crown' of OspC and contains the portion of OspC that gives rise to the different phyletic types (Earnhart *et al.*, 2010). In contrast to LBD1, the residues that make up LBD2 are highly variable. In addition to these two binding domains, other portions of OspC may also be important in its function. A cysteine residue at position 130 (C130) may play a role in the formation of inter-dimeric bonds, forming higher order OspC oligomers on the bacterial cell surface. Also the last ten amino acids (C10) of the C-terminal end of OspC are highly conserved in sequence and have been theorized to be important in plasminogen binding and/or formation of important structural components, (Lagal *et al.*, 2006; Mathiesen *et al.*, 1998b). Investigation of these different OspC domains and residues could help lead to a better understanding of the function of this critical *Borrelia* protein.

In this dissertation, the role of OspC in the establishment of mammalian infection, host species specificity/association, and tissue dissemination were investigated through the creation of many different OspC mutant strains of *B. burgdorferi*. The following regions of OspC and their role in OspC function investigated:

(1) C130 – OspC harbors two cysteine residues, the N-terminal cysteine that helps tether the protein to the cell surface and a cysteine at position 130. This residue was mutated to an alanine in order to assess the role of C130 in the formation of inter-dimeric bonds between OspC dimers on the cell surface, resulting in higher-order oligomers. Additionally, the in vivo significance of oligomer formation was assessed by infectivity and dissemination studies.

(2) C10 – The last ten amino acids of OspC are highly conserved and have been hypothesized to have a variety of functions. A mutant strain of *B. burgdorferi* was created in which these residues have been deleted. This strain was used to assess the role of these C-terminal residues in plasminogen binding, infectivity, and host immune response to B. *burgdorferi* infection.

(3) LBD1 – The high level of conservation of residues within LBD1 indicate that this region of OspC may play a role in the function of OspC that is necessary for infection establishment. Specific residues within LBD1 were chosen for site-directed mutagenesis in order to assess the role of this region of OspC in the ability of the spirochetes to establish a mammalian infection.

(4) LBD2 – The most amino acid variability of OspC occurs in the LBD2 region. This variability results in marked differences in electrostatic surface charge. Specific residues within LBD2 were chosen for site-directed mutagenesis in order to produce a more positive or negative charge on the LBD2 surface. The role of surface charge on this portion of OspC in dissemination and spirochete persistence was assessed.

(5) OspC type – Strains of *Borrelia* that express different OspC types also contain other differences in their genome. In order to assess the role of OspC type alone, whole *ospC* genes of different types were inserted into the same genetic background. These mutants were then used to assess the role of OspC type in host species association and infectivity.

(6) OspC type A/PKo hybrids – OspC types A (from *B. burgdorferi* B31-5A4) and PKo (from *B. afzelii* PKo) are expressed by strains that have been shown to have differing host species specificity. *B. burgdorferi* mutants were generated that express hybrids of OspC types A and PKo in order to assess the role of different regions of OspC in host specificity.

Chapter 2: Methods and Materials

2.1 *Borrelia* strains and nomenclature

All strains were cultured in Barbour-Stoener-Kelley (BSK) media supplemented with 6% rabbit serum in a 37°C incubator with 5%CO_2 (Table 1). Mutants generated for this study were named with a common nomenclature that includes: (1) the name of the parental strain (for all mutants in this document, the parental strain was *B. burgdorferi* strain B31-5A4) (2) the gene targeted for mutagenesis and/or allelic exchange (in all cases this gene was *ospC*), and (3) the amino acid, position, and substitution, OspC type, or OspC hybrid chosen for allelic exchange (Table 2).

2.2 Site-directed mutagenesis of *ospC*

Site-directed mutations were introduced into the type A-*ospC* gene derived from *B. burgdorferi* B31-5A4 by overlap extension using mutagenic primers (Table 3) as previously described (Hovis *et al.*, 2008). The following mutants were generated using this method: B31::*ospC* (E61Q/E63Q), B31::*ospC* (E61Q), B31::*ospC* (E63Q), B31::*ospC* (K60Y), B31::*ospC* (C130A), B31::*ospC* (N85G/D88N/E90Q), and B31::*ospC* (H82D/N84D/N92D). Polymerase chain reaction (PCR) was used to amplify the type A-*ospC* gene with the introduced amino acid change(s) using the mutagenic primers in

Table 1. Description of *Borrelia* isolates and strains

Species/Isolate	Source	Geographic Origin
B. burgdorferi		
B31MI (clone 5A4)	*Ixodes scapularis*	New York
DRI-83a	Dog skin	Rhode Island
DRI-40g	Dog skin	Rhode Island
LDS79	Human serum	Baltimore, MD
Veery	Veery bird	Connecticut
B. garinii		
PBi	Human CSF	Germany
B. afzelii		
PKo	Human Skin	Germany
B. andersonii		
MOD-1	*Ixodes dentatus*	Bollinger County, MO
MOK-3a	*Ixodes dentatus*	Bollinger County, MO
MOS-1b	*Ixodes dentatus*	Stodderd County, MO

Table 2. Description of *B. burgdorferi* allelic exchange mutants

Allelic Exchange Mutant	Vector	Missing Plasmids	Parental Strain
B31Δ*ospC*	pCR2.1		B31-5A4
B31Δ*ospC* kan^R	pCAEV3		B31-5A4
B31::*ospC* (wt)	pCAEV1	U	B31-5A4
B31::*ospC* (C130A)	pCAEV1		B31-5A4
B31::*ospC* (K60Y)	pCAEV1	U	B31-5A4
B31::*ospC* (E61Q/E63Q)	pCAEV1	U	B31-5A4
B31::*ospC* (E61Q)	pCAEV1		B31-5A4
B31::*ospC* (E63Q)	pCAEV1		B31-5A4
B31::*ospC* (type E)	pCAEV1	U	B31-5A4
B31::*ospC* (type F)	pCAEV3		B31-5A4
B31::*ospC* (type H)	pCAEV1		B31-5A4
B31::*ospC* (type M)	pCAEV3		B31-5A4
B31::*ospC* (type PBi)	pCAEV1		B31-5A4
B31::*ospC* (type PKo)	pCAEV1	T	B31-5A4
B31::*ospC* (type MOD1)	pCAEV1	T	B31Δ*ospC* kan^r
B31::*ospC* (type MOK3a)	pCAEV1		B31Δ*ospC* kan^r
B31::*ospC* (type MOS1b)	pCAEV1		B31Δ*ospC* kan^r
B31::*ospC* (H82D/N84D/N92D)	pCAEV1		B31-5A4
B31::*ospC* (N85G/D88N/E90Q)	pCAEV1		B31-5A4
B31::*ospC* (hybrid Aap)	pCAEV1		B31-5A4
B31::*ospC* (hybrid Apa)	pCAEV1	U	B31-5A4
B31::*ospC* (hybrid App)	pCAEV1		B31Δ*ospC* kan^r
B31::*ospC* (hybrid Paa)	pCAEV1		B31-5A4
B31::*ospC* (hybrid Pap)	pCAEV1	C, U	B31Δ*ospC* kan^r
B31::*ospC* (hybrid Ppa)	pCAEV1	U	B31Δ*ospC* kan^r
B31::*ospC* (Δ200)	pCAEV1	U	B31-5A4

conjunction with full length *ospC* primers (OspC LIC +/-) at a final concentration of 0.75 pmol μL^{-1}. These reactions were run using 10 ng of B31-5A4 genomic DNA (isolation described below) as template and Phusion Master Mix HF (Finnzymes) in 30 μL reactions in a MJ Research PTC-100 thermal cycler with the following conditions: 1 cycle of 98°C for 30 sec followed by 34 cycles of 98°C for 5 sec, 61°C for 10 sec, 72°C for 30 sec, and finally 1 cycle of 72°C for 4 minutes with a indefinite 4°C hold. The products from these reactions were then used as template for another round of PCR in 60 μL reactions using Phusion Master Mix HF and full length OspC LIC +/- *ospC* primers following the same parameters and conditions described above.

2.3 Ligase-independent cloning and sequencing of site-directed mutants

Site-directed mutant *ospC* genes were PCR amplified as described above. For the introduction of different *ospC* types into the B31-5A4 type A-*ospC* background, the desired types were PCR amplified from genomic DNA using full length gene primers corresponding to the correct sequence of the different types (Table 3). The following OspC types were chosen for allelic exchange into B31-5A4: (types A (from strain *B. burgdorferi* B31-5A4), E (*B. burgdorferi* DRI83a), F (*B. burgdorferi* DRI40g), H (*B. burgdorferi* LDS79), M (*B. burgdorferi* Veery), PKo (*B. afzelii* PKo), PBi (*B. garinii* PBi), MOD-1 (*B. andersonii* MOD-1), MOK-3a (*B. andersonii* MOK-3a), and MOS-1b (*B. andersonii* MOS-1b) (Table 2). Hybrid genes of OspC types A and PKo were synthesized by an independent company (GenScript). After PCR amplification, the full reactions were run out on a 1% GTG agarose gel and visualized with ethidium bromide. The bands corresponding to the size of *ospC* were excised from the gel and the PCR products purified using a Quiagen Gel Extraction kit. The *ospC* types, *ospC* hybrids,

Table 3. Oligonucleotide primers

Primer	Description	Sequence
pCAEV1 Upstream (+)	pCAEV1 vector construction	CAGAATGAGTTACTTCTGGATGG
pCAEV1 OspC 5' (-) BspEI/MluI	pCAEV1 vector construction	GTACGCGTTTTTCCGGAATTATTACAAGATATAAATA
pCAEV1 OspC 3' (+) BspEI	pCAEV1 vector construction	TCCGGATCAATATTATAAGATTAATTTGTTTTAAA
pCAEV1 BBB21 3' (-) AatII	pCAEV1 vector construction	GACGTCATCTCACATAAAACCAAAGAAACTAC
Spec/Strep (+) SalI	pCAEV1 vector construction	GTCGACTAATACCCGAGCTTCAAGGA
Spec/Strep (-) AatII	pCAEV1 vector construction	GACGTCATTATTTGCCGACTACCTTGG
pCAEV1 Downstream (+) SalI	pCAEV1 vector construction	GTCGACTTTAAAAAGTTGTTAAATAGACTTAACTAT
pCAEV1 Downstream (-) MluI	pCAEV1 vector construction	ACGCGTGGATATATGCAATCTTTAGTCCAG
flaB-GFP (+) XhoI	pCAEV3 vector construction	CTCGAGCCGGAGGAGTTATTTATAATAAAATAAAGAATTAC
flaB-GFP (-) XhoI	pCAEV3 vector construction	CTCGAGGTGGCGGCCGCTCTAG
Insert type A, F, M (+) BspEI	Amplification of insert	TCCGGAAAAGATGGGAATACATCTGCA
Insert type E, H (+) BspEI	Amplification of insert	TCCGGAAAAGATGGGAATGCATCTGCA
Insert PKo (+) BspEI	Amplification of insert	TCCGGAAAAGGTGGGGATTCTGCA
Insert PBi (+) BspEI	Amplification of insert	TCCGGAGGGGATTCTGCATCTACTAATC
Insert type A, E, F, H, M, PKo, PBi (-) BspEI	Amplification of insert	TCCGGATTAAGGTTTTTTTGGACTTTCTG
Upstream confirm (+)	Transformation confirmation	CCTACGTTGTGATGAGACTTGATTT
Spec/strep 3' (+)	Transformation confirmation	GGCGAGATCACCAAGGTAGTC
Insert sequencing (+)	OspC sequence confirmation	TAAAAAGGAGGCACAAATTAATG
Deletion upstream (+)	Deletion vector construction	GCAACAATCCAGTGTTTACAAAAACG
Deletion upstream (-)	Deletion vector construction	ACCGGTTCTGACGTCTAATTTGTGCCTCCTTTTTATTTATGA
Deletion downstream (+)	Deletion vector construction	GACGTCGTTGTGGCAGAAAGTCCAAAAAAACC
Deletion downstream (-)	Deletion vector construction	ACCGGTGCTGTTTAACGATTTATTTGATACTTTGGGC
OspC (+) LIC	Amplification of OspC for pET46	GACGACGACAAGATTAATAATTCAGGGAAAGATGGG
OspC (-) LIC	Amplification of OspC for pET46	GAGGAGAAGCCCGGTTTAAGGTTTTTTTGGACTTTCTGC
OspC E61Q/E63Q (+)	Mutagenic primer	GTGAAACAGGTTCAAGCGTTG
OspC E61Q/E63Q (-)	Mutagenic primer	CAACGCTTGAACCTGTTTCAC
OspC K60Y (+)	Mutagenic primer	CAACGCTTCAACCTCATACAC

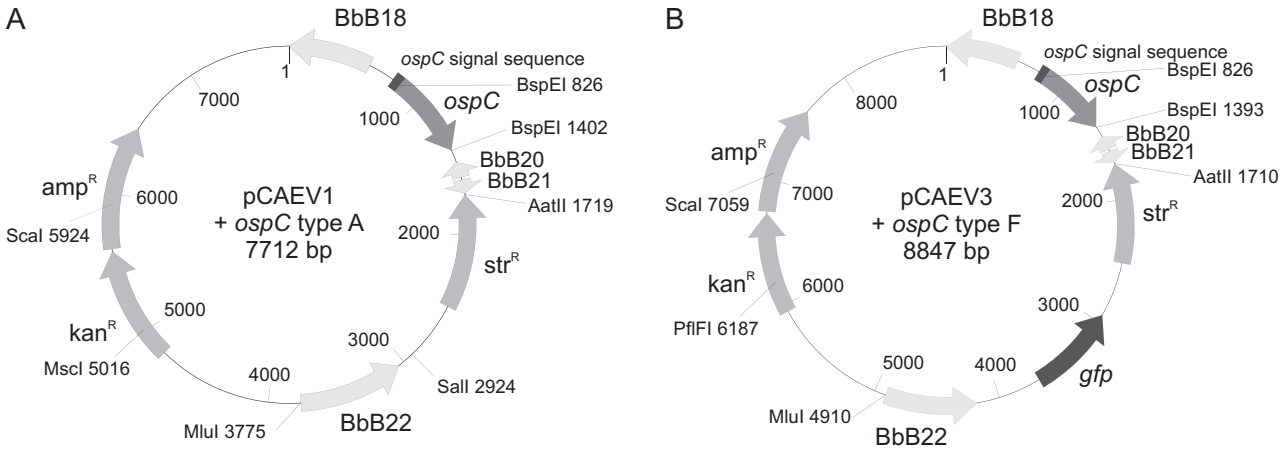
OspC K60Y (-)	Mutagenic primer	GTGTATGAGGTTGAAGCGTTG
OspC E61Q (+)	Mutagenic primer	GTGAAACAGGTTGAAGCGTTG
OspC E61Q (-)	Mutagenic primer	CAACGCTTCAACCTGTTTCAC
OspC E63Q (+)	Mutagenic primer	GTGAAAGAGGTTCAAGCGTTG
OspC E63Q (-)	Mutagenic primer	CAACGCTTGAACCTCTTTCAC
OspC C130A (+)	Mutagenic primer	GCGGCTAAGAAAGCTTCTGAAAC
OspC C130 (-)	Mutagenic primer	GTTTCAGAAGCTTTCTTAGCCGC
OspC N85G/D88N/E90Q (+)	Mutagenic primer	ATACACCAAAATGGTGGTTTGAATACCCAA AATAATCAC
OspC N85G/D88N/E90Q (-)	Mutagenic primer	GTGATTATTTTGGGTATTCAAACCACCATT TTGGTGTAT
OspC H82D/N84D/N92D (+)	Mutagenic primer	GCTAAAGCTATTGGTAAAAAAAGAGATCAA GATAATGGTTTGGATACCGAAAATGATCAC
OspC H82D/N84D/N92D (-)	Mutagenic primer	GTGATCATTTTCGGTATCCAAACCATTATC TTGATCTCTTTTTTTACCAATAGCTTTAGC
FlaB q-PCR primer (+)	q-PCR primer	CAGGTAACGGCACATATTCAGATGC
FlaB q-PCR primer (-)	q-PCR primer	CTTGGTTTGCTCCAACATGAACTC
Nid1 q-PCR primer (+)	q-PCR primer	CCAGCCACAGAATACCATCC
Nid1 q-PCR primer (-)	q-PCR primer	GGACATACTCTGCTGCCATC
OspC 20 (+) LIC	Amplification of *ospC* for pET vectors	GACGACGACAAGATTAATAATTCAGGGAA AGATGGG
OspC 210 (-) LIC	Amplification of *ospC* for pET vectors	GAGGAGAAGCCCGGTTTAAGGTTTTTTTG GACTTTCTGC
OspC 200 (+) LIC	Amplification of *ospC* for pET vectors	GACGACGACAAGATTCCTGTTGTGGCAGA AAGTCC
OspC 200 (-) LIC	Amplification of *ospC* for pET vectors	GAGGAGAAGCCCGGTTTAGCTTGTAAGCT CTTTAACTGAATT
Insert type A 20 (+) BspEI	Amplification pCAEV1 insert	TCCGGAAAAGATGGGAATACATCTGCA
Insert type A 200 (-) BspEI	Amplification pCAEV1 insert	TCCGGATTAGCTTGTAAGCTCTTTAACTGA ATTAG

and site-directed mutant genes were then prepared for and annealed into the pET46 Ek/LIC vector. To accomplish this, the purified PCR products from above were first treated with T4 DNA polymerase to generate single-stranded overhangs and then annealed into the pET46 vector. The resulting plasmids were then transformed into both Novablue and BL21 (DE3) strains of *E. coli.* Colonies were selected for ampicillin resistance (50 μg mL^{-1}) and PCR with the OspC LIC +/- primer set was used to screen the colonies for the presence of *ospC*. These PCR reactions were carried out using GoTaq Master Mix (Promega) in 30 μL reactions and the following PCR conditions: 1 cycle of 95°C for 2 min followed by 34 cycles of 94°C for 15 sec, 50°C for 30 sec, 72°C for 1 min, and finally 1 cycle of 72°C for 4 minutes with a indefinite 4°C hold. Several colonies containing *ospC* were used to start 6 mL cultures in LB broth with ampicillin (50 μg mL^{-1}) and allowed to grown over night in a shaking 37°C incubator. The plasmids were then isolated from the *E. coli* using a HiSpeed Plasmid Mini kit (Qiagen). All procedures were performed as directed by the manufacturer (Novagen). The resulting constructs were validated by automated DNA sequencing (MWG Biotech or GENEWIZ).

2.4 Generation of allelic exchange vectors

A master allelic-exchange vector designated as pCAEV1 was generated using a pCR2.1 backbone (Figure 6A). A second allelic exchange vector called pCAEV3 was constructed that is identical to pCAEV1 with the addition of the gene for production of green fluorescent protein (*gfp*) (Figure 6B). To create pCAEV1 the following regions of *B. burgdorferi* B31-5A4 cp26 were amplified: (1) region 5' of *ospC* (includes BbB18 and extends through the signal sequence and first four amino acids of OspC; the amplicon harbors 3' tandem BspEI and MluI sites), (2) region 3' of *ospC* (includes BbB20 and

Figure 6. Schematic of allelic exchange vectors. The (A) pCAEV1 and (B) pCAEV3 vectors were generated as described in the text. Restriction sites necessary for creation are shown.



BbB21 and flanking sequences; amplicon harbors 5' BspEI and 3' AatII sites) and (3) region 3' of BbB21 (includes a portion of BbB22; amplicon harbors 5' SalI and 3' MluI sites). To allow for streptomycin/spectinomycin resistance selection, *aadA* was amplified from pKFSS1 using primers with 5' SalI and 3' AatII sites (Frank *et al.*, 2003) (Table 3). All amplicons were individually annealed into the pCR2.1 TOPO plasmid and the plasmids were propagated in and isolated from Novablue *E. coli* as described above. The plasmids were digested with the appropriate restriction enzymes to release the inserts. The inserts were ligated into the plasmid carrying the 5' *ospC*-containing fragment in the pCR2.1 TOPO vector and transformed into *E. coli* (C2925; New England Biolabs). The pCAEV3 vector was created by amplifying the constitutively active *gfp* gene from pCE323 and ligating it 5` of the streptinomycin resistance cassette in pCAEV1 (Eggers *et al.*, 2006).

2.5 Allelic-exchange replacement of wild type *ospC*

To create the *ospC* allelic-exchange vectors, wild-type, *ospC* types, *ospC* hybrids, and *ospC* genes with site-directed mutations (generated as detailed above) were amplified with primers possessing 5' and 3' BspEI sites (Table 3). The amplicons were cut with the appropriate restriction enzyme and ligated into pCAEV1 or pCAEV3. The plasmids were propagated in Novablue *E. coli* cells (Novagen) and purified using HiSpeed Plasmid Midi kits (Qiagen). All mutant strains of *B. burgdorferi* were made using pCAEV1 with the exception of the B31-5A4 mutants containing OspC types F and M, which were created using pCAEV3.

2.6 Transformation of *B. burgdorferi*

The plasmids generated as described above were introduced into *B. burgdorferi* B31-5A4 (kindly provided by Dr. Jon Skare) or B31Δ*ospC* kan^r (described below) by electroporation essentially as previously described with some modifications (Samuels and Garon, 1993; Samuels *et al.*, 1994). *B. burgdorferi* B31-5A4 and B31Δ*ospC* kan^r cultures were grown at 37°C to mid-log phase in BSK-H medium with 6% rabbit serum (Sigma). The cells were collected by a series of centrifugation steps. All centrifugations were performed at 4°C and the cultures were always kept on ice. The cultures were spun at 4,000 x g for 20 min, washed with 10 mL of cold Dulbecco's PBS (dPBS), spun at 3,000 x g for 10 min, washed with 10 mL of cold EPS buffer (93 g L^{-1} sucrose, 15% glycerol), spun at 2,000 x g for 10 minutes, washed with 10 mL of cold EPS buffer, and finally spun again at 2,000 x g and resuspended in 150 μL of cold EPS. The pCAEV1/3 plasmids containing the mutant *ospC* gene were linearized using MscI or PflFI and ScaI-HF (New England Biolabs). A small amount each reaction was run on a 1% agarose gel to ensure correct cutting. The linearized plasmids were purified through two rounds of phenol/chloroform/isoamyl alcohol (25:24:1) (Fisher Scientific), precipitated with ice cold absolute ethanol, washed with 70% isopropanol, allowed to dry, resuspended in 6 μL of mili-Q water, and quantified using UV spectrophotometery. Twenty μg of linearized plasmid was then mixed with 50 μL of the cell suspension. After 5 min on ice, the cells were electroporated (0.2 cm cuvette, 2.5 kV, 25 μFD, 200 Ω), transferred to 10 mL BSK medium with 6% rabbit serum, and incubated overnight at 37°C (5%CO_2). The culture volumes were increased to 50 mL with fresh BSK media, streptomycin (50 μg mL^{-1}) was added, and the cultures were maintained at 37°C for 2 to 4 weeks.

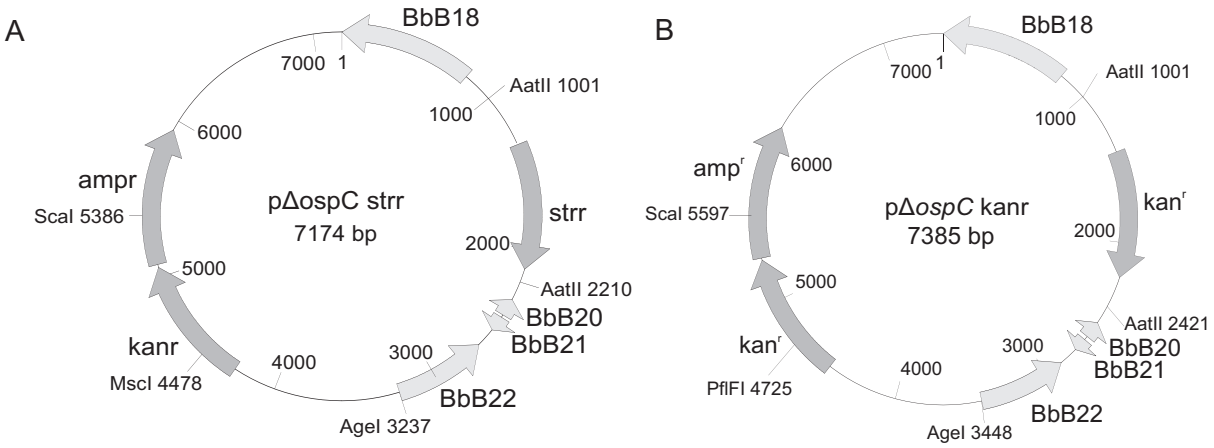
2.7 Subsurface plating of *B. burgdorferi*

Clonal populations of all transgenic strains were obtained by subsurface plating. Briefly, 2% GTG agarose was autoclaved and placed in a 55°C water bath while BSK media supplemented with 6% rabbit serum was placed in a 45°C water bath. The bottom layers of plates were poured first with a final agarose concentration of 0.6%. Three serial dilutions of the transformant cultures were prepared and mixed with the top layer of the plates (final agarose concentration of 0.8%). The top layers were poured over the bottom layers and the plates were allowed to cool and solidify before being placed in sterile bags and incubated at 37°C (5%CO_2) for 2-4 weeks until colonies became visible. Clonal colonies were punched from plates using sterile pipettes, placed in 2 mL of BSK-H media, and allowed to grow until dense. One mL of each clonal culture was spun down (14,000 x g, 10 min) and cell pellets were resuspended in 40 mL of mili-Q water. These pellets were boiled and used as template for PCR to determine the plasmid contents of the transformants using plasmid-specific primer sets (McDowell *et al.*, 2001; Rogers *et al.*, 2009). Additionally, to confirm the sequence of the DNA that crossed over into cp26, the desired region was PCR amplified using Phusion Taq as described above and sequenced (MWG Biotech or GENEWIZ).

2.8 Generation of *B. burgdorferi* B31Δ*ospC* str^r and B31Δ*ospC* kan^r strains

A pΔ*ospC* str^r knockout vector was created by amplification of the upstream (1000 bp) and downstream (1025 bp) regions of *ospC* with primers harboring appropriate restriction sites (Figure 7A). The upstream amplicon contains tandem AatII and AgeI sites at its 3' end while the downstream amplicon has 5' AatII and 3' AgeI sites

Figure 7. Schematic of *ospC* knock-out vectors. The (A) p∆*ospC* str^r and (B) p∆*ospC* kan^r vectors were generated as described in the text. Restriction sites are indicated.



(Table 3). The amplicons were cloned into pCR2.1 and the plasmids were propagated in and isolated from Novablue *E. coli* as described above. The plasmids were digested with AatII and AgeI, and the downstream fragment was ligated to the upstream-containing plasmid. Finally, the *aadA* gene for streptomycin/spectinomycin resistance was excised from the pKFSS1-AatII plasmid (Frank *et al.*, 2003) and ligated between the upstream and downstream sequences. The pΔ*ospC* kan^r was created by excising the *aadA* gene from pΔ*ospC* str^r using AatII, digesting the PFligBaphIT7T kanamycin resistance gene (kindly provided by Dr. Scott Samuels) with AatII, and ligating the two together (Figure 7B). Transformation, subsurface plating and plasmid screening were conducted as described above.

2.9 Assessment of growth curves

Either 10 or 15 mL tubes of BSK-H complete media were seeded with approximately 5 x 10^5 cells mL^{-1} of actively growing cells. Cultures for determining LBD1 mutant growth curves were seeded lower than other transgenic strains. Triplicate cultures were maintained at 37°C (5% CO_2) and cell counts were conducted each day using darkfield microscopy with an Olympus BX51 fluorescence microscope.

2.10 Production of recombinant OspC proteins

For recombinant protein production, BL21 (DE3) cells were transformed with the pET46 Ek/LIC plasmids carrying wild type or mutated *ospC* genes as described above. The pET46 plasmid utilizes an IPTG-inducible promoter and contains an N-terminal His-tag to allow for protein purification. PCR was used to ensure that individual BL21 (DE3) colonies contained *ospC*. Once the correct insert was confirmed, protein was

induced and purified. For protein induction, an individual colony was grown in 100 mL of LB broth containing ampicillin (50 μg mL^{-1}) in a 37°C shaking incubator. Once the culture density reached an OD_{600} of between 0.5 and 0.8, protein expression was induced with IPTG (1 mM). After incubating for an additional 3 hours, cells were collected by centrifugation (6,000 x g for 15 min), lysed using Bug Buster HT (Novagen), and proteins purified by nickel chromatography using His-Bind resin (Novagen). The purified proteins were then dialyzed against three changes of PBS and quantified by the BCA assay (Pierce).

2.11 Generation of anti-OspC antiserum

To generate antiserum, C3H/HeJ mice were immunized with 10 μg of wild type recombinant OspC protein adsorbed to Imject alum (Pierce), with boosts at weeks 4 and 6. Serum was collected by tail bleed at weeks 5 and 7. Mice were sacrificed at week 12 and blood was collected. The serum was tested by immunoblot and shown to be specific. Week 7 blood had the greatest reactivity and was used in all analyses.

2.12 OspC immunoblot

To ensure that all mutant strains correctly produce OspC, Western blot analysis was utilized. Cultures were cultivated at 27°C and then transferred to 37°C for 3 days to induce *ospC* expression. The cells were then harvested by centrifugation (5,000 x g, 20 min), washed twice with PBS, and the equivalent of 1 OD_{600} of culture was suspended in 150 μL of reducing SDS-PAGE sample buffer (Laemmli and Favre, 1973). The cells were then boiled and the cell lystates were subjected to SDS-PAGE using a 15% gel (Criterion, Bio-Rad) (200 volts, 1 hr) and were transferred to PVDF membranes

(Milipore) by electroblotting (100 volts, 1 hr). The membranes were blocked (5% milk in PBS-T (PBS with 0.2% Tween 20)) for 1 hour before being screened with mouse-anti-OspC (1:1,000). Washes with PBS-T occurred between each incubation. Antibody binding was detected using peroxidase-conjugated goat-anti-mouse IgG (1:40,000) and chemiluminescence (Supersignal West; Pierce).

2.13 Proteinase K proteolysis

To assess the presentation of OspC at the *B. burgdorferi* cell surface, strains were cultivated at 27°C and then transferred to 37°C for 3 days to upregulate *ospC* expression. The cells were harvested (5,000 x g, 20 min), washed twice with dPBS, and the equivalent of 1 mL of culture at 0.3 or 0.2 OD_{600} was suspended in 1 mL of PBS with or without proteinase K (20 mg mL^{-1}). The cell suspensions were incubated at 22°C for 1 hr before phenylmethylsulfonyl fluoride (PMSF) was added (0.5 mg mL^{-1} methanol). The cells were then harvested by centrifugation (14,000 x g, 10 min) and suspended in 120 µL of SDS-PAGE sample buffer. The cell lysates (2 µL) were subjected to SDS-PAGE using 15% precast gels (Criterion, BioRad) and then transferred to PVDF membranes (Millipore) by electroblotting. After being allowed to dry, the membranes were blocked using 5% milk in PBS-T (PBS with 0.2% Tween 20) for 1 hour before being screened with mouse-anti-OspC (1:20,000) or with mouse-anti-FlaB (1:400,000). Washes with PBS-T occurred between each incubation. Antibody binding was detected as described above.

2.14 Immunofluorescence assays (IFA)

The distribution of OspC on *Borrelia* cells was assessed by immunofluorescent microscopy. Cells were collected by centrifugation (3,000 x g, 10 min), washed with 10 mL of dPBS, spun again, and resuspended in 1 mL of dPBS. The OD_{600} of the cells was determined and a 0.005 OD of each culture was suspended in 100 μL of dPBS and immobilized on slides (Superfrost Plus; Fisher). The slides were then blocked with 3% BSA in PBS-T for 1 hour in a humidified chamber, washed with PBS-T, and probed with mouse-anti-OspC antiserum (1:2,000) for 1 hour. Alexafluor 488 or 568-conjugated rabbit-anti-mouse IgG (1:200) served as the secondary antibody. The slides were mounted with Prolong Gold with DAPI (Invitrogen) and the cells visualized using an Olympus BX51 fluorescence microscope.

2.15 Infectivity analyses

2.15.1 Subcutaneous C3H/HeJ mouse inoculation

To test infectivity of the different *B. burgdorferi* strains generated, C3H/HeJ mice were inoculated with $1x10^4$ spirochetes of each strain by subcutaneous injection. After four weeks, the mice were euthanized and the heart, tibiotarsal joint, ear, bladder and blood were harvested. Blood was spun down (14,000 x g, 15 min) to collect the serum which was used for ELISA and Western blot analyses to assess the humoral response generated by the host (described below). Portions of the ear and bladder biopsies or portions of all organs collected were placed in BSK medium (supplemented with rifampicin, fosfomycin, and amphotericin B) and incubated at 37°C under 5% CO_2. Each sample was visually assessed for spirochetes by darkfield microscopy after 2 and 4 weeks of incubation as a means of determining infectivity.

2.15.2 Intradermal C3H/HeJ mouse inoculation

In order to investigate the effects of site-directed mutation of LBD2 on dissemination, C3H/HeJ mice were inoculated intradermally with 1 x 10^4 spirochetes of strains B31::*ospC* (wt) and B31::*ospC* (N85G/D88N/E90Q). The purpose of intradermal inoculation was to restrict the inoculum and avoid passive dissemination of spirochetes. Three mice were sacrificed 1, 3, 7, 14, and 21 days post-inoculation and blood, injection site skin, distal skin approximately one cm from the injection site, heart, tibiotarsal joint, ear and bladder tissues were collected. Dissemination of spirochetes was assessed by organ culture and serum was assessed for humoral response as described above.

2.16 DNA isolation

2.16.1 Genomic DNA isolation from *Borrelia*

To isolate genomic DNA from *B. burgdorferi* cells, a 50 mL culture was grown to high density and the cells were collected by centrifugation (6,000 x g, 20 min) and washed twice with PBS. The cells were resuspended in 1 mL of TES solution (50 mM Tris-HCl pH 8. 0, 40 mM EDTA, 25% sucrose) with 2% SDS and proteinase K (3 mg mL^{-1}) and incubated at 37°C for 45 minutes to lyse the cells. DNA was then extracted using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and spun down at room temperature (14,000 x g, 30 sec) to separate the layers. The upper aqueous phase was transferred to a new 1.7 mL tube. To the aqueous phase, 3 volumes of cold absolute ethanol and 1/10 volume of 7.5 M sodium acetate were added, mixed, and chilled overnight at -80°C. The next day, the tubes were spun in a refrigerated centrifuge (14,000 x g, 15 min) to pellet the DNA and the ethanol was removed. The

resulting DNA pellets were then washed twice with 500 μL of 70% ethanol and allowed to air dry before being resuspended in 750 μL of TE solution (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and treated with RNase (0.03 mg mL^{-1}), SDS (1%), and proteinase K (0.3 mg mL^{-1}). After incubating for 30 minutes at 37°C, the DNA was again extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with absolute ethanol, and allowed to chill overnight as described above. The pellets were then spun down (14,000 x g, 10 min, 4°C) and washed with 500 μL of 70% ethanol three times before allowing the pellets to completely air dry. The DNA was resuspended in 300 μL of mili-Q water and quantified using UV spectrophotometery.

2.16.2 Total DNA isolation from animal tissues

To isolate DNA from mouse tissues, approximately 0.5 g of each tissue or organ was digested with collagenase (2 mg mL^{-1}) for 4 hours at 37°C and then with proteinase K (0.1 mg mL^{-1}) over night at 55°C. The samples were spun down to remove left over animal debris and the supernatant was transferred to a clean 1.7 mL tube. DNA was extracted from the supernatant by treatment with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by centrifugation (14,000 x g, 30 sec) and removal of the top aqueous phase to a clean tube. This step was performed twice. Next, the DNA was precipitated from the aqueous phase by using chilled absolute ethanol and 1/10 volume of sodium acetate and allowed to sit at -80°C for 30 min. The DNA was then spun down (14,000 x g, 10 min) and the pellet was washed with 70% ethanol twice, allowed to air dry, and resuspended in mili-Q water. The concentration of DNA was quantified by UV spectrophotometery.

2.17 Quantitative PCR analysis

Quantitative PCR (q-PCR) analysis was performed on LBD1 mutants (B31::*ospC* E61Q/E63Q, B31::*ospC* (E61Q), B31::*ospC* (E63Q), and B31::*ospC* (K60Y)) and the B31::*ospC* (C130A) mutant. For q-PCR, the *Borrelia flaB* gene and the mouse nidogen 1 (*nid1*) gene were amplified with Sybr Green PCR master mix (Applied Biosystems) using an Opticon 2 DNA engine (MJ Research). PCR cycling conditions were: 94°C for 10:00, 40 cycles of 94°C for 15 sec, 56°C for 30 sec, 72°C for 30 sec, with fluorescence reads following the extension cycle. Relative numbers of gene copies were determined using a standard curve of *Borrelia* genomic DNA. To determine the extent of non-specific, background amplification, qPCR was also conducted using DNA extracted from the organs and tissues of uninfected mice. All samples were run in three replicates of triplicates, and the organ-specific background amplification was subtracted from the mean ratios. Statistical significance was assessed by ANOVA analysis of log-transformed data, with post-hoc testing by the Holm-Sidak method (SigmaPlot).

2.18 Enzyme-linked immunosorbant assay (ELISA)

The humoral response to inoculation was assessed by whole cell ELISA and Western blot analyses. For the ELISA analyses, *B. burgdorferi* B31-5A4 cells were pelleted, washed with PBS, suspended at 10^6 cells mL^{-1} (carbonate coating buffer) and immobilized onto ELISA plates (100 µL $well^{-1}$; 4°C; overnight) (Costar 3590). The plates were blocked with 1% BSA in PBS-T blocking buffer for 2 hours at room temperature. Serial dilutions of sera in blocking buffer were applied in duplicate and allowed to incubate for 1 hour. Prior to application, sera were thoroughly mixed with a

vortex. Antibody binding was detected using peroxidase-conjugated goat-anti-mouse IgG (1:20,000) and ABTS chromogen. Titers were calculated as the inverse dilution corresponding to one third of the plateau optical density.

2.19 Western blot

For Western blot analyses of the host immune response, *B. burgdorferi* B31-5A4 whole cell lysates were separated by SDS-PAGE (Criterion, Bio-Rad) and blotted to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in PBS-T and screened with mouse serum (1:1000) for 1 hour at room temperature. Antibody binding was detected using peroxidase-conjugated goat-anti-mouse IgG (1:40,000) and chemiluminescence (SuperSignal West Pico; Pierce).

2.20 Circular dichroism analysis of OspC

Far-UV circular dichroism spectra of wild type and mutated recombinant OspC proteins were measured at 20°C in a Jasco J-715 spectropolarimeter (Jasco, Easton, MD, USA) (Chen *et al.*, 1974). The protein samples (10 μM) were scanned in a 1 mm path-length cuvette using a 1 nm bandwidth, 8 sec response time, and a scan rate of 20 nm/min. Three independent scans were made of each protein. Circular dichroism spectra were subtracted from background (buffer alone) and converted from circular dichroism intensity to molar ellipiticity (deg cm^2 $dmol^{-1}$).

2.21 Blue-native PAGE

To determine if the recombinant wild type, OspC site-directed mutant and OspC hybrid proteins undergo dimerization, 1 μg of each protein was equilibrated in BN-PAGE

loading buffer (15% glycerol, 50 mM Bistris pH 7.0) containing 0.02% Coomassie brilliant blue G-250 (CBB-G250), and electrophoresed in a Novex Bis/Tris NativePAGE gels 4-16% (Schagger *et al.*, 1994). The cathode buffer (50mM tricine, 15mM Bis/tris, pH 7.0) contained 0.002% CBB-G250 for the first half of the electrophoresis run (100 volts). Once the proteins entered the gel and ran approximately one third of the way down the gel, the cathode buffer containing 0. 002% CBB-G250 was removed and replaced with cathode buffer with no CBB-G250 and the gel was allowed to run to the end (200 volts). The anode buffer was 50mM bis/tris pH 7.0. To visualize proteins, the gel was stained using Coomassie Blue stain solution (R-250). The molecular mass of the recombinant OspC proteins was interpolated from a standard curve generated with NativeMark (Invitrogen) marker mix.

2.22 Plasminogen binding assay

Plasminogen binding by recombinant OspC was assessed by ELISA (Lagal *et al.*, 2006). ELISA plates (Costar 3590) were coated with 500 ng plasminogen (P7999, Sigma) in carbonate buffer (pH 9.6). The wells were blocked with 1% BSA in PBS-T for 1 hour at room temperature. Recombinant OspC proteins (1 µg $well^{-1}$ in blocking buffer) were incubated in triplicate wells for two hours at room temperature. Secondary detection of bound OspC was by mouse-anti-His-tag monoclonal antibody (1:1,000) (Pierce), then by peroxidase-conjugated goat-anti-mouse IgG. Binding was quantified using ABTS chromogen.

2.23 Structural modeling

Electrostatic surface charge maps of the site-directed mutants B31::*ospC* (N85G/D88N/E90Q) and B31::*ospC* (H82D/N84D/N92D) were created using Deep View Swiss PdbViewer 4.0.1. The Poisson-Boltzman method was used and the color blue denotes positive charge while red signifies negative charge. The prototypical OspC model used in all structural models in this thesis is a surface projection of the IGGQ OspC crystal structure (Kumaran *et al.*, 2001b). All plasmids maps were generated using BioEdit.

Chapter 3: Results

3.1 In vitro assessment of *B. burgdorferi* OspC transgenic strains

3.1.1 Generation of mutant and control strains

A master allelic-exchange construct (designated as pCAEV1) was generated (Earnhart *et al.*, 2010) and *ospC* genes harboring amino acid substitutions, whole *ospC* genes of different types, and *ospC* type hybrids were inserted. Selection was accomplished with a streptomycin resistance (str^R) cassette downstream of the BbB21 open reading frame. In pCAEV1, the modified *ospC* genes are under the transcriptional control of a *B. burgdorferi* type *A-ospC* promoter (Alverson *et al.*, 2003; Gilmore *et al.*, 2001; Marconi *et al.*, 1993a; Xu *et al.*, 2007; Yang *et al.*, 2005). The endogenous cp26-encoded wild type-type A *ospC* gene of *B. burgdorferi* B31-5A4 was replaced with the mutated genes or with a wild type copy of *ospC.* This strain, designated B31::*ospC* (wt), served as a control to verify that the general genetic manipulation procedures employed and the expression of the antibiotic resistance cassette did not alter the properties of *B. burgdorferi* B31-5A4 (detailed below). As a negative control, the cp26-carried wild type copy of *ospC* was deleted from *B. burgdorferi* B31-5A4 and replaced with a resistance marker using the pΔ*ospC* str^r vector. An additional *ospC* knockout, B31Δ*ospC* kan^r, was generated to expedite the generation of mutants.

3.1.2 Assessment of plasmid content of transformant strains

B. burgdorferi B31-5A4 retains a large number of circular and linear plasmids that are easily lost during transformation (Barbour, 1988; Casjens *et al.*, 2000; Eggers and Samuels, 1999; Eggers *et al.*, 2000; Fraser *et al.*, 1997; McDowell *et al.*, 2001; Persing *et al.*, 1994; Schwan *et al.*, 1988; Zhang and Marconi, 2005). After obtaining clonal populations of each mutant or control, plasmid content was determined through PCR using plasmid specific primer sets (data not shown) (Rogers *et al.*, 2009). The full list of allelic exchange strains and the plasmids lost can be found in Table 3. Sixteen of the 26 strains created retained the full B31-5A4 plasmid repertoire. The only plasmids lost by any of the strains are cp9, lp5, and lp21. Strains B31::*ospC* (wt), B31::*ospC* (K60Y), B31::*ospC* (E61Q/E63Q), B31::*ospC* (type E), B31::*ospC* (hybrid Apa), B31::*ospC* (hybrid Ppa), and B31::*ospC* (ΔC10) lost lp21 which has been shown to be is inconsequential as it is not required for infectivity (Purser and Norris, 2000). Lp5, which is missing from strains B31::*ospC* (type PKo) and B31::*ospC* (type MOD-1), contains very few open reading frames, none of which encode genes necessary for infection establishment. B31::*ospC* (hybrid Pap) is missing two plasmids, cp9 and lp21, neither of which had any effect on the ability of this strain to cause mammalian infection.

3.1.3 Verification of sequence of the inserted *ospC* gene

Generating *B. burgdorferi* mutants is a difficult process due to many factors including the slow growth rate of these organisms, how easily they lose plasmids, and their low frequency of transformation. Additionally, during recombination, different portions of the *ospC* genes located on pCAEV1/3 can cross over with the type A-*ospC*

gene on cp26, making it difficult to introduce the exact changes desired. In order to avoid this problem of incomplete crossover, the B31::*ospC* kan^r mutant was developed. Using this mutant, the whole correct *ospC* gene was inserted into cp26. Twenty of the mutants in this study were generated by transformation of B31-5A4 while six strains were created using the B31::*ospC* kan^r mutant. Following transformation and subsurface plating, the introduced copy of *ospC* was amplified from individual colonies and the amplicons were sequenced to ensure that the desired sequence changes occurred and that no other changes were introduced into the cp26 *ospC* gene. No sequence alterations, other than those intended, were detected in any of the allelic exchange strains created.

3.1.4 Assessment of mutant strain growth rates

To ensure that the production of the mutant OspC proteins and the expression of the antibiotic resistance cassette have no effect on the growth rate of the allelic exchange strains, growth curves were determined by dark field microscopy. None of the allelic exchange or *ospC* deletion mutants displayed a growth phenotype that differed from the parental B31-5A4 strain (Figure 8A-G).

3.1.5 Verification of OspC production and surface exposure

Western blot, proteinase K digestion, and indirect immunofluorescence assays (IFA) were used to demonstrate that all allelic exchange strains (except B31Δ*ospC*) are able to export, present, and distribute OspC on the cell surface in a manner consistent with the parental B31 strain. Cells were exposed (or not exposed) to proteinase K, which is known to cleave OspC from the outer membrane of *Borrelia*, and western blots

Figure 8. Analysis of growth rate of OspC allelic exchange mutant strains. The rate of growth of all mutants was determined by triplicate counts of each culture using dark field microscopy. (A) C130A (B) ΔC10 (C) LBD1 mutants (D) LBD2 mutants (E) OspC type allelic exchange mutants (F) OspC type allelic exchanges from *B. andersonii* (G) OspC A/PKo hybrids

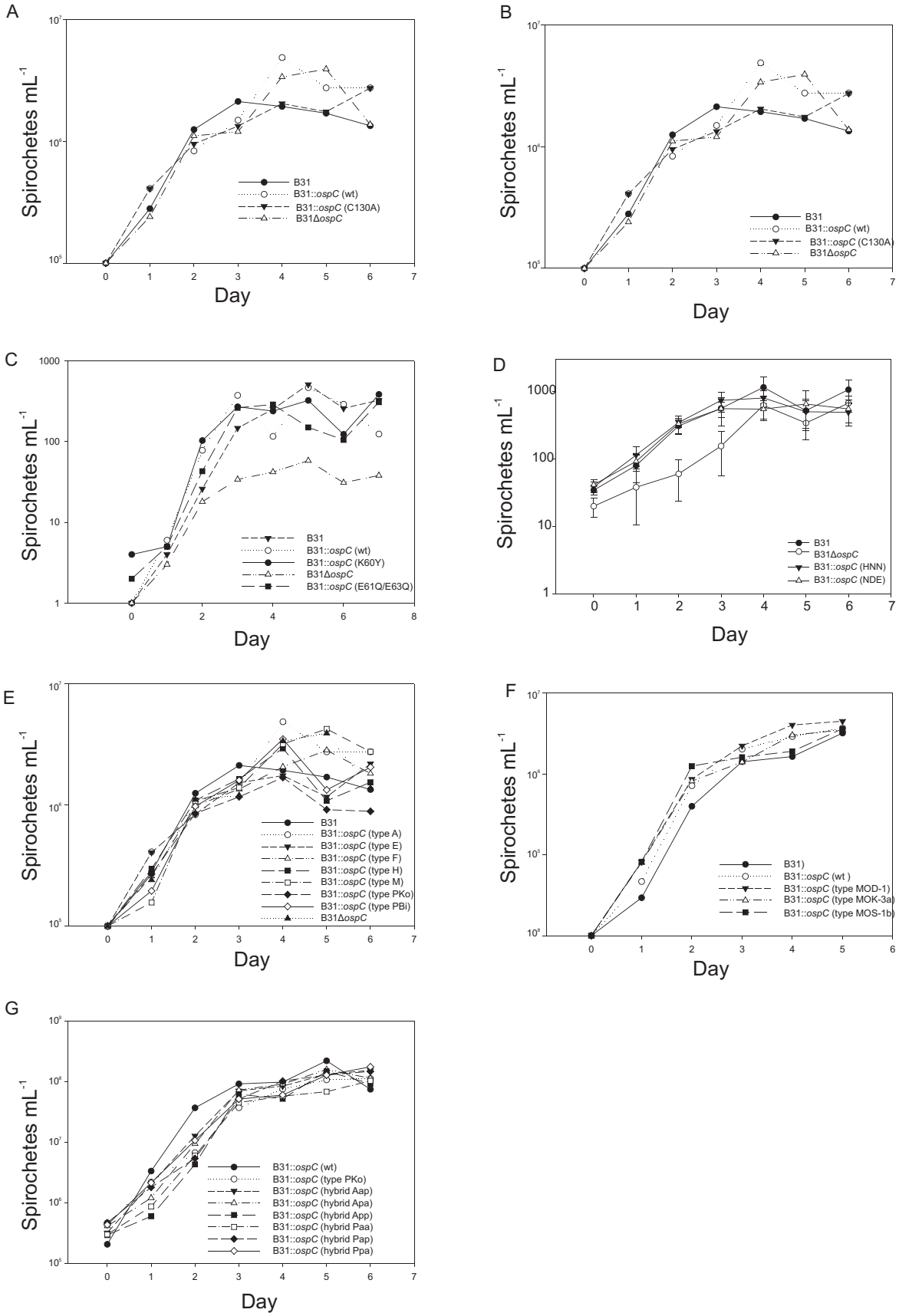
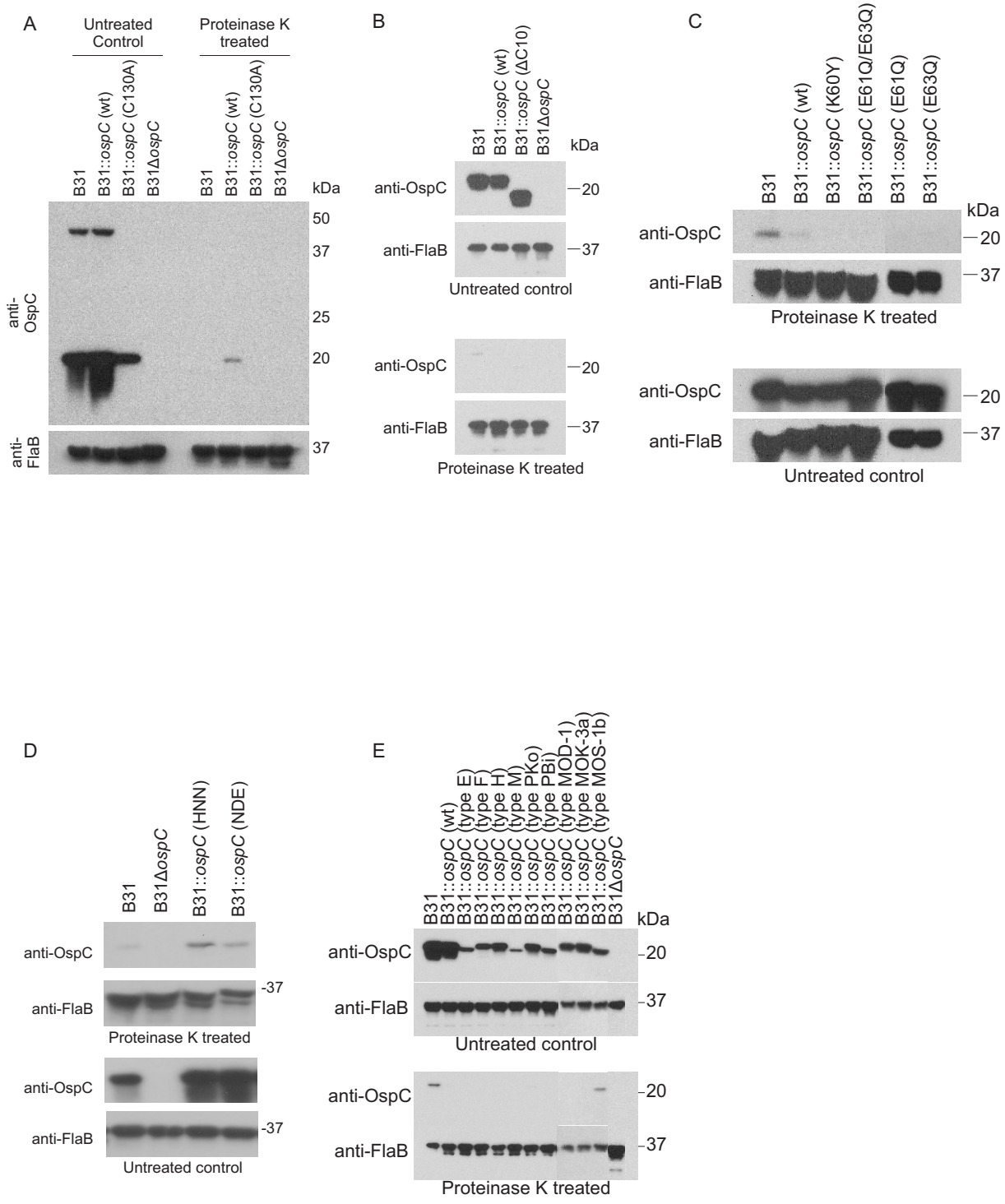


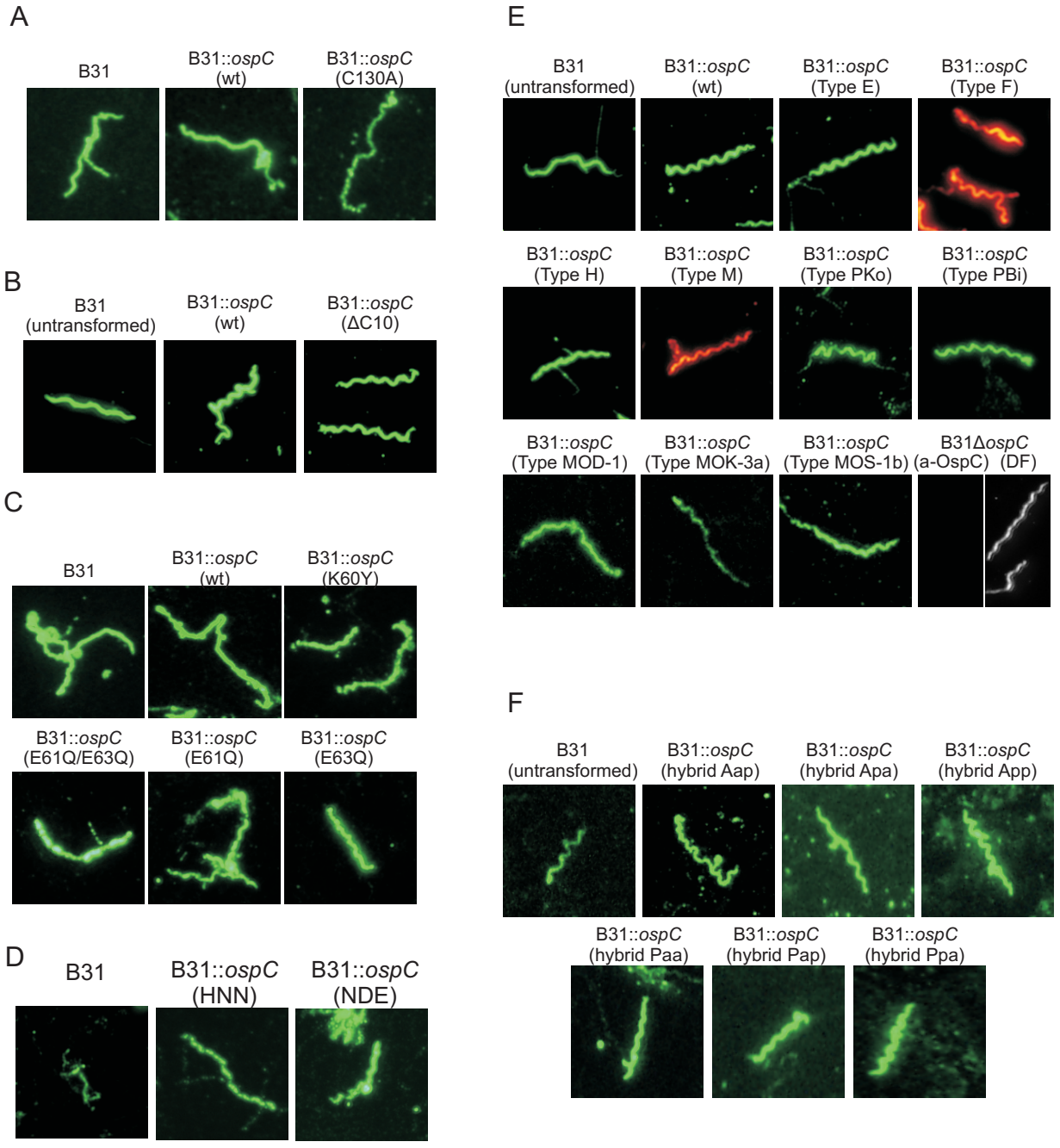
Figure 9. Analysis of OspC expression and presentation by allelic exchange strains. Anti-OspC western blot was used to show OspC expression and proteinase K digestion was used to show OspC cell surface presentation. (A) C130A (B) ΔC10 (C) LBD1 mutants (D) LBD2 mutants (E) OspC type allelic exchange mutants



of the cell lysates were screened with anti-OspC or anti-FlaB antisera, generated against a periplasmic flagellar protein. The western blot of the cells not treated with proteinase K show that all the mutant strains produce OspC (Figure 9A-F). Although there appear to be differences in the expression levels of OspC between the allelic exchange mutants and B31-5A4, these differences are most likely due to the type-specific nature of the immune response. The immunoblots were screened using anti-OspC antiserum generated against OspC type A. This antiserum does not react optimally to the different OspC types used for allelic exchange, accounting for the differences in the band strengths. Exposure to proteinase K resulted in the loss of detection of OspC, but not the periplasmic FlaB (Figure 9A-E). Digestion with proteinase K showed that the allelic exchange mutants present OspC on the outer membrane. Proteinase K analysis of the OspC type A/PKo hybrid strains is still ongoing.

IFA analyses were used to assess the surface presentation and distribution of OspC on the outer membrane of the transgenic strains. These analyses demonstrated no visible differences in surface labeling patterns between the allelic exchange mutants and B31-5A4 (Figure 10A-F). Additionally, the distribution of OspC on the surface of these strains appears to be the same as B31-5A4. For strains B31::*ospC* (type F) and B31::*ospC* (type M) an antibody tagged with red fluorescent protein was used due to the fact that these mutants were created using pCAEV3 and already produce green fluorescent protein. Based on the western blot, proteinase K, and IFA analyses, it can be concluded that none of the *ospC* site-directed mutations, *ospC* type allelic exchanges, *ospC* hybrid genes or the genetic manipulation of these strains influence

Figure 10. Analysis of OspC surface presentation and distribution by allelic exchange strains. IFA analysis was used as described in the text. (A) C130A (B) ΔC10 (C) LBD1 mutants (D) LBD2 mutants (E) OspC type allelic exchange mutants (F) OspC A/PKo hybrids



the production, surface presentation, and distribution of OspC. Additionally, deletion of C10 does not appear to alter OspC expression or outer membrane export (Kumru *et al.*, 2011). In all of the above assays, no OspC signal was detected in the B31Δ*ospC* str^r or the B31Δ*ospC* kan^r strain.

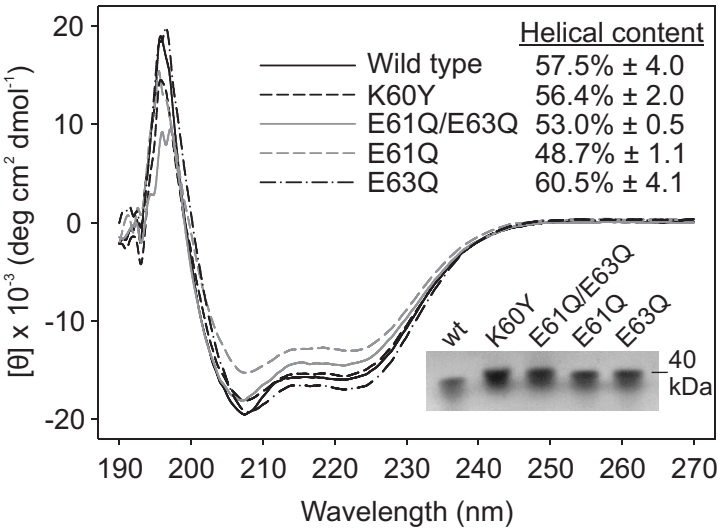
3.1.6 Assessment of dimerization ability of recombinant mutated OspC proteins by blue-native PAGE

To determine if the LBD1 or LBD2 site-directed mutations influenced the ability of OspC to dimerize, blue-native PAGE (BN-PAGE) analyses were performed (Schagger *et al.*, 1994) using recombinant protein. BN-PAGE analysis was also conducted using recombinant C130A protein (r-OspC (C130A)) and will be discussed in greater detail below. All site-directed mutant recombinant proteins efficiently dimerized and no residual monomer was detected (Figure 11 inset, and data not shown). Collectively, these analyses indicate that the substitutions introduced into OspC did not significantly alter the secondary structure or ability of the mutant strains to dimerize.

3.1.7 Determination of alpha helical content of recombinant mutated OspC proteins

The possible effect of each substitution of the LBD1 mutations and C130A on OspC alpha helical content was directly assessed using circular dichroism spectroscopic analyses (Figure 11). The estimated alpha helical content of the wild type OspC protein (57.5% ± 4%) was found to be in agreement with that determined by X-ray crystallography (~58%) (Eicken *et al.*, 2001; Huang *et al.*, 1999; Kumaran *et al.*, 2001b). Estimated percent alpha helical content values are indicated in Figure 11.

Figure 11. Blue-native PAGE and circular dichroism analysis of the LBD1 mutants. Circular dichroism analysis was used to analyse the helical content of the LBD1 mutants. BN-PAGE analysis (inset) demonstrated dimerization of all LBD1 mutants.



While the alpha helical content of E61Q decreased to some degree, the circular dichroism spectra of all other mutants were largely unaffected.

3.2 OspC residue C130 plays a role in oligomerization

3.2.1 Site-directed mutagenesis of *B. burgdorferi* OspC C130 and production of C130A mutant r-OspC

Full length native OspC harbors two cysteine residues: an N-terminal tripalmitoyl glyceryl modified Cys and C130 (Figure 12A). To determine if OspC dimers form disulfide bonds that mediate higher order oligomerization in vivo, *B. burgdorferi* B31 *ospC* was replaced with *ospC* (C130A) by allelic exchange using the pCAEV1 vector. Recombinant OspC was generated that lacked the leader sequence and the N-terminal Cys residue. In the native protein, the modification of the N-terminal Cys prevents it from playing a role in disulfide bond mediated oligomerization. To determine if C130 is involved in OspC oligomerization, recombinant protein harboring a C130A substitution was generated.

3.2.2 Assessment of oligomerization of OspC in vitro and in vivo

To assess the formation of disulfide bonds, r-OspC proteins and cell lysates of each strain were analyzed by SDS-PAGE under reducing (β-mercaptoethanol) and non-reducing conditions (Figure 13). The monomeric native OspC and His-tagged r-OspC proteins have masses of 20.3 kDa and 22 kDa, respectively. Under non-reducing conditions, r-OspC (wt) existed in both monomeric and dimeric form, while r-OspC (C130A) existed only in monomeric form (Figure 12B). Under reducing conditions only monomers were detected (see conceptual model presented in Figure 12C-D). Dimeric

Figure 12. Location of residue C130 and conceptual model of OspC oligomers. Figure 12A shows the hydrophobically bound OspC dimer (with residue C130 shown), which is maintained under native conditions while in (B) the OspC monomer that is found under denaturing conditions is shown. (C) Two OspC dimers can be covalently linked by disulfide bonds formed between C130 residues. Under denaturing conditions, the covalently bound OspC oligomers can be dissociated into monomers and covalently bound dimers (D). These covalent dimers can only be dissociated into monomers under reducing conditions.

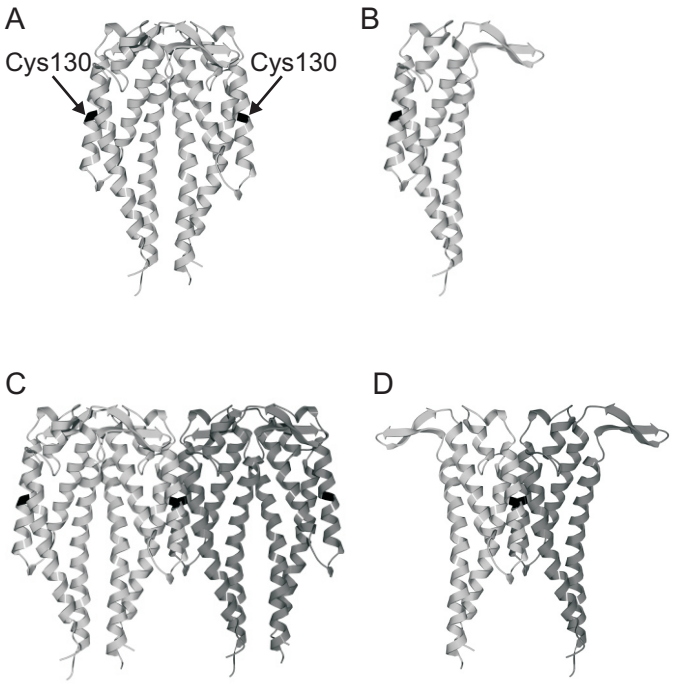
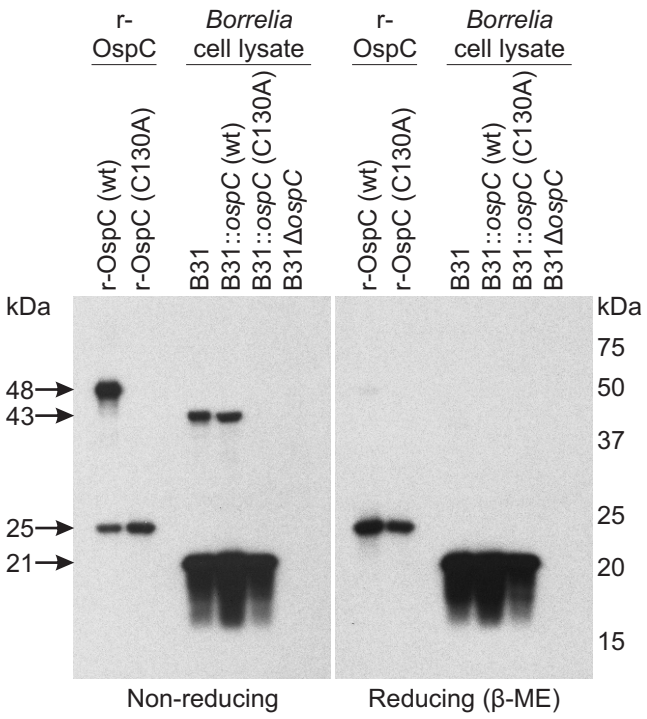


Figure 13. SDS-PAGE analysis of OspC oligomers under non-native conditions. Recombinant wild-type (r-OspC(wt)) and C130A (r-OspC(C130A)) protein and *Borrelia* cell lysates were separated using SDS-PAGE under conditions to reduce disulfide bonds or nonreducing conditions, transferred to PVDF, and then probed with anti-OspC antisera. The calculated molecular mass of the proteins are shown on the left.



OspC was also detected in B31 and B31::*ospC* (wt) cell lysates when separated under non-reducing conditions but not in the B31::*ospC* (C130A) cell lysate.

To determine if r-OspC dimers can oligomerize, r-OspC (wt) and r-OspC (C130A) were separated under non-denaturing (both reducing and non-reducing) conditions using BN-PAGE. Under non-reducing conditions five immunoreactive bands were detected, all with molecular masses consistent with OspC oligomers from single dimers to chains of 5 dimers (Figure 14). Only dimers maintained by hydrophobic interactions were observed for the C130A protein. Under reducing conditions all OspC was dimeric (Figure 14).

3.2.3 Infectivity and dissemination of strains expressing wild-type or C130A OspC

The ability of each strain to establish infection in mice was assessed using needle inoculation in two trials (5 mice per group). Four weeks post-inoculation, blood was collected and tissue biopsies of the heart, tibiotarsal joint, ear, and bladder were harvested and placed in media. In the first infection trial, only ear and bladder tissues were placed in media for spirochete cultivation. Serum was collected from the blood and used to determine the immune response mounted by the mice against the strains. Positive cultures were found in all mice, except those infected with the negative control B31Δ*ospC* strain. Cultivation of each infectious strain from mouse tissues did not reveal differences in dissemination patterns among strains (Table 4).

To assess the antibody response elicited by each strain (i.e., seroconversion), anti-OspC and anti-*B. burgdorferi* IgG titers were determined by ELISA using r-OspC or

Figure 14. BN-PAGE analysis of OspC oligomers under native conditions. Recombinant wild-type (r-OspC(wt)) and C130A (r-OspC(C130A)) protein were separated by blue native PAGE under reducing and nonreducing conditions, blotted to PVDF, and probed using anti-OspC antiserum. Calculated molecular masses are shown to the left.

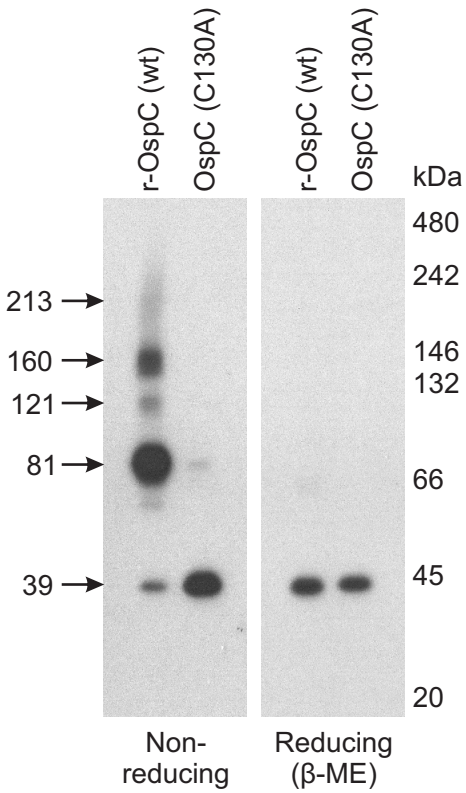


Table 4. Summary of culture results from tissues of mice inoculated with B31, *ospC* transgenic control strains, and B31::*ospC* (C130A).

	#Infected/Total					
	Ear	Bladder	Brain	Heart	Joint	Kidney
B31 (untransformed)	10/10	10/10	1/5	3/5	1/5	1/5
B31::*ospC* (wt)	10/10	9/10	0/5	5/5	3/5	0/5
B31::*ospC* (C130A)	5/10	10/10	0/5	4/5	2/5	0/5
B31Δ*ospC*	0/10	0/10	0/5	0/5	0/5	0/5

B. burgdorferi whole cells as the immobilized antigen. Whole cell and OspC-specific IgG titers were similar for mice infected with B31, B31::*ospC* (wt) and B31::*ospC* (C130A) cells (Figure 15).

3.3 The highly conserved C-terminal domain of *B. burgdorferi* OspC is not required for OspC function in the mouse model

3.3.1 Deletion of the C-terminal domain of OspC and generation of recombinant protein

The final ten amino acids (PVVAESPKKP) of OspC are highly conserved and have been theorized to have multiple functions. In order to investigate the potential functional roles and antigenicity of the conserved OspC C-terminus, *ospC* was replaced in its native location with *ospC* lacking the C-terminal ten amino acids (C10) using the pCAEV1 vector. Recombinant protein lacking the C10 amino acids was also generated.

3.3.2 Analysis of plasminogen binding by the B31::*ospC* (ΔC10) strain

It has been hypothesized that conservation in the C10 sequence is due to a functional constraint on sequence variation. Several functions for this domain have been proposed. Lagal et al. (2006) hypothesized that the C-terminal lysine residues of OspC are involved in binding plasminogen, which could aid in spirochete dissemination by proteolysis of extracellular matrix proteins. In this study, a small decrease in plasminogen binding by r-OspC (ΔC10) was noted when compared with wild-type (Figure 16); however, it is clear that C10 is not required for plasminogen binding.

Figure 15. Immune response of mice inoculated with B31::*ospC* (C130A). ELISAs were conducted using serum collected from mice four weeks post-inoculation. The infecting strain is indicated along the x axis. The bars show the geometric mean antibody titer while the triangles represent each individual mouse. The sera were serially diluted, screened against whole *B. burgdorferi* 5A4 cells immobilized on ELISA plates, and calculated as the inverse dilution corresponding to one-third of the plateau optical density.

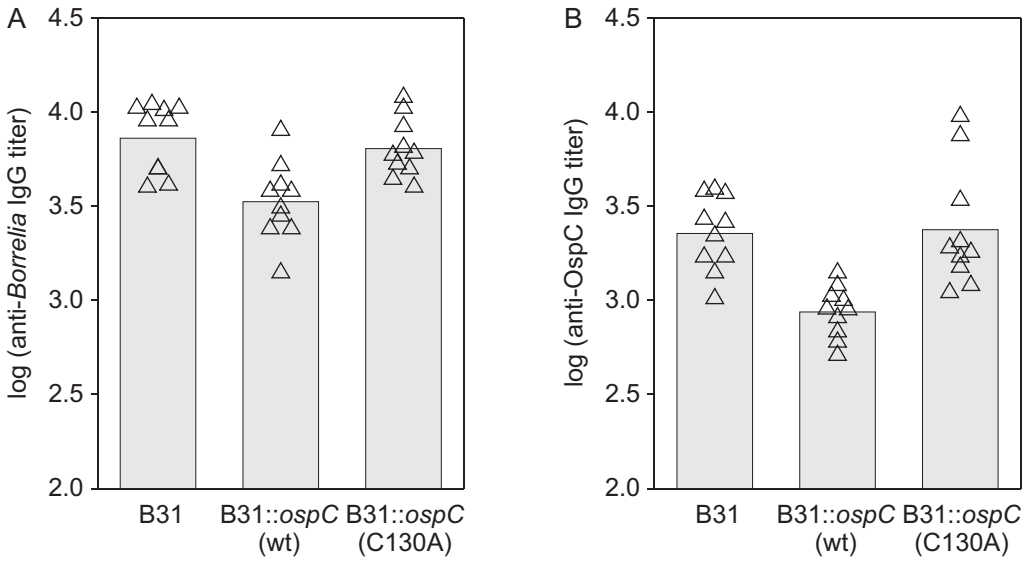
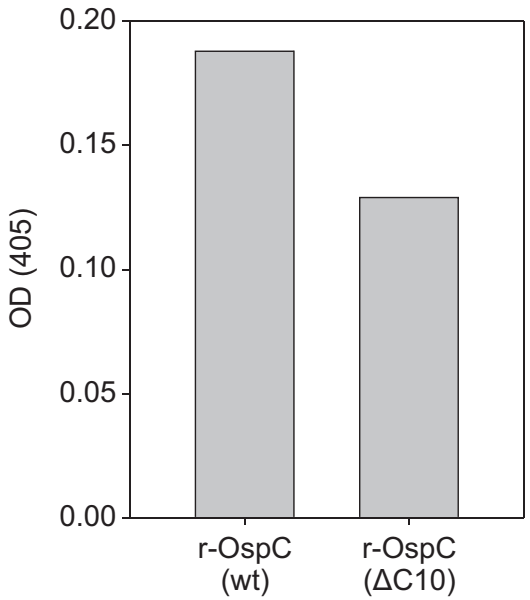


Figure 16. Ability of B31::*ospC* (ΔC10) to bind human plasminogen. Human plasminogen was immobilized in ELISA plate wells and screened with His-tagged r-OspC (wt) or r-OspC (ΔC10), with bound OspC detected by mouse anti-His-tag antibody and peroxidase-conjugated goat-anti-mouse IgG.



3.3.3 Assessment of infectivity of B31::*ospC* (ΔC10) in mice

Mathiesen et al. (1998b) suggested that C10 adopts a polyproline II helical conformation – a secondary structure associated with protein-protein interactions and bacterial adhesins (Brady *et al.*, 2010). C10 also has sequence similarity with the consensus recognition sequence for src homology domain 3 (SH3) proteins, which is a potential bacterial virulence mechanism (Aitio *et al.*, 2010; Ren *et al.*, 1993). The potential effect of disruption of these interactions on infection establishment was necessarily accomplished by assessing the functional significance of the C10 domain during infection. C3H/HeJ mice (n=5 per strain) were inoculated with control or transgenic strains. After four weeks organs were harvested and used to assess infectivity. Spirochetes were recovered from all mice infected with B31, B31::*ospC* (wt) and B31::*ospC* (ΔC10) strains (Table 5). The negative control B31Δ*ospC* strain was non-infectious. Furthermore, there were no significant differences in induced anti-whole *Borrelia* cell or anti-OspC IgG titers between the three infectious strains (Figure 17). Thus, by this route of infection, the C10 domain is not required for OspC function.

3.4 OspC LBD1 plays a crucial role in the ability of *B. burgdorferi* to establish mammalian infection

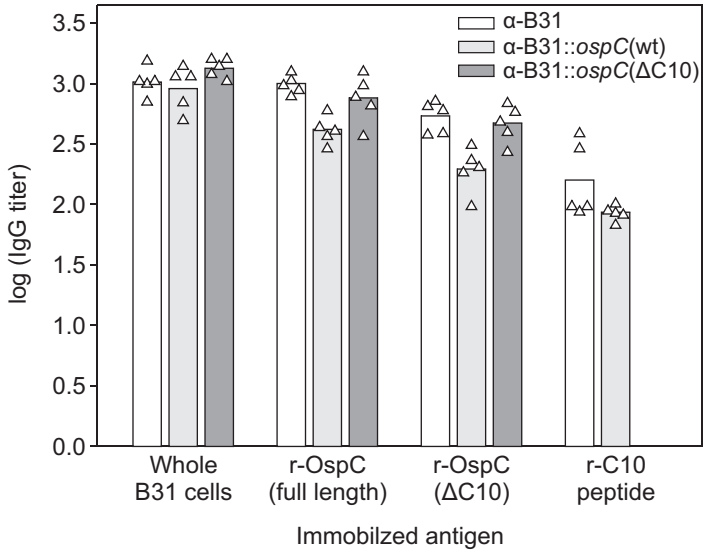
3.4.1 Site-directed mutagenesis of LBD1 of OspC of *B. burgdorferi* B31-5A4

To test the hypothesis that residues within LBD1 are involved in the in vivo functional activities of OspC that are required for the establishment of infection in mammals, residues K60, E61 and E63, which are charged, highly conserved, and possess side chains that extend into the solvent accessible pocket of LBD1, were

Table 5. Summary of culture results from tissues of mice inoculated with B31, *ospC* transgenic control strains, and B31::*ospC* (ΔC10).

	#Infected/Total	
	Ear	Bladder
B31 (untransformed)	4/5	5/5
B31::*ospC* (wt)	5/5	5/5
B31::*ospC* (ΔC10)	3/5	5/5
B31Δ*ospC*	0/5	0/5

Figure 17. Analysis of the specific IgG titer of mice infected with B31::*ospC* (ΔC10). Serum was harvested from mice four weeks after needle inoculation. The sera were serially diluted and screened against immobilized whole *B. burgdorferi* cells, r-OspC (wt), r-OspC (ΔC10), or r-C10 peptide. Titers (indicated by triangles) were calculated as the inverse dilution corresponding to one third of the plateau optical density. Geometric mean titers are indicated by the bar.



targeted for site-directed mutagenesis (Figure 18). These residues may serve as ligand contact points or be important in defining the physiochemical properties of LBD1 required for ligand binding. Residues E61 and E63, which are negatively charged and hydrophilic, and the positively charged and hydrophilic residue, K60, were replaced with Q and Y residues (polar and hydrophilic), respectively. These changes were chosen based on the reasoning that these substitutions would alter charge distribution, a key parameter in ligand binding, without significantly affecting secondary structure. The master allelic-exchange construct pCAEV1 was used to create *B. burgdorferi* strains containing these site-directed mutations to OspC. Additionally, recombinant OspC proteins harboring these mutations were generated.

3.4.2 Analysis of the infectivity of isogenic strains expressing LBD1 site-directed mutations of OspC

To determine if the production of mutated OspC proteins affects the ability of each strain to infect and disseminate, recombinant strains were administered to mice using needle inoculation (n=5 mice per group). Ear punch biopsies and urinary bladders were obtained 4 weeks after inoculation. Positive cultures were obtained from all mice infected with B31-5A4 (untransformed control), B31::*ospC* (wt), B31::*ospC* (E63Q) and B31::*ospC* (K60Y) (Table 6). In contrast, none of the mice injected with B31Δ*ospC*, B31::*ospC* (E61Q/E63Q) or B31::*ospC* (E61Q) yielded positive cultures (Table 6). Since the sole genetic difference in strain B31::*ospC* (E61Q) relative to the wild type parental strain was a single amino acid substitution, it can be concluded that E61 is a critical determinant of OspC function in mammals.

Figure 18. Location of residues chosen for LBD1 site-directed mutagenesis. A ribbon diagram and surface projection of OspC is used to show the location of the residues within LBD1 chosen for site-directed mutagenesis. The region containing LBD1 is highlighted by a yellow box and magnified in the right half of the figure to show the spatial location of these residues. Residue K60 is shown in red, E61 in blue, and E63 in green.

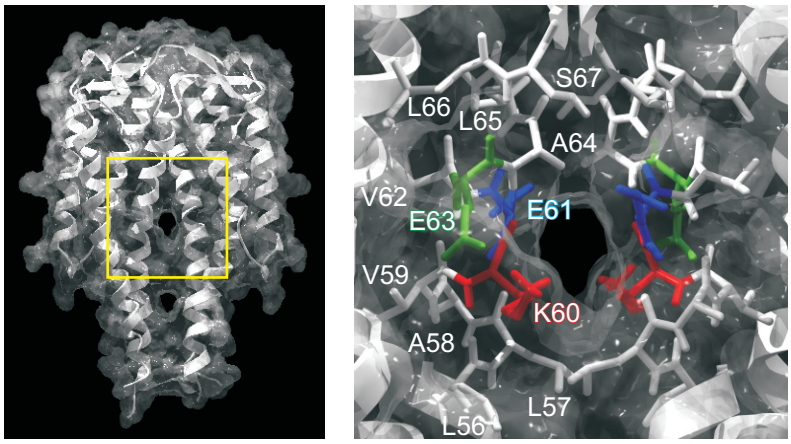


Table 6. Summary of culture results from tissues of mice inoculated with B31, *ospC* transgenic control strains, and LBD1 site-directed mutants of OspC.

	#Infected/Total	
	Ear	Bladder
B31-5A4 (not transformed)	5/5	5/5
B31::*ospC* (wt)	5/5	5/5
B31*ospC*Δ	0/5	0/5
B31::*ospC* (K60Y)	4/5	5/5
B31::*ospC* (E61Q/E63Q)	0/5	0/5
B31::*ospC* (E61Q)	0/5	0/5
B31::*ospC* (E63Q)	5/5	5/5

3.4.3 Analysis of the immune response to strains expressing LBD1 site-directed mutations of OspC

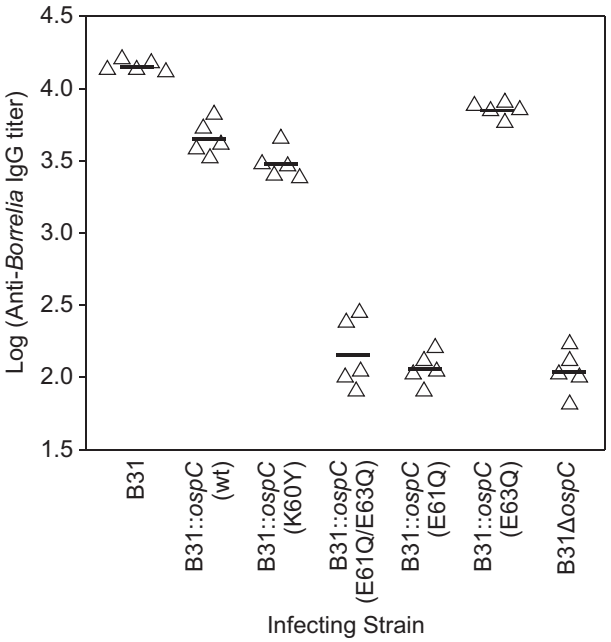
To determine if the inoculated mice developed a significant IgG response to *B. burgdorferi*, anti-*B. burgdorferi* IgG titers were determined by ELISA. Immobilized B31-5A4 whole cells served as the test antigen. All strains that were infectious, based on the cultivation results, displayed high level IgG titers whereas strains that yielded negative cultivation results had low titers (Figure 19A). The low titer antibody response observed in these strains most likely reflects a response to the inoculum ($1x10^4$ cells). Immunoblot analyses yielded results consistent with the ELISAs (Figure 19B). Sera from all infected mice reacted strongly with multiple *B. burgdorferi* proteins while no significant reactivity of the sera derived from cultivation negative mice was observed. The ELISA and immunoblot results are consistent with the infectivity data and serve to further establish that specific site-directed substitutions within LBD1 result in a non-infectious phenotype.

3.4.4 Quantitative PCR (q-PCR) analysis of the dissemination of infectious strains expressing LBD1 site-directed OspC mutations

To assess the influence of site-directed substitutions within LBD1 on dissemination, spirochete burdens in tissues and organs of all infected mice were determined by q-PCR. The parental B31-5A4 wild-type (untransformed), B31::*ospC* (wt) and B31::*ospC* (E63Q) strains disseminated to bladder, heart and joint (Figure 20). The numbers of B31::*ospC* (E63Q) spirochetes in the bladders of infected mice were higher than those observed for all other strains; these differences were statistically

Figure 19. Immune response of mice inoculated with LBD1 site-directed mutants. ELISAs were conducted using serum collected from mice four weeks post-inoculation. The infecting strain is indicated along the x axis. The bars show the geometric mean antibody titer while the triangles represent each individual mouse. The sera were serially diluted, screened against whole *B. burgdorferi* 5A4 cells immobilized on ELISA plates, and calculated as the inverse dilution corresponding to one-third of the plateau optical density.

A



B

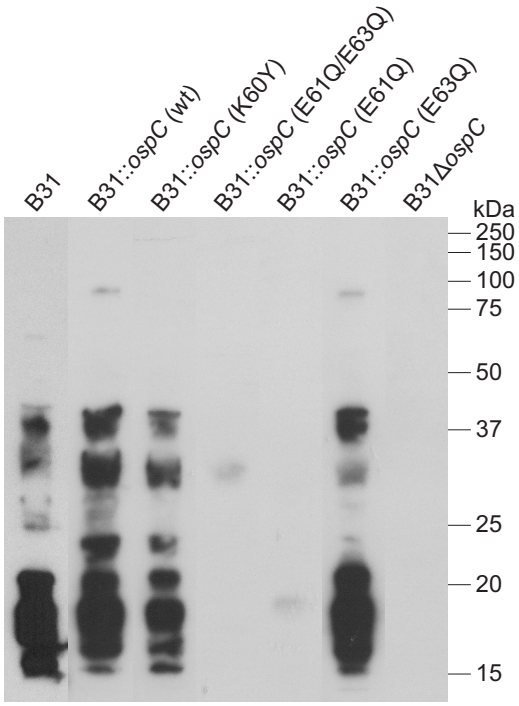
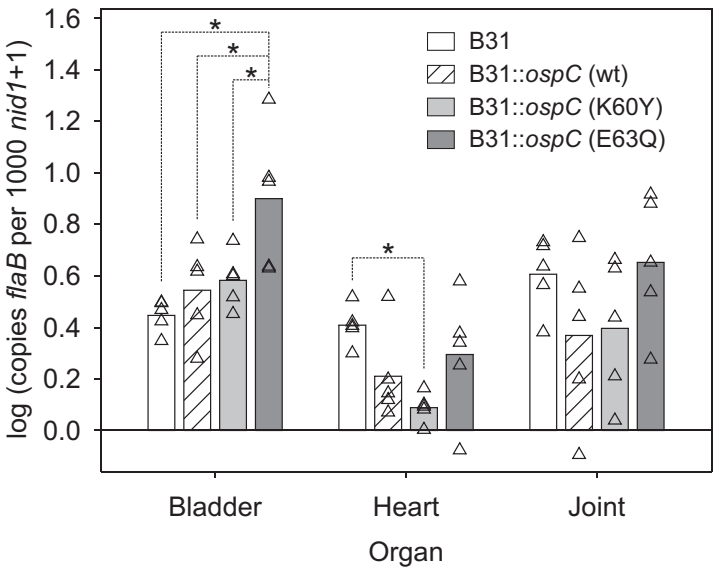


Figure 20. Spirochete burden in tissues of mice infected with LBD1 site-directed mutants. Quantitative PCR was used to analyze the number of spirochetes in the bladders, hearts, and joints of mice infected with LBD1 site-directed mutants. Organs were harvested 4 weeks after needle inoculation and DNA was isolated from each organ. The spirochete burden was determined by amplification of the *B. burgdorferi flaB* gene normalized to copies of the mouse *nid1* gene. The mean is represented by the bar while each triangle represents an individual mouse. Statistical significance ($P<0.05$) between the data sets is denoted by an asterisk (*).



significant. No significant differences in spirochete burdens were observed among the strains in tibiotarsal joints. Interestingly, very few B31::*ospC* (K60Y) spirochetes were detected in the heart (Figure 20). However, the difference was statistically significant only when compared with the wild type strain and hence some caution is in order in assessing this data. Nonetheless, this observation is noteworthy in light of findings suggesting that OspC is a determinant required for colonization of the heart (Antonara *et al.*, 2007).

3.5 Alteration of surface charge on LBD2 of *B. burgdorferi* OspC results in a failure to persist in the mouse model

3.5.1 Site-directed mutagenesis of OspC LBD2 of *B. burgdorferi* B31-5A4

In order to assess the potential role of the LBD2 region of OspC in *B. burgdorferi* infectivity and/or dissemination, site directed mutations were made within loops 2 and 3 in order to alter the surface charge of LBD2 (Figure 21). The amino acids selected for substitution occur in the same positions in other OspC types. Two mutant strains of were produced: B31::*ospC* (H82D/N84D/N92D) and B31::*ospC* (N85G/D88N/E90Q). For the B31::*ospC* (H82D/N84D/N92D) strain, positive and polar residue H82 was changed to an acidic and negative D residue while the polar residues N84 and N92 were also changed to D. These mutations resulted in an expansion of negative charge across the dome of OspC (Figure 22). For the B31::*ospC* (N85G/D88N/E90Q) mutant, residue N85 was changed to G, negative and acidic residue D88 was changed to a neutral N, and acidic residue E90 was changed to a neutral Q. These mutations resulted in a loss of much of the negative charge across LBD2 which

Figure 21. Alignment of OspC loop 2/3 region showing residues chosen for LBD2 site-directed mutagenesis. Three residues each were chosen for mutation in the region of loop 2 – loop 3 of OspC for the two LBD2 mutants. The residues chosen for mutation in the type A *ospC* gene were substituted with amino acids that occur in the same in at least one other OspC type. Residues chosen for mutation are highlighted with boxes.

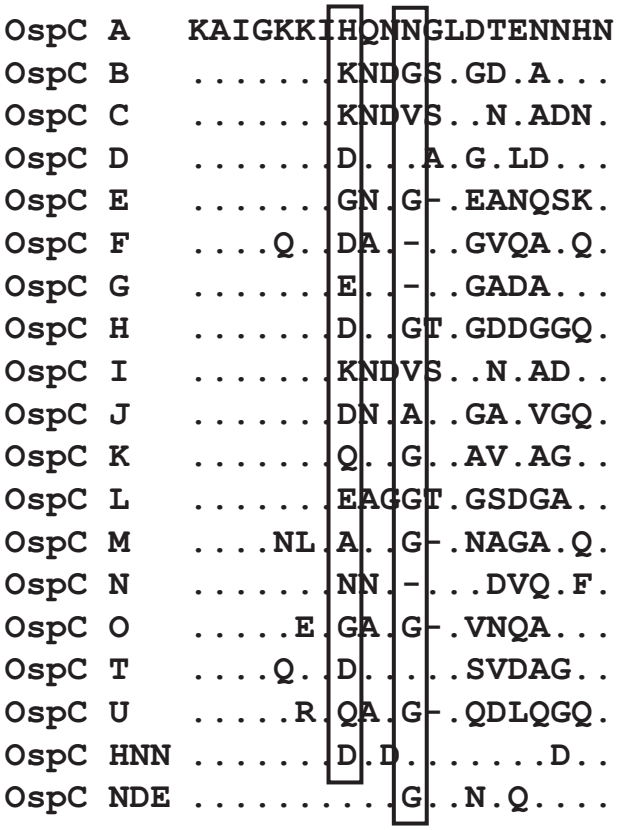
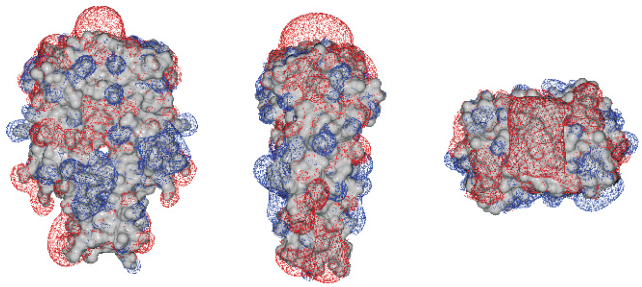
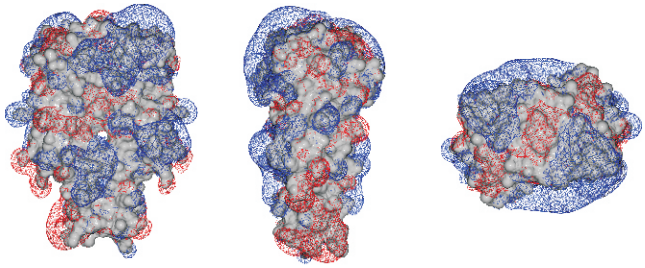


Figure 22. Electrostatic charge maps of LBD2 site-directed mutants. The OspC dimer is shown in two rotations and from the top with the calculated electrostatic potential superimposed on the molecular surface using Swiss-PdbViewer 4.0.1. Negative charge is depicted by the color red while positive charge is shown with blue. The charge maps for the mutants were super imposed on the structure of wild-type type A OspC.

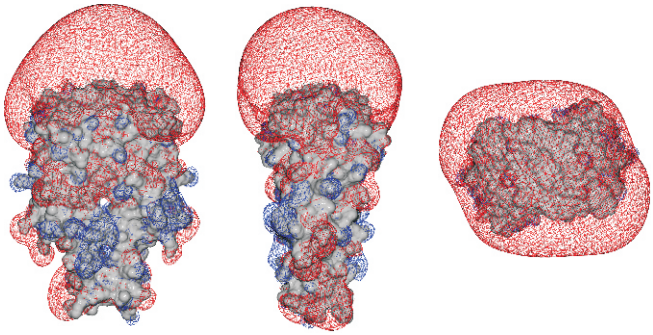
B31



B31::*ospC* (NDE)



B31::*ospC* (HNN)



allowed for an expansion of the positive charge along the surface of the whole of OspC molecule (Figure 22). Recombinant OspC protein was generated containing these site-directed mutations. For the rest of this manuscript, these two mutants will be referred to as B31::*ospC* (HNN) and B31::*ospC* (NDE).

3.5.2 Analysis of the infectivity of isogenic strains expressing OspC LBD2 site-directed mutations

In order to test the infectivity of the LBD2 mutant strains, mice (n=5 per strain) were injected subcutaneously with the *B. burgdorferi* strains B31 wild-type, B31Δ*ospC*, B31::*ospC* (HNN) and B31::*ospC* (NDE). Ear and bladder tissues were collected four weeks post-injection and placed in BSK complete medium for spirochete culture. Spirochetes were cultivated from organs of mice inoculated with all strains except B31Δ*ospC* str^r and B31::*ospC* (NDE). Mice infected with these two strains produced no spirochetes from either the ear or bladder tissues (Table 7).

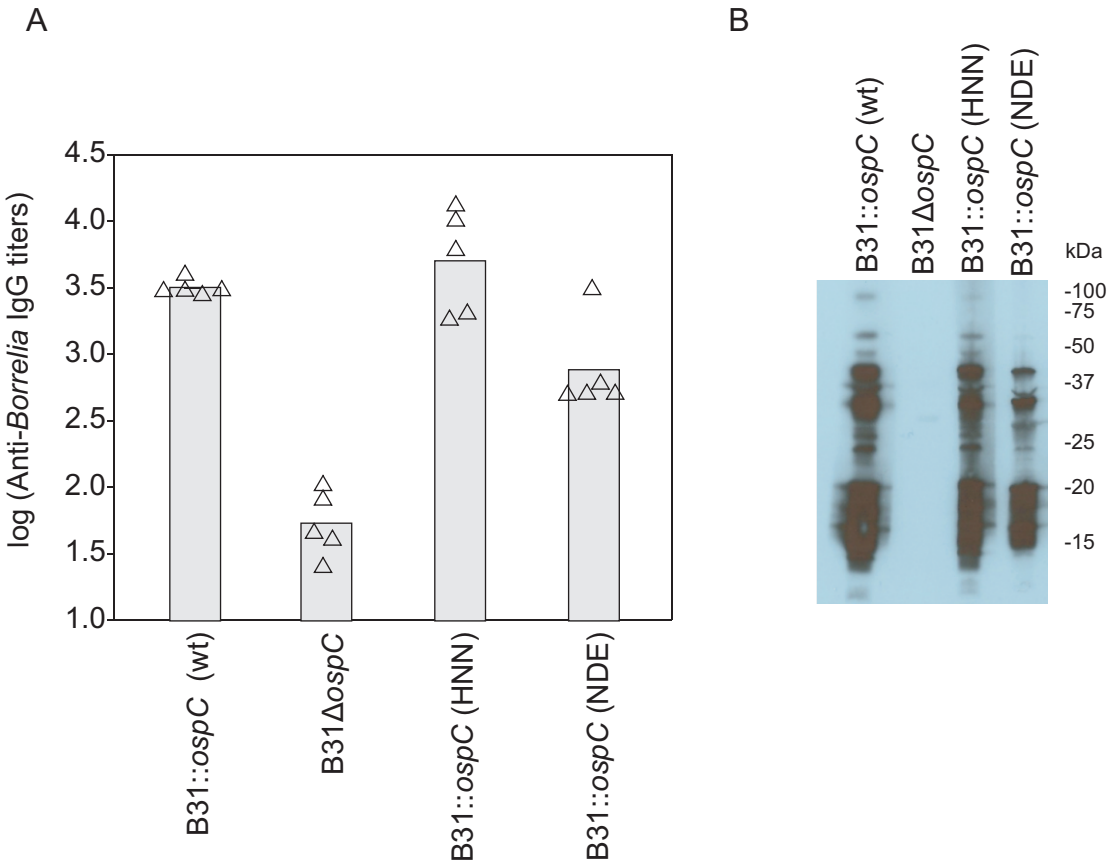
3.5.3 Analysis of the immune response to strains expressing OspC LBD2 site-directed mutations

ELISA and western blot analysis were used to investigate the humoral immune response produced by mice inoculated with the *B. burgdorferi* strains described above. All strains found to be infectious displayed high IgG titers. Despite the absence of spirochetes cultured from organs, mice inoculated with B31::*ospC* (NDE) produced antibody titers that were approximately one log fold lower than those of the wild type-infected mice and much higher than those of the noninfectious B31Δ*ospC* str^r (Figure 23A). Western blot analysis similarly showed antibody generated against B31::*ospC*

Table 7. Summary of culture results from tissues of mice inoculated subcutaneously with B31, *ospC* transgenic control strains, and LBD2 site-directed mutants of OspC

	#Infected/Total	
Strain	Ear	Bladder
B31	4/5	5/5
B31::*ospC* (wt)	5/5	5/5
B31Δ*ospC*	0/5	0/5
B31::*ospC* (H82D/N84D/N92D)	5/5	5/5
B31::*ospC* (N85G/D88N/E90Q)	0/5	0/5

Figure 23. Immune response of mice inoculated subcutaneously with LBD2 site-directed mutants. ELISAs were conducted using serum collected from mice four weeks post-inoculation. The infecting strain is indicated along the x axis. The bars show the geometric mean antibody titer while the triangles represent each individual mouse. The sera were serially diluted, screened against whole *B. burgdorferi* 5A4 cells immobilized on ELISA plates, and calculated as the inverse dilution corresponding to one-third of the plateau optical density.



(NDE) binding to B31-5A4 cell lysate, indicating that, while uncultivable from ear and bladder tissues, this mutant was able to elicit an immune response (Figure 23B).

3.5.4 Analysis of dissemination/persistence of isogenic strains expressing OspC LBD2 site-directed mutations

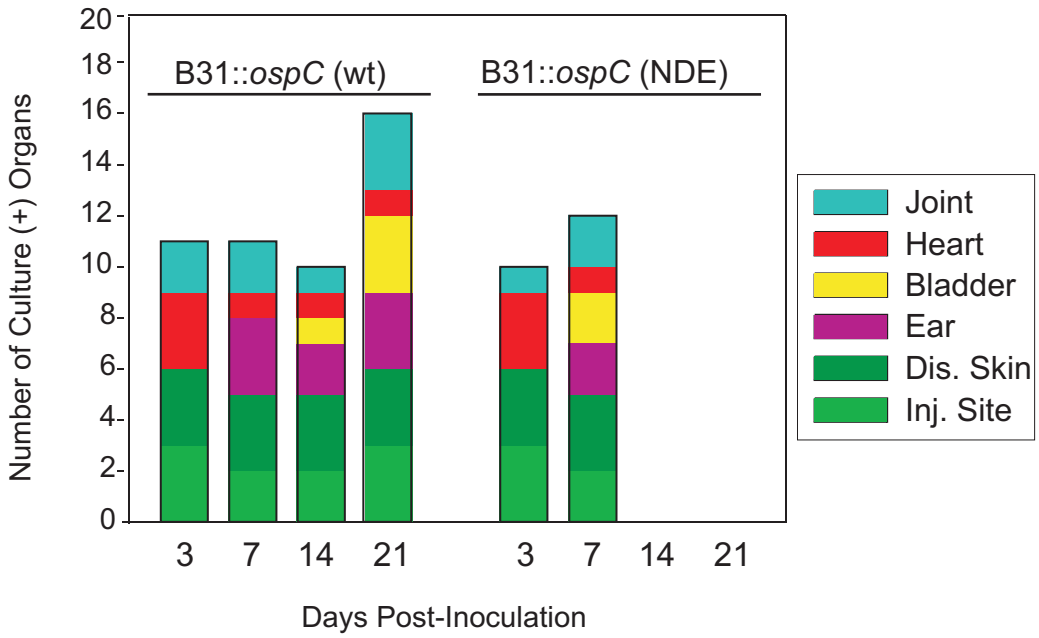
The inability to culture strain B31::*ospC* (NDE) from ear and bladder tissues while still detecting an antibody response against this mutant posed the question of whether this result was due to lack of dissemination of the mutant to these tissues or an inability of this mutant to persist in the mouse for 28 days. In order to answer this question, intradermal inoculations were performed. Mice were inoculated with strains B31::*ospC* (wt) and B31::*ospC* (NDE). Culture of organs taken from 1, 3, 7, 14, and 21 days post-inoculation showed that mutant B31::*ospC* (NDE) was able to disseminate to all organs harvested but between days 7 and 14, this mutant was no longer cultivable (Figure 24). This result indicates that the B31::*ospC* (NDE) mutant is unable to persist in the mouse model.

3.6 Allelic exchange replacement of *ospC* demonstrates the role of OspC phyletic type in establishment of murine infection

3.6.1 Allelic exchange of the *ospC* genes of different types

To assess the role of OspC type in infection establishment in mice, a number of allelic exchanged strains were created which express different *ospC* phyletic types. Nine different types were chosen for allelic exchange: OspC type E from *B. burgdorferi* strain DRI-83a, OspC type F from *B. burgdorferi* strain DRI-40g, OspC type H from *B. burgdorferi* strain LDS79, OspC type M from *B. burgdorferi* strain Veery, OspC type PBi

Figure 24. Analysis of dissemination/persistence of LBD2 site-directed mutants. Mice were intradermally inoculated with LBD2 site-directed mutants. Three mice per time point were sacrificed and multiple organs were harvested and placed in media to assess infectivity.



from *B. garinii* strain PBi, OspC type PKo from *B. afzelii* strain PKo, OspC type MOD-1 from *B. andersonii* strain MOD-1, OspC type MOK-3a from *B. andersonii* strain MOK-3a, and OspC type MOS-1b from *B. andersonii* strain MOS-1b (Tables 1 and 2). Controls included previously described strains in which the wild type gene (type A) was introduced in the same genetic context as the mutant (B31::*ospC* (wt)), and in which the gene was deleted entirely (B31Δ*ospC* str^r) (Earnhart *et al.*, 2010). An alignment of the different allelic exchange strains shows the amino acid variability between the OspC types chosen for analysis in this study (Figure 25). Following transformation, streptomycin selection, and establishment of a clonal line by subsurface plating, *ospC* was confirmed by DNA sequencing and the plasmid content of each transgenic strain was determined.

3.6.2 Analysis of infectivity and dissemination of strains expressing wild-type or OspC type switch mutants

In order to test infectivity of the transgenic strains, mice (n=5 per group) were inoculated and four weeks later organs were harvested. Additionally, mice were inoculated with seven of the wild type strains in order to confirm the infectivity of these strains. Spirochetes were re-cultivated for all transgenic strains except B31::*ospC* (type F) and B31::*ospC* (type MOS-1b) in which only a single mouse was culture positive, and B31::*ospC* (type PKo), B31::*ospC* (type MOD-1), and B31Δ*ospC* strains, in which none were positive. Positive cultures were obtained from all mice infected by wild type strains, except *B. afzelii* PKo and *B. andersonii* MOD-1, MOK-3a, and MOS-1b in which none were positive (Table 8).

Figure 25. Alignment of OspC types chosen for allelic exchange into the B31-5A4 background.

```
                                10        20        30        40        50        60        70        80
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B31::ospC(wt)          ............................-T.....................D....L.......A..S....I.A.....
B31::ospC(type E)      MKKNTLSAILMTLFLFISCNNSGKDGN-ASANSADESVKGPNLTEISKKITESNAVVLAVKEVETLLASIDELATKAIGK
B31::ospC(type F)      ............................-T.................................I....S...........Q
B31::ospC(type H)      ............................-.............................................NQ..-.....
B31::ospC(type M)      ............................-T.........................................V.K....N
B31::ospC(type PKo)    .........................G.DS..T.P....A.............D...F..........VL......K....Q
B31::ospC(type PBi)    .........................--G.DS..T.P-...A......V......D...FL.......A..S.....S-.....
B31::ospC(type MOD-1)  ............................S..N.............V.......D........Q...I.........K....Q
B31::ospC(type MOK-3a) ............................S..N......A.....I.......D..........I....S.....GN.....
B31::ospC(type MOS-1b) ............................S..T.PV.........I.......D........Q............I.K....Q
Consensus              ************************  .*: :* *. ***.*****  ******:***.:***:*:* *: **:::. ****:

                                90        100       110       120       130       140       150       160
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B31::ospC(wt)          ..HQ....DTENNH.G...A......T..KQ..DG...-.G......A..K..ET......EK.TD..KEG--V..AD.K
B31::ospC(type E)      KIG-NNGLEANQSKNTSLLSGAYAISDLIAEKLNVLKN-EELKEKIDTAKQCSTEFTNKLKSEHAVLGLDN--LTDDNAQ
B31::ospC(type F)      ..DA.G-.GVQANQ.G...A......T..TQ..SA.N-S.D....VAKV.K..ED......NGN.Q...AA--A.....K
B31::ospC(type H)      ..DQ.GT.GDDGGQ.G...A......TV.I...ST...V.......TK..D..EK.AG...N...S..KKD--A...D.K
B31::ospC(type M)      L.A-Q...N.GANQ.G...A...V..T......DG...S.......ED..K.NKA..D....S..E..IA.GAA..A..K
B31::ospC(type PKo)    ..DN....A.LNNQ.G...A......T..T...SK...L....TE.AK..K..E........G..D..KQD--A...H.K
B31::ospC(type PBi)    ..KNDGT.DNEANR.E..IA...E..K..TQ..S..N-S.......KE..D..EK..T...DS..E..IQS--VQ....K
B31::ospC(type MOD-1)  R.DQG.K.A.DADH.G...A.V.T..T..T....S..IS.......EK..KY.....K..SDS.G...QVGGNA..V..K
B31::ospC(type MOK-3a) ..Q-G...QDL.N..G...A......A..TQ..SA.N-S.......EK..K......K..SDSN.E..IE.--A.....K
B31::ospC(type MOS-1b) R.APG.T.T.DA.R.G..IV.V.T..T..TG...G..DL.D.....EK..K......K..LDS..E..QADGAV.....K
Consensus               *   . *    .:* **: *.* ** :*  **. *:  * ** ::  .*. .  *: ** . :  **       * .*:

                                170       180       190       200       210
                       ....|....|....|....|....|....|....|....|....|....|....
B31::ospC(wt)          E....TNGT.T...E..G...ES..V.....KEM.A.S.......V........
B31::ospC(type E)      RAILKKHANKDKGAAELEKLFKAVENLSKAAQDTLKNAVKELTSPIVAESPKKP
B31::ospC(type F)      A....TNGTN....K..KD.SDS..S.V....VM.T.S.......V........
B31::ospC(type H)      K....T.G.T....K..KD.SDS..S.V...KEM.T.S.......V........
B31::ospC(type M)      A....TNGT.....Q......ES.K.......E..N.S.......V........
B31::ospC(type PKo)    A....T..TT....K.FKD..ES..G.L....VA.T.S.......V........
B31::ospC(type PBi)    K....T.GT.....K...E...SL.S......AA.T.S.....N.V........
B31::ospC(type MOD-1)  A....TNQTT....K..KE.SES..S.A...KEM.NDSI....K.V.T.A....
B31::ospC(type MOK-3a) .....T.S.T...TK..KE.SES..S.A...KEM.N.S.....K.V.T.A....
B31::ospC(type MOS-1b) .....T.N.T....K..KD.SES..S.A...KEM.N.......K.V.T.T....
Consensus               ****.: .. **: *: .* .::: * ***:  * ::*****.*:*:*:****
```

Table 8. Summary of culture results from tissues of mice inoculated with parental strains, *ospC* transgenic control strains, and B31::*ospC* strains expressing different *ospC* types.

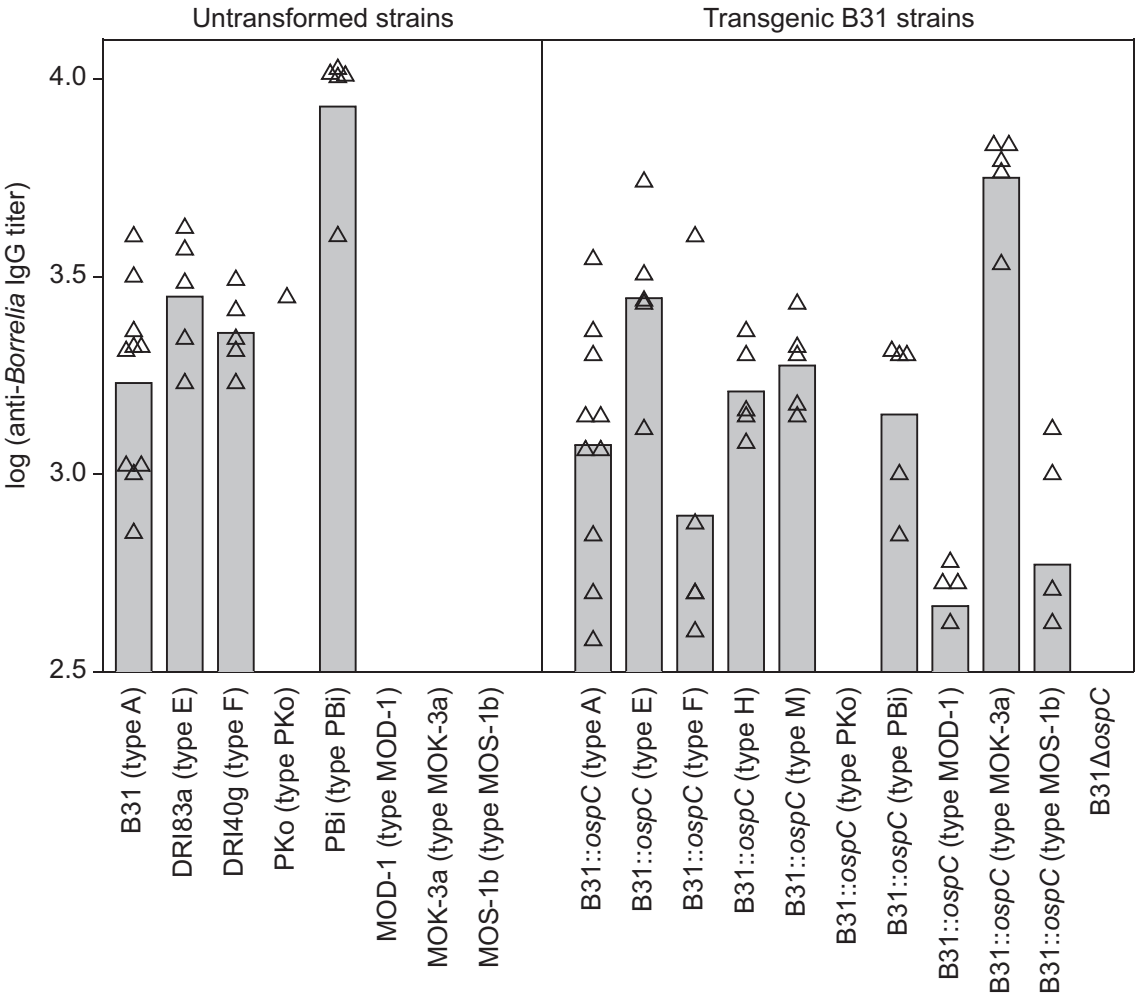
	#Infected/Total	
	Ear	Bladder
Wildtype strains		
B31(type A)	5/5	5/5
DRI-83a (type E)	5/5	4/5
DRI-40g (type F)	5/5	5/5
PKo (type PKo)	0/5	0/5
PBi (type PBi)	5/5	2/5
MOD-1 (type MOD-1)	0/5	0/5
MOK-3a (type MOK-3a)	0/5	0/5
MOS-1b (type MOS-1b)	0/5	0/5
Transformed strains		
B31::*ospC* (wt)	5/5	5/5
B31::*ospC* (type E)	4/5	5/5
B31::*ospC* (type F)	1/5	1/5
B31::*ospC* (type H)	5/5	4/5
B31::*ospC* (type M)	5/5	5/5
B31::*ospC* (type PKo)	0/5	0/5
B31::*ospC* (type PBi)	4/5	5/5
B31::*ospC* (type MOD-1)	0/5	0/5
B31::*ospC* (type MOK-3a)	4/5	3/4
B31::*ospC* (type MOS-1b)	1/5	1/5
B31Δ*ospC*	0/5	0/5

3.6.3 Analysis of the immune response to strains expressing wild-type or OspC type switch mutants

The antibody response to infection was quantified by whole cell ELISA. Antibody titers for sera from mice inoculated with transgenic strains were similar in all mice except those infected with B31::*ospC* (type PKo), B31::*ospC* (type MOD-1), B31::*ospC* (MOS-1b), and B31Δ*ospC* str^r (Figure 26). No mouse inoculated with B31::*ospC* (type PKo) seroconverted. The mice inoculated with strains B31::*ospC* (MOD-1) and B31::*ospC* (MOS-1b) seroconverted but at a lower level than mice inoculated with other strains. The titer for the single culture positive mouse infected with B31::*ospC* (type F) was approximately a log-fold higher than the titer from the culture negative mice, though the titer for these mice was similar to that seen in sera from culture positive animals infected with other strains.

In the analysis of titers produced by mice inoculated with *B. afzelii* PKo wild type, one mouse had a titer similar to that seen in mice inoculated with strains known to be infectious (e.g., B31). PCR amplification and sequencing of *ospC* from DNA extracted from tissue in that mouse demonstrated the presence of type A and E *ospC.* Thus, this infection was likely due to the establishment of infection in that mouse by other strains in the non-clonal primary *B. afzelii* PKo isolate. It should be noted that these strains were not re-cultivated from this mouse. All mice inoculated with *B. andersonii* wild type strains showed no seroconversion.

Figure 26. Immune response of mice inoculated with OspC type allelic exchange mutants. ELISAs were conducted using serum collected from mice four weeks post-inoculation. The infecting strain is indicated along the x axis. The bars show the geometric mean antibody titer while the triangles represent each individual mouse. The sera were serially diluted, screened against whole *B. burgdorferi* 5A4 cells immobilized on ELISA plates, and calculated as the inverse dilution corresponding to one-third of the plateau optical density.



3.7 Investigation of the domain of OspC that influences host specificity of the Lyme *Borrelia*

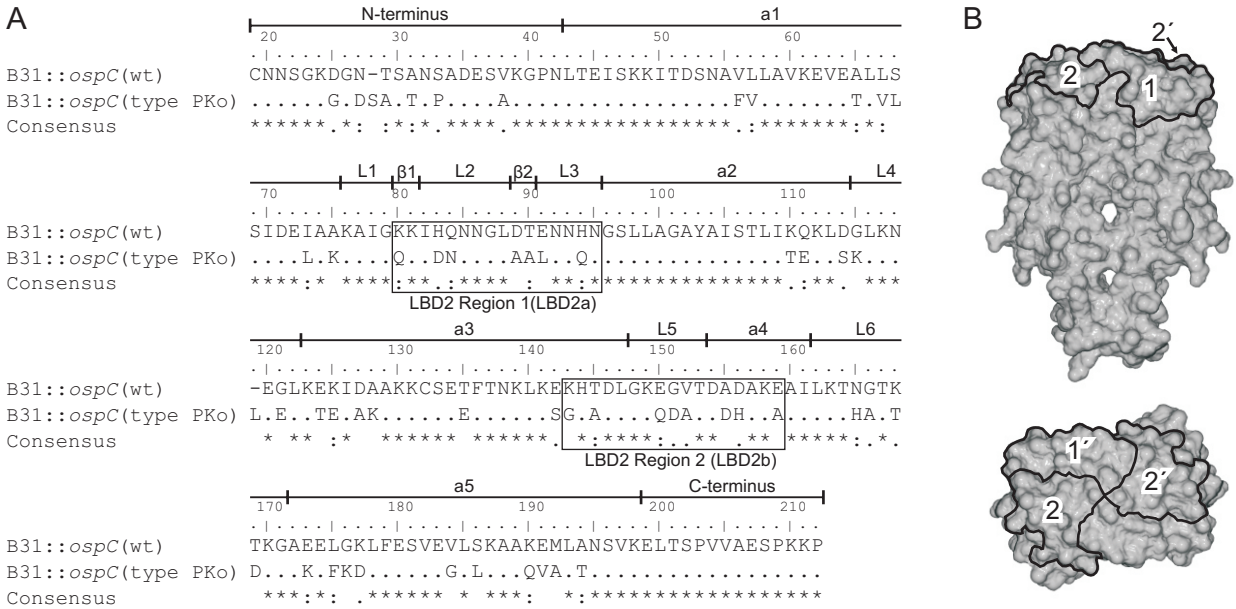
3.7.1 Design and allelic exchange of OspC type A/PKo hybrids

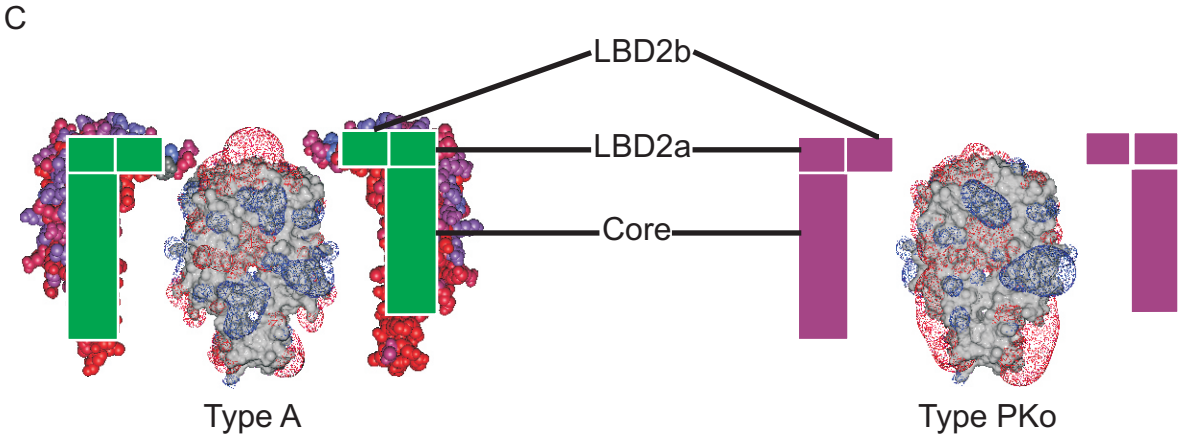
In order to determine the domain of OspC responsible for host species specificity, mutant strains of *B. burgdorferi* were generated that express hybrids of OspC types A and PKo. OspC was divided into three portions, LBD1/OspC core (amino acids 19-78, 94-140, and 158-210), LBD2a (amino acids 79-93), and LBD2b (amino acids 141-157) (Figure 27 A-B). Complete *ospC* genes were produced that contained all possible combinations of these three portions. Three mutants each contained the core of either ospC type A or PKo. The different hybrids were denoted with a common nomenclature that included the first letter of the OspC type that comprised the core, either an upper case A or P, followed by the first letter (lower case) of the type that made up the LBD2a portion and then the first letter of the type of the LBD2b region. For example, B31::*ospC* (hybrid Aap) contains the OspC type A core and LBD2a regions and the OspC type PKo LBD2b region (Figure 27 C). The complete genes were ligated into the pCAEV1 vector and transformed into *B. burgdorferi* to generate OspC hybrid mutant strains (Earnhart *et al.*, 2010). Recombinant protein of the different hybrids was also generated.

3.7.2 Analysis of infectivity of OspC type A/PKo hybrid strains in mice

To determine which portion(s) of OspC is necessary for infectivity in mice, CeH/HeJ mice were inoculated subcutaneously with spirochetes by needle injection. Four weeks post-inoculation the mice were sacrificed and the ear, heart, bladder and

Figure 27. Design of OspC type A/PKo hybrid genes. (A-B) OspC was divided into three portions, LBD1/OspC core (amino acids 19-78, 94-140, and 158-210), LBD2a (amino acids 79-93), and LBD2b (amino acids 141-157). Boxes and an OspC model are used to highlight the two portions of LBD2 chosen. (C) Schematic of the OspC hybrids created.





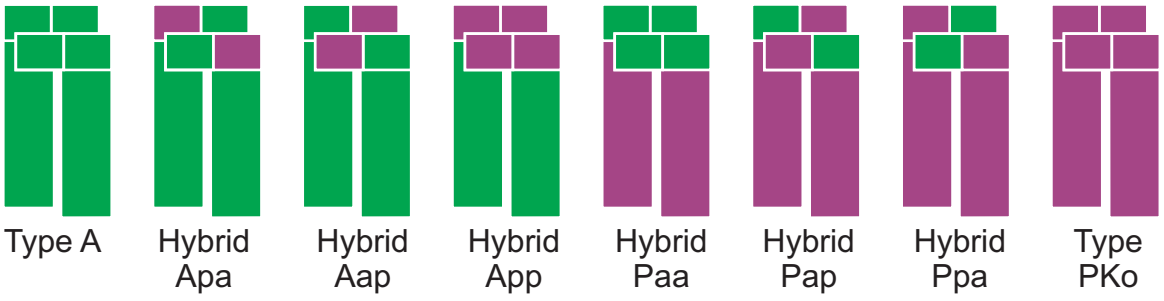


Table 9. Summary of culture results from tissues of mice inoculated with *ospC* transgenic control strains and B31::*ospC* (A/PKo) hybrid strains.

	#Infected/Total				
Strain	Ear	Bladder	Heart	Joint	Total[a]
B31::*ospC* (wt)	4/4	4/4	2/4	3/4	4/4
B31::ospC (type PKo)	0/4	0/4	0/4	0/4	0/4
B31::*ospC* (hybrid Aap)	2/4	2/4	2/4	2/4	4/4
B31::*ospC* (hybrid Apa)	4/4	2/4	4/4	2/4	4/4
B31::*ospC* (hybrid App)	4/4	3/4	2/4	3/4	4/4
B31::*ospC* (hybrid Paa)	0/4	0/4	0/4	0/4	0/4
B31::*ospC* (hybrid Pap)	4/4	2/4	3/4	3/4	4/4
B31::*ospC* (hybrid Ppa)	4/4	2/4	4/4	1/4	4/4

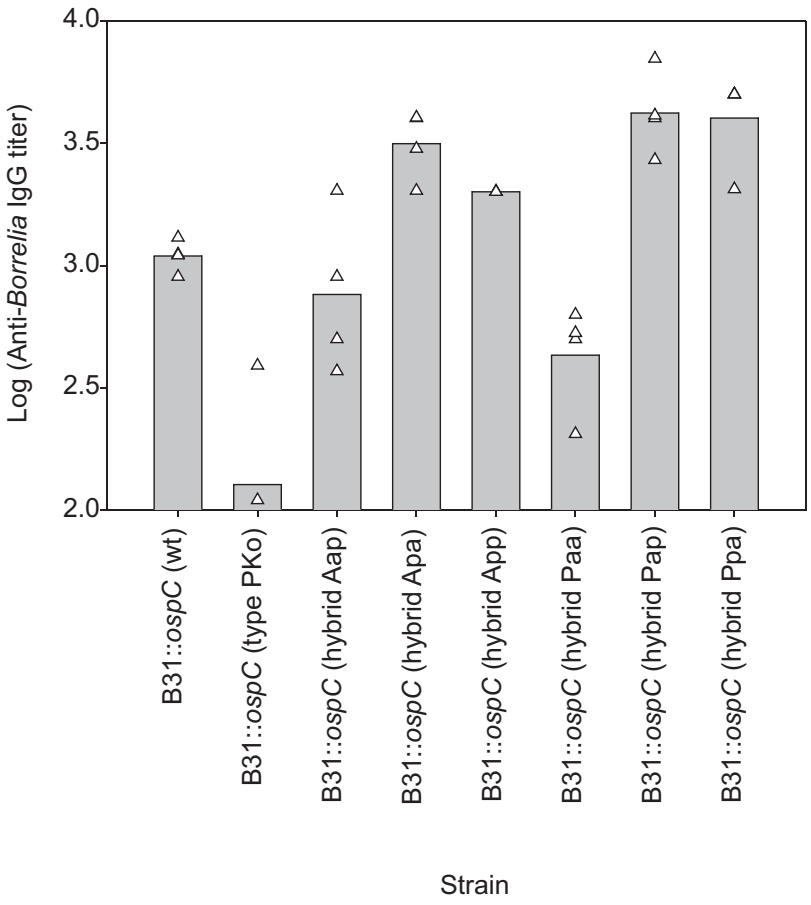
[a] Total mice infected column included due to the fact that organ data does not accurately depict number of infected animals.

tibiotarsal joint tissues were harvested and cultured in order to assess infectivity. The strains B31::*ospC* (type PKo) and B31::*ospC* (hybrid Paa) showed no spirochetes in any of the organs cultivated (Table 9). All other strains were able to be cultivated out of at least one organ cultured.

3.7.3 Analysis of immune response to OspC A/PKo hybrid strains generated by mice.

ELISA using immobilized whole *B. burgdorferi* cells were used to assess the humoral response generated by both mice and hamsters inoculated with the OspC type A/PKo hybrid mutants. All mutants that were culture positive also displayed high IgG titers. Mice inoculated with B31::*ospC* (type PKo) had a detectable but extremely low titer while mice inoculated with B31::*ospC* (hybrid Paa) produced a titer that was similar to infectious strains (Figure 28).

Figure 28. Immune response of mice inoculated with OspC type A/PKo hybrids. ELISAs were conducted using serum collected from mice four weeks post-inoculation. The infecting strain is indicated along the x axis. The bars show the geometric mean antibody titer while the triangles represent each individual mouse. The sera were serially diluted, screened against whole *B. burgdorferi* 5A4 cells immobilized on ELISA plates, and calculated as the inverse dilution corresponding to one-third of the plateau optical density.



Chapter 4: Discussion

4.1 OspC residue C130 plays a role in oligomerization

OspC is an important virulence factor for the Lyme *Borrelia* (Earnhart *et al.*, 2007; Earnhart and Marconi, 2007b, c; Grimm *et al.*, 2004; Pal *et al.*, 2004; Stewart *et al.*, 2006; Tilly *et al.*, 1997; Tilly *et al.*, 2006; Tilly *et al.*, 2007). Efforts to define the function of OspC have been significantly enhanced by recent analyses of OspC structure-function relationships (Earnhart *et al.*, 2010; Eicken *et al.*, 2001; Kumaran *et al.*, 2001a; Kumaran *et al.*, 2001b; Wang *et al.*, 1999). A postulate that has not yet been tested is that OspC may form functionally and immunologically significant arrays in the *Borrelia* outer membrane (Lawson *et al.*, 2006). While freeze-fracture microscopic analyses indicate the existence of membrane protein arrays in *B. burgdorferi,* the composition of these arrays have not yet been determined (Radolf *et al.*, 1994). Here we test the hypothesis that hydrophobic based-OspC dimers form biologically relevant higher order oligomers that result from interdimeric disulfide bonding mediated by residue C130. To assess this, a recombinant site-directed OspC mutant protein (r-OspC (C130A)) and a *B. burgdorferi* strain that produces this site-directed mutant (B31::*ospC* (C130A)) were generated and OspC oligomerization tested (Earnhart *et al.*, 2011). Recombinant wild type OspC formed oligomeric chains of two to five dimers (Figure 14). Formation of these oligomers was eliminated by reducing conditions and proved to be strictly

dependent on C130 (Figure 13). While technical limitations prevented the direct assessment of OspC oligomeric length at the *Borrelia* cell surface, the occurrence of disulfide bonded OspC at the cell surface and the data obtained with recombinant proteins support the notion that disulfide-dependent oligomers form in vivo.

Direct functional assays for OspC have not yet been defined. However, due to the essential nature of OspC, mutations that perturb critical determinants of the protein can be identified by assessing infectivity in mice. To determine if OspC oligomerization is required for OspC to carry out its in vivo function, wild type and B31::*ospC* (C130A) strains were inoculated into mice and infectivity and dissemination assessed. While substitution of C130 with Ala prevents higher order oligomerization, it did not abolish infectivity as inferred by seroconversion and the ability to cultivate spirochetes from ear punch biopsies of inoculated mice. Dissemination was assessed by cultivation of biospy samples harvested at sites distal from the original inoculation site (Table 4). An apparent difference in dissemination potential was not observed. These analyses definitively demonstrate that disulfide bond mediated oligomerization is not required for survival and dissemination within the mammalian host.

In summary, it can be concluded that OspC is able to form oligomers on the cell surface of *B. burgdorferi*. It has also been shown through this study that disulfide bond mediated oligomerization is not required for OspC to function in the establishment of infection, dissemination or the generation of a robust and potentially productive antibody response(Earnhart *et al.*, 2011).

4.2 The highly conserved C-terminal domain of OspC is not required for OspC function in the mouse model

The highly conserved C-terminal 10 amino acids of OspC have been hypothesized to have multiple functions. Here the potential functional role and antigenicity of the conserved C-terminus of OspC is investigated. To accomplish this, *ospC* was replaced in its native location with *ospC* lacking the C-terminal ten amino acids (C10). The resultant B31::*ospC* (ΔC10) strain was screened for plasmid loss, growth rate, and surface expression and presentation of OspC, all of which are similar to wild type. It can be concluded that deletion of C10 does not alter expression or export of OspC to the outer membrane (Kumru *et al.*, 2011) (Figures 9 and 10).

Targeted mutations in conserved, surface-exposed OspC residues have been successfully used to define structurally and functionally important domains and residues (Earnhart *et al.*, 2010; Earnhart *et al.*, 2011). In this study it is hypothesized that conservation in the C10 sequence (PVVAESPKKP) is due to functional constraints on sequence variation. Several functions for this domain have been proposed. It has been suggested that C10 adopts a polyproline II helical conformation (Mathiesen *et al.*, 1998b) – a secondary structure associated with protein-protein interactions and bacterial adhesins (Brady *et al.*, 2010). C10 also has sequence similarity with the consensus recognition sequence for src homology domain 3 (SH3) proteins – a potential bacterial virulence mechanism (Aitio *et al.*, 2010; Ren *et al.*, 1993). Lagal et al. (2006) hypothesized that the C-terminal lysine residues are involved in binding plasminogen. Cell surface associated plasminogen could aid in spirochete dissemination through the proteolysis of extracellular matrix proteins. To determine if

C10 participates in plasminogen binding, r-OspC (ΔC10) was used in binding assays to determine if the C10 amino acids facilitate this interaction (Figure 16). Deletion of the C10 domain did not have a significant impact on plasminogen binding, therefore it can be concluded that C10 is not required for plasminogen binding.

To determine if C10 domain is required for OspC function in vivo, mice were inoculated with control or transgenic strains. All mice inoculated with B31, B31::*ospC* (wt) and B31::*ospC* (ΔC10) strains yielded positive cultures. As expected, the negative control B31Δ*ospC* strain was non-infectious. The immune response generated by mice inoculated with these strains confirmed infectivity. Thus, by this route of infection, the C10 domain is not required for OspC function in vivo (Table 5).

OspC is of considerable interest as a diagnostic antigen because of its early and high level expression during mammalian infection. A number of studies have used peptides based on the conserved C-terminal 7 to 10 amino acids of OspC as an immobilized antigen to detect early *Borrelia*-specific immune responses (Bacon *et al.*, 2003; Du *et al.*, 2007; Jobe *et al.*, 2003; Jobe *et al.*, 2008; Lovrich *et al.*, 2005; Lovrich *et al.*, 2007; Mathiesen *et al.*, 1996; Mathiesen *et al.*, 1998a; Mathiesen *et al.*, 1998b; Nagel *et al.*, 2008; Panelius *et al.*, 2002; Porwancher *et al.*, 2011; Qiu *et al.*, 2000). The majority of these studies have found an early, predominantly IgM response to this epitope, and it has been suggested that this may be due to a T-cell-independent response mediated by the presentation of this epitope in a repetitive fashion at the cell surface (Du *et al.*, 2007; Earnhart *et al.*, 2011; Mathiesen *et al.*, 1998b). Alternatively, it has been suggested that the predominant IgM response is associated with infection in certain niches (e.g., the central nervous system in neuroborreliosis) (Fung *et al.*, 1994;

Mathiesen *et al.*, 1998b). The data presented herein demonstrate that the C-terminus can induce an IgG response following infection by needle inoculation in mice (Figure 17). Comparison of the reactivity of sera generated during infection with B31, B31::*ospC* (wt), or B31::*ospC* (ΔC10) demonstrates that while C10 induces an IgG response, it is not an immunodominant epitope. This is clearly demonstrated by the similarity in IgG titers of mice infected with B31 versus B31::*ospC* (ΔC10) when screened against r-OspC (wt) and r-OspC (ΔC10). The response to the C10 domain is in agreement with previous epitope mapping studies, though the minor relative contribution of the C10 domain was not evident in the non-quantitative maps (Earnhart *et al.*, 2005). Mapping studies have also demonstrated an early response to linear epitopes in the loop 5 and alpha helix 5 regions (Earnhart *et al.*, 2005). Based on the current data, the sensitivity of an OspC-based diagnostic would likely be further improved by inclusion of epitopes derived from regions outside of C10. Antibodies generated against the OspC C10 epitope have been found to be bactericidal against *Borrelia* in vivo (Lovrich *et al.*, 2005; Lovrich *et al.*, 2007). In contrast with earlier reports of poor immunogenicity of this domain in mice (Lovrich *et al.*, 2005), in the current study there was clearly an induction of C10 specific IgG during infection. The induced antibodies are specific to the C-terminus, as evidenced by the lack of any anti-C10 reactivity in mice infected with B31::*ospC* (ΔC10).

4.3 OspC LBD1 plays a crucial role in the ability of *B. burgdorferi* to establish mammalian infection

While gene deletion analyses have advanced the understanding of when OspC participates in the enzootic cycle, its biological function remains unknown (Earnhart *et*

al., 2010; Fingerle *et al.*, 2007; Gilmore and Piesman, 2000; Grimm *et al.*, 2004; Pal *et al.*, 2004; Tilly *et al.*, 2006; Tilly *et al.*, 2007). In this study, the hypothesis that LBD1 is a key determinant of OspC function in the mammalian environment was tested. Site-directed mutations were introduced into the type A *ospC* gene of *B. burgdorferi* and the mutated genes were transformed into an isogenic background. Residues K60, E61, and E63, which are invariant or highly conserved (Earnhart and Marconi, 2007a) and which possess side chains that extend into the solvent accessible pocket of LBD1, were targeted for mutagenesis. Analysis of the resulting recombinant strains revealed that the expression, export, and presentation of the modified proteins on the cell surface were similar to the wild type strain. All strains maintained wild type growth rate and the full complement of plasmids (with the exception of the non-essential lp21 in some strains). Infectivity was assessed in mice through needle inoculation with cultivation and seroconversion serving as the read out. The parental wildtype B31-5A4 strain (untransformed), B31::*ospC* (wt), B31::*ospC* (E63Q), and the B31::*ospC* (K60Y) strains were infectious while B31Δ*ospC*, B31::*ospC* (E61Q/E63Q), and B31::*ospC* (E61Q) strains were non-infectious. It is striking that a single amino acid substitution at position 61 proved sufficient to render cells non-infectious, presumably by inactivating the critical functions associated with the OspC protein during the establishment of infection. For those strains that retained infectivity, the possibility that the amino acid substitutions introduced into LBD1 affect dissemination characteristics was assessed by determining the number of spirochetes present at different anatomical sites by q-PCR. Higher numbers of B31::*ospC* (E63Q) in the urinary bladder and lower numbers of B31::*ospC* (K60Y) in the heart were observed (Earnhart *et al.*, 2010). These results are consistent

with the hypothesis that dissemination characteristics can be attributed, at least in part, to the physiochemical properties of OspC (Alghaferi *et al.*, 2005; Brisson and Dykhuizen, 2004; Earnhart *et al.*, 2005; Jones *et al.*, 2006; Seinost *et al.*, 1999; Wormser *et al.*, 2008).

There are several possible explanations for the inability of the B31::*ospC* (E61Q) and B31::*ospC* (E61Q/E63Q) strains to establish infection. Since these strains are isogenic derivatives of the parental strain, the most direct explanation is that the loss of infectivity is a direct result of the modification of the OspC itself. The loss of infectivity would imply that OspC has a specific function during early stage infection that cannot be fulfilled by any other *Borrelia* protein. As discussed above, the biological properties of these and control strains suggest that they are not compromised in any way. At the molecular level, the amino acid substitutions could cause structural changes that ablate function. However, this possibility is not supported by circular dichroism and dimerization analyses that indicate that OspC structure and dimerization are largely unaffected. A second possibility is a direct role for residue E61 as a ligand contact point. Alternatively, E61 may indirectly influence the function of LBD1 through alteration of its local surface charge. While this possibility cannot be ruled out, it is noteworthy that the CD spectra for the E63Q and E61Q mutants which are associated with infectivity and loss of infectivity, respectively, have nearly identical surface charge maps (data not shown). In either case, it is clear that LBD1 and E61 are central in OspC's in vivo function.

This data is the first to provide direct evidence that a specific domain of OspC is required for in vivo function. Specifically, it has been demonstrated that residue E61 is

required for infectivity and suggestive evidence has been provided that residues E63 and K60 influence dissemination. While these may not be the only functionally important residues of OspC, the data firmly support the importance of LBD1 in OspC function. The results presented here challenge recent hypotheses regarding the role of OspC in *Borrelia* pathogenesis. Consistent with the phylogenetic, structural, and charge properties of OspC, our lab favors the hypothesis that OspC binds a small ligand that may be present in blood, extracellular fluid or possibly tissue that functions to facilitate the adaptive changes required for survival during the early stages of infection. Once the spirochetes adapt, OspC is no longer required and is down-regulated. Such a model is consistent with the requirement of OspC for establishing infection in mice by tick or needle inoculation routes but not by tissue transplantation (Tilly *et al.*, 2006). In the latter case, the spirochetes would already be "host-adapted" and would not require OspC. With wild type and "loss of function" OspC mutant proteins in hand, it may be possible to devise high-throughput screening assays designed to identify OspC ligands that interact with LBD1 and thus unravel the function of OspC in *Borrelia* biology.

4.4 Alteration of surface charge on LBD2 of *B. burgdorferi* OspC results in a failure to persist in the mouse model

The largest amount of intertype variability of OspC occurs in LBD2. This variation results in great changes in the electrostatic potential of the protein in this area. In order to investigate the role of surface charge of OspC LBD2 in *B. burgdorferi* pathogenesis, two site-directed mutants were generated, B31::*ospC* (HNN) and B31::*ospC* (NDE). Both of these mutants contained all the B31 plasmids, typical growth rates, and express OspC normally. Upon infection in mice, it was found that the

B31::*ospC* (HNN) mutant retained full infectivity and was able to disseminate to both ear and bladder tissues. Conversely, the B31::*ospC* (NDE) mutant, while able to disseminate normally, was completely cleared from mice by fourteen days post-inoculation. This result indicated that the B31::*ospC* (NDE) mutant is unable to persist in the mouse model.

There are several physicochemical differences between the B31::*ospC* (NDE) mutant and the known *B. burgdorferi* OspC types, including differences in predicted electrostatic surface charge and isoelectric point (pI). Electrostatic charge maps were generated for both LBD2 mutants. The B31::*ospC* (HNN) mutant was found to have a large dome of negative charge on LBD2 while the B31::*ospC* (NDE) mutant has very little negative charge and an expansion of positive charge around LBD2. Charge maps have been generated by our lab for several additional OspC types including OspC types A, E, F, H, M, PKo and PBi. None of the OspC types that have been mapped have as much positive charge on LBD2 as the B31::*ospC* (NDE) mutant. Additionally, the pI of LBD2 was calculated for the OspC types commonly found in *B. burgdorferi* and for the LBD2 mutants. While the pI of the B31::*ospC* (HNN) mutant (pI = 4.35) fell within the range of the other OspC types (4.03 – 5.37), the pI of B31::*ospC* (NDE) LBD2 was greater, being 6.28. These differences in surface charge and pI may account for the differences in observed with the B31::*ospC* (NDE) mutant in vivo.

There have been other studies in which *B. burgdorferi* mutants were able to cause infection but not persist in the animal model. For example, a previous study expressed *ospC* in an *ospC* deletion strain on a nonessential plasmid. In this study *ospC* was under the control of a constitutively active *flaB* promoter. When

immunocompetent mice were inoculated with this strain, the only recoverable spirochetes had lost this plasmid. Due to the fact that OspC is highly antigenic during infection, a robust immune response is generated against this protein. It was suggested that the anti-OspC immune response mounted by the host provides a selective pressure against the spirochetes expressing OspC, clearing all spirochetes that did not lose the plasmid (Tilly *et al.*, 2006). This study was able to show that when OspC is not downregulated in the correct timeframe, the humoral immune response is able to clear the infection. Additionally, it has been shown that *B. burgdorferi* strains lacking the plasmid lp28-1, which harbors the VlsE gene system, are cleared from immunocompetent mice between seven and fourteen days post-inoculation (Embers *et al.*, 2008). It has also been demonstrated that spirochetes lacking lp28-1 showed higher and sustained OspC production during the shortened infection in immunocompetent mice, infection in SCID mice, and when cultured in dialysis membrane chambers (DMCs). This study hypothesized that a repressor encoded on lp28-1 is responsible for *ospC* downregulation and when this plasmid is missing, continued OspC expression results in clearance by the humoral immune response (Embers *et al.*, 2008). Due to the fact that the surface charge of the B31::*ospC* (NDE) mutant is changed significantly from that of other OspC types, the change in charge could result in disrupted or altered interaction with this hypothesized repressor. If a change in this interaction occurred, it would account for the lack of persistence of the B31::*ospC* (NDE) mutant strain.

The research presented here on LBD2 suggests that the surface charge of the variable portion of OspC plays an important role in the ability of *B. burgdorferi* to persist

in mice during infection. The spirochetes are able to disseminate to multiple organs/tissues in the body and induce an antibody response in the mice but the infection is cleared between seven and fourteen days post-inoculation. This is the first study to demonstrate the role of a specific portion of OspC in *B. burgdorferi* persistence. In the future, studies to confirm antibody clearance of the B31::*ospC* (NDE) mutant by the immune response will be performed in order to begin investigating the role of this portion of OspC in *ospC* downregulation.

4.5 Allelic exchange replacement of *ospC* demonstrates the role of OspC phyletic types in establishment of murine infection

Multiple OspC phyletic types have been identified from the *Borrelia* strains *B. burgdorferi*, *B. garinii*, and *B. afzelii*. These different OspC types have been shown to have type-specific differences in reservoir host specificity/association and in the presence of human disseminated infection. In this study, multiple OspC types were chosen to be inserted into the B31-5A4 genetic background using allelic exchange, replacing the native OspC type A. For this study, OspC types were selected from a variety of different *Borrelia* species. OspC types E, F, H, and M are found in the *B. burgdorferi* strains DRI-83a, DRI-40g, LDS79, and Veery, respectively. OspC types PBi and PKo were amplified from the European Lyme disease strains *B. garinii* strain PBi and *B. afzelii* strain PKo and finally, the *B. andersonii* OspC types MOD-1, MOK-3a, and MOS-1b were cloned from the strains of the same name. There were varying reasons for the selection of these different OspC types including differences in tissue dissemination, isolate origin, and host species specificity/association. To confirm the infectivity status of the strains from which the OspC types were chosen, these strains

were injected into mice and all were found to be infectious except for *B. afzelii* PKo and all three *B. andersonii* strains. Once the selected OspC types were inserted into B31-5A4 in place of the native type A *ospC* gene, the resulting transgenic strains were used to investigate the effect of OspC type alone on infectivity in the mouse. Most of the transgenic strains were found to retain full infectivity in mice (B31::*ospC* (wt), B31::*ospC* (E), B31::*ospC* (H), B31::*ospC* (M), B31::*ospC* (PBi), and B31::*ospC* (MOK-3a)) but four strains showed altered infectivity. Two of the OspC type switch strains, B31::*ospC* (F) and B31::*ospC* (MOS-1b), were cultivable from only one out of five mice inoculated with these strains while another two transgenic strains, B31::*ospC* (PKo) and B31::*ospC* (MOD-1), were unable to infect mice. This is the first study to show a relationship between OspC type and infectivity in an animal model, regardless of the rest of the genetic background of the infecting strain.

The wild type strains used in this analysis have varying host specificities. The parental strain *B. burgdorferi* B31-5A4 is used extensively in vitro and is known to be highly infectious in mice. This strain was originally isolated from a tick collected in New York (Fraser *et al.*, 1997). Several strains in this analysis were obtained from humans with Lyme disease, including *B. afzelii* strain PKo, *B. garinii* strain PBi, and *B. burgdorferi strain* LDS79. All these strains were found to infect mice except *B. afzelii* PKo. This strain has previously been shown to be unable to cause murine infection (Bockenstedt *et al.*, 1997) but is able to infect gerbils (Preac-Mursic *et al.*, 1992) and hamsters (data not shown). Several of the wild type strains used in these analyses were isolated from animals. The strains from which OspC types E and F were amplified, DRI-83a and DRI-40g, were isolated from dogs experimentally infected with

B. burgdorferi. OspC type M comes from the Veery strain which was isolated from a bird (Anderson *et al.*, 1986). Additionally, all of the *B. andersonii* strains used were isolated from ticks obtained from rabbits (Lin *et al.*, 2002). The differing locations of origins of these isolates made them attractive candidates for an analysis into the role of OspC in host specificity.

The cause for the lack of infectivity or reduced infectivity of the PKo, MOD-1, type F, and MOS-1b transgenic strains could be attributed to multiple possibilities. The B31::*ospC* (type PKo) and B31::*ospC* (type MOD-1) support the hypothesis that the OspC protein mediates infectivity in different hosts. The B31::*ospC* (type PKo) strain did not grow out of any organs nor did the mice inoculated with this strain show any seroconversion. As the only difference between this strain and the parental B31-5A4 is the presence of OspC type PKo instead of OspC type A, it is possible that it is the presence of OspC type PKo that renders this previously infectious strain non-infectious in mice. The B31::*ospC* (MOD-1) strain is also noncultivable, yet the mice inoculated with this strain did seroconvert at a very low level. The B31::*ospC* (MOS-1b) strain was only able to be cultivated out of one mouse and showed a low level of seroconversion. With the wild type *B. andersonii* MOD-1 and MOS-1b strains both being noninfectious, these results indicate that perhaps these two strains were able to set up but not maintain a long term infection in mice. These three transgenic strains support the hypothesis that OspC plays a role in the ability of *B. burgdorferi* to infect mice.

The infection data from two transgenic and wild type strains contradict the data presented above. First, the wild type strain for OspC type F was found to be fully infectious in mice while the transgenic strain, B31::*ospC* (type F), showed reduced

infectivity. Of the five mice inoculated with this strain, only one mouse showed spirochete grow out and several others produced low level seroconversion. This could be due to altered dissemination. Only ear and bladder tissues were used for infectivity analysis. It is possible that the B31::*ospC* (type F) strain has altered dissemination and was not present in detectable numbers in the tissues that we collected. Repeating this infection and sampling more tissues might help to clarify this result. The data obtained from the wild type and transgenic strains harboring OspC type MOK-3a produced results that are actually the opposite of the hypothesis. The wild type *B. andersonii* strain was noninfectious in mice but the transgenic strain was both cultivable and induced a robust immune response. This result could be due to problems with the wild type strain. This strain was isolated from a nymphal tick collected off of a rabbit. The full plasmid repertoire of *B. andersonii* is not known so it is possible that the wild type *B. andersonii* MOK-3a strain is missing plasmids necessary for infection. If this is the case and lack of infectivity of the wild type strain is due to a factor other than the OspC type, full infectivity of the transgenic strain would be expected. Further analysis of these wild type and transgenic strains is warranted to clarify these results.

In summary, B31-5A4 mutants expressing different OspC types have been used to show a relationship between OspC type and infectivity in mice. These analyses have shown that OspC type alone is enough to render a previously infectious strain non-infectious. While further research is needed to fully understand this relationship, it is clear that OspC type does play a role in the ability of *B. burgdorferi* to infect certain hosts.

4.6 Investigation of the domain of OspC that influences host specificity of the Lyme *Borrelia*

OspC types A and PKo have been shown to have differing host specificities. As discussed above, the *B. burgdorferi* B31-5A4 strain that expresses OspC type A is highly infectious in mice while the *B. afzelii* strain PKo that expresses OspC type PKo is not. As described in the above section, an allelic exchange mutant of B31-5A4 expressing OspC type PKo instead of type A completely lost the ability to cause infection in mice. In order to further investigate this finding, B31-5A4 mutants were created that express OspC hybrids of types A and PKo. Infectivity analysis showed that in addition to the B31::*ospC* (type PKo) strain only one OspC hybrid-expressing strain, B31::*ospC* (hybrid Paa), lost the ability to maintain an infection in mice.

There are a variety of reasons that this particular transgenic strain was uncultivable from mice. The strains B31::*ospC* (type PKo) and B31::*ospC* (hybrid Paa) have both similarities and differences. Both strains contain the exact same genetic background and only have one plasmid difference. B31::*ospC* (type PKo) is missing plasmid lp5, which has been shown to have no effect on in vitro cultivation. Both of these strains were shown to export and present OspC at the same levels as all other strains tested while also growing in vitro at the same rate. Lastly, both strains, along with the fully infectious hybrids B31::*ospC* (hybrid Pap) and B31::*ospC* (hybrid Ppa), contain the core of OspC type PKo, which includes LBD1. The major difference between these strains exists on the LBD2 portion of the protein. The B31::*ospC* (hybrid Paa) strain expresses an OspC protein with the full type A LBD2. B31-5A4, when expressing OspC type A, is highly infectious in mice. Therefore, all portions of the

hybrid Paa OspC protein can be found in strains that are infectious in the mouse. This data suggests that there is not one clear potion of OspC that is involved in infectivity in a particular host.

The mice inoculated with the B31::*ospC* (hybrid Paa) strain generated a low but detectable immune response (Figure 28). This suggests that, like many of the previously described mutant strains, the B31::*ospC* (hybrid Paa) mutant was able to briefly establish an infection but was then cleared. It is thought that OspC plays multiple roles in the pathogenesis of *B. burgdorferi* and mutants such as the B31::*ospC* (hybrid Paa) strain indicate that perhaps not all OspC proteins are able to carry out the different functions of this protein. This could be the cause for very little observed recombination of OspC in nature. Perhaps recombination events occur more regularly than previously thought but the new OspC types are not functional. In summary, based on these results it appears that no one portion of OspC mediates the ability of *B. burgdorferi* to infect different animals.

4.7 Summary

The data presented in this dissertation reflects the great body of knowledge about OspC that has been generated by our lab. OspC is one of the few known virulence factors of *B. burgdorferi* and the study of the structure/function relationships of this protein is complicated by the lack of an identified functional ligand and/or a defined mechanism of action. Our lab has developed a novel approach to study this protein by creating allelic exchange mutants that express any OspC type or mutant from the native location of *ospC* in the *B. burgdorferi* genome. Most studies focusing on OspC utilize

deletion strains that express *ospC* mutations from a plasmid (Pal *et al.*, 2004; Tilly *et al.*, 2006; Tilly *et al.*, 2007; Yang *et al.*, 2005). The method used in our lab allows us to study OspC that is expressed in the same way as wild type and without differences in plasmid copy number. Also, creating allelic exchange mutants has allowed for the targeted study of different portions of OspC and how these portions play a role in OspC function without having a defined ligand. Using this allelic exchange method and generating many different mutations to the conserved and variable portions of OspC has helped our lab to create a comprehensive map of the structure function relationships of OspC. These mutants provide an ideal tool for deciphering the ligand and/or function of OspC.

The calculated size of the solvent accessible pocket of LBD1 suggests its potential ligands would be relatively small (Eicken *et al.*, 2001; Kumaran *et al.*, 2001b). It was postulated in an earlier study that the region of OspC, designated by our lab as LBD1, may be an aspartate binding domain (Eicken *et al.*, 2001). The *Salmonella typhimurium* aspartate receptor, which is involved in chemotaxis and signaling (Yeh *et al.*, 1996), possesses a four alpha helical bundle similar to that present in OspC. However, other than structural similarity there is no discernable sequence homology between these proteins. While it seems unlikely that OspC binds aspartate, the general hypothesis that LBD1 is involved in the binding of small ligands is supported by its structural properties, the results presented herein. While the identity of the ligands that interact with LBD1 remain unknown, there is now strong and compelling evidence that LBD1 is a key functional determinant of OspC that plays an essential role in the establishment of infection in mammals.

It is important to note that OspC has been reported to bind at least two other ligands, the Salp15 protein of *Ixodes* ticks (Das *et al.*, 2001; Hovius *et al.*, 2007) and the mammalian-derived protein plasminogen (Lagal *et al.*, 2006). The domains and residues of OspC that are involved in these interactions have not been identified. In this dissertation, the OspC interaction with Salp-15 was not specifically assessed. Plasminogen binding assays were conducted with all mutant strains described in this dissertation (Figure 16 and data not shown). None of the mutants displayed attenuated plasminogen binding, therefore it does not appear that plasminogen is the critical ligand for OspC. Interestingly, the E61Q/E63Q protein, a mutant strain that was shown to be noninfectious, bound plasminogen at a higher level than wild type protein. The extent to which the OspC-plasminogen interaction contributes to pathogenesis remains to be directly assessed and such analyses are complicated by the fact that *Borrelia* produce several plasminogen binding proteins including OspE and CspZ (factor H binding proteins) (Brissette *et al.*, 2009; Hovis *et al.*, 2006).

Several opposing hypotheses have been offered regarding OspC function (Radolf and Caimano, 2008). It has been postulated that OspC does not have a specific or unique function and instead, by simple virtue of its abundance, serves to maintain membrane integrity (Xu *et al.*, 2008). It was reported that the introduction of random lipoprotein genes (OspE, OspA, VlsE and DbpA) on an autonomously replicating plasmid restored infectivity to an *ospC* knock out strain in SCID mice (but less so in immunocompetent mice). From this it was concluded that structurally unrelated proteins are functionally equivalent (i.e., the “surrogate hypothesis”). If OspC can be replaced by randomly selected, unrelated *Borrelia* lipoproteins, it is not clear why

OspC variants with single amino acid substitutions that are unaltered in structure and expressed at wild type levels, cannot complement the putative membrane integrity maintenance function of OspC. In addition, if OspC could be replaced by other lipoproteins, it could be argued that *ospC*-deficient strains would have emerged in nature since there would be no selective advantage in maintaining such a gene. Analyses of hundreds of Lyme disease isolates have showed that OspC is universal in all Lyme *Borrelia* species and homologs are carried by all relapsing fever spirochete species and isolates [(Marconi *et al.*, 1993b; Margolis *et al.*, 1994). These facts, coupled with *ospC* phylogenetic analyses which indicate selective constraints on OspC sequence variation within specific domains, including LBD1, C10, and C130 (Attie *et al.*, 2007; Baranton *et al.*, 2001; Earnhart and Marconi, 2007a; Lagal *et al.*, 2002; Lin *et al.*, 2002; Qiu *et al.*, 2008; Theisen *et al.*, 1993), strongly argue against the "surrogate hypothesis".

In this dissertation, we have presented the first studies to (a) demonstrate the presence of OspC oligomers in vitro and in vivo while also identifying the residue that mediates oligomer formation, (b) identify a specific domain and a single amino acid critical to OspC function, (c) demonstrate that changes in OspC surface charge can alter persistence, and (d) abolish infectivity in a certain host by changing only the OspC type expressed by the inoculating strain. We have also shown that the C10 portion of OspC does not appear to interact with plasminogen or have a role in infectivity. In conclusion, it is clear that OspC plays a vital role in the ability of *B. burgdorferi* to cause infection and that the many functional roles of OspC are mediated by domains/residues in both the conserved and variable portions of this protein. The research presented in

this dissertation has added significantly to the body of knowledge of the role of OspC in *B. burgdorferi* pathogenesis.

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VITA

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Manuscripts

Earnhart, C. G., **LeBlanc, D. V**., Alix, K. E., Desrosiers, D. C., Radolf, J. D., and Marconi, R. T. 2010. Identification of residues within ligand binding domain 1 (LBD1) of the *B. burgdorferi* OspC protein required for function in the mammalian environment. Molecular Microbiology 76(2):393-408.

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