REGULATION OF OUTER SURFACE LIPOPROTEIN A IN THE LYME DISEASE SPIROCHETE *BORRELIA BURGDORFERI*

Tara Lynn Oman

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X. Frank Yang, Ph.D. - Chair

Margaret E. Bauer, Ph.D.

Doctoral Committee

Mark G. Goebl, Ph.D.

April 16, 2012

Stanley M. Spinola, M.D.

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ABSTRACT

Tara Lynn Oman

REGULATION OF OUTER SURFACE LIPOPROTEIN A IN THE LYME DISEASE SPIROCHETE *BORRELIA BURGDORFERI*

Borrelia burgdorferi, a bacterium which causes Lyme disease, is maintained in nature through a cycle involving two distinct hosts: a tick vector and a mammalian host. To adapt to these two diverse environments, *B. burgdorferi* undergoes dramatic alterations in its surface lipoprotein. Two essential lipoproteins, outer surface protein A (OspA) and outer surface protein C (OspC), are reciprocally regulated throughout the *B. burgdorferi* lifecycle. Very little is known about the regulation of OspA. These studies elucidate the regulatory mechanisms controlling the expression of OspA. Various truncations of the *ospA* promoter were created and then studied in our novel *in vitro* model of *ospA* repression or grown within the host-adapted model. A T-Rich region of the *ospA* promoter was determined to be a *cis*-element essential for both the full expression and full repression of *ospA*.

X. Frank Yang, Ph.D.- Chair

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LIST OF ABBREVIATIONS

AF	Accessory factor
Amp	Ampicillin
BSK	Barbour-Stoenner-Kelly media
˚C	Degrees Celsius
CAT	chloramphenicol acetyltransferase
CCLR	Cell culture lysis reagent
CDC	Center for Disease Control and Prevention
c-di-GMP	Bis-(3′-5′)-cyclic dimeric guanosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
cp	circular plasmid
Ct	Cycle threshold
Dbp	Decorin-binding protein
DMC	Dialysis membrane chamber
DNA	Deoxyribonucleic acid
EBP	Enhancer binding protein
EPS	Electroporation solution
HK1	Histadine kinase 1
HK2	Histadine kinase 2
IR	Inverted repeat
Kan	Kanamycin
Kb	Kilobases
LB	Luria-Bertani
lp	linear plasmid
LPS	Lipopolysaccharide

min	Minute
ml	Milliliter
ms	Millisecond
NtrC	Nitrogen regulatory protein C
Osp	Outer surface lipoprotein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
Rep	Repressor
Rept	Direct Repeat
RLU	Relative light unit
RNA	Ribonucleic acid
RNAP	RNA polymerase
rps	Revolutions per second
Rrp1	Response regulator protein 1
Rrp2	Response regulator protein 2
SCID	Severe combined immunodeficient
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	Seconds
Strep	Streptomycin
TLR1	Toll-like Receptor 1
TLR2	Toll-like Receptor 2
TLR4	Toll-like Receptor 4
T-Rich	Thymine rich
VlsE	Vmp-like sequence E

Vmp	Variable major protein
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranosid

CHAPTER ONE: INTRODUCTION

History of Lyme disease

Lyme disease is the most common arthropod-borne disease in the United States. In 1975, in Lyme, Connecticut, an unusual number of children presented symptoms of Juvenile Rheumatoid Arthritis (Steere et al., 1977). Although the definitive cause had not been confirmed, an infectious agent was suggested to be responsible for the arthritis displayed in the children. It was suspected that the unidentified infectious agent was being spread via an arthropod vector given that the patients lived near wooded areas and the highest frequency of outbreaks occurred in the summer (Steere et al., 1979; Steere et al., 1977). Around this same period, outbreaks of spotted fever spread by *Rickettsia*-infected ticks were occurring on Long Island, New York. In 1984, scientist Willy Burgdorfer was collecting tick samples in attempts to isolate a virulent strain of *Rickettsia rickettsii*. Incidentally, during this endeavor, Willy Burgdorfer discovered the spirochete *Borrelia burgdorferi*. It was suspected that this newly discovered bacterium was the causative agent of the arthritis cases in Lyme Connecticut (Burgdorfer, 2006; Burgdorfer et al., 1985). To confirm this notion, sera were taken from the patients in Lyme, Connecticut that were misdiagnosed with Juvenile Rheumatoid Arthritis and the presence of *B. burgdorferi* was confirmed.

Epidemiology of Lyme disease

B. burgdorferi is the primary causative agent of Lyme disease (Burgdorfer et al., 1985). Twelve of the thirty-seven known *Borrelia* species are capable of causing Lyme disease (Hengge et al., 2003). Lyme disease is an emerging infectious disease throughout Europe, Asia, South America, and Canada as well as the United States. According to the Center for Disease Control and Prevention (CDC), Lyme disease is the most common

vector-borne illness in the United States (2011). The number of reported Lyme disease cases has increased over the past 14 years. In 1996, there were 17,730 confirmed cases of Lyme disease while in 2010 there were 22,572 confirmed cases and 597 probable cases of Lyme disease in the United States as reported by the CDC (**Figure 1**).

Lyme disease is endemic in several areas of the United States, but is concentrated in the Northeastern and the upper Northeastern states (**Figure 2**). According to the CDC, 94% of the total Lyme disease cases were reported from just the following 12 states: Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Jersey, New Hampshire, New York, Pennsylvania, Virginia and Wisconsin (2010). Lyme disease is most prevalent in these states because they have a wooded, grassy environment which supports *B. burgdorferi*'s vector, the deer tick *Ixodes scapularis*, as well as the mammalian hosts required for the complete *B. burgdorferi* natural lifecycle.

Natural Lifecycle of *B. burgdorferi*

In nature, *B. burgdorferi* is maintained via an enzootic cycle being transmitted between a tick vector, the *Ixodes* tick, and a mammalian host, usually small animals like the white-footed mouse, rabbit and some birds (Anderson, 1998). In the United States, *I. scapularis* is the main arthropod vector in the upper Midwestern and the Northeastern parts of the country while the Western blacklegged tick, *Ixodes pacificus*, is the main arthropod vector in the western part of the country. The tick's lifespan is only two years. During their lifespan, the tick matures from an egg and then transforms into larval, nymph, and adult stages (**Figure 3**). The tick takes three blood meals throughout its entire life- once during each of its last three stages of development.

The adult female ticks lay eggs during the spring time, and then the eggs hatch during the summer into larvae. Upon hatching, the naïve larvae acquire *B. burgdorferi* when the tick larva takes a blood meal from a *Borrelia*-infected small mammal, usually a white-footed mouse. Because *B. burgdorferi* cannot be transmitted transovarially, infected ticks cannot pass the bacterium to its offspring and therefore the offspring must acquire the bacterium via the blood meal of an infected animal (Burgdorfer et al., 1985; Magnarelli et al., 1992; Piesman et al., 1986).

In the subsequent spring, the *Borrelia*-infected larva molts into a nymph. During the summer, the nymph is able to feed a second time. During this feeding, the nymph takes a blood meal from a small, uninfected mammal and will transmit the bacteria to the mammal thus completing the transmission cycle (Lane and Loye, 1991; Spielman, 1994; Steere et al., 2004). It is also during this nymphal stage that the infected tick is most likely to transmit *B. burgdorferi* to humans, which serve as accidental hosts (Lane and Loye, 1991). After molting into an adult, the tick feeds for the third and final time during the fall, usually on a large mammal such as a deer. Although deer are not competent reservoirs for spirochetes, larger animals are needed to feed a large number of adult ticks to support tick mating (Matuschka et al., 1993).

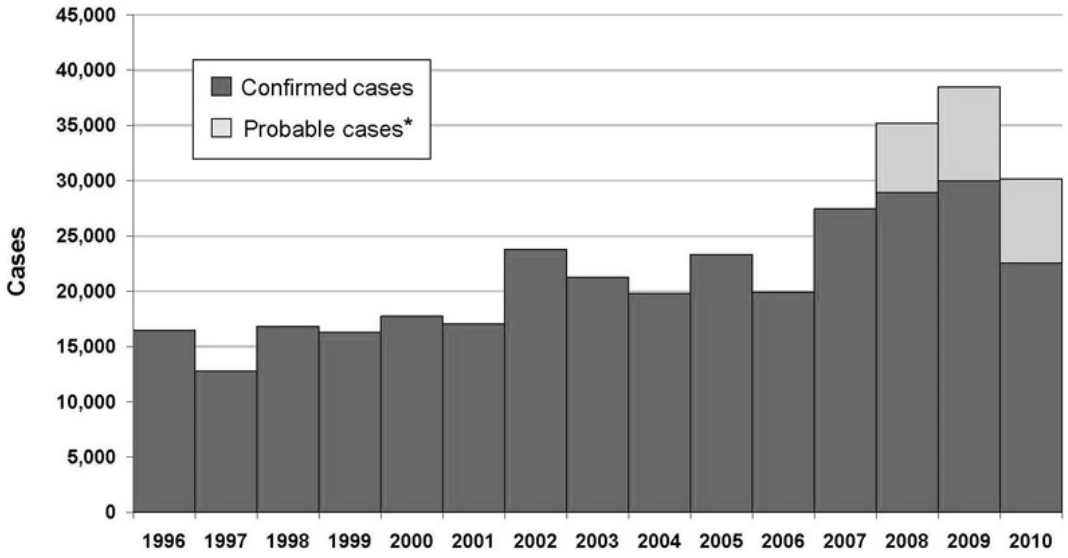

Figure 1. Reported Cases of Lyme Disease by Year in the United States, 1996-2010 (CDC Division of Vector-borne Infectious Diseases).

The number of confirmed cases and estimated probable cases of Lyme disease, as reported by the State Health Departments, shows an increase of incidences over time. There were 22,572 confirmed cases of Lyme disease in 2010, a 37% increase from 1996.

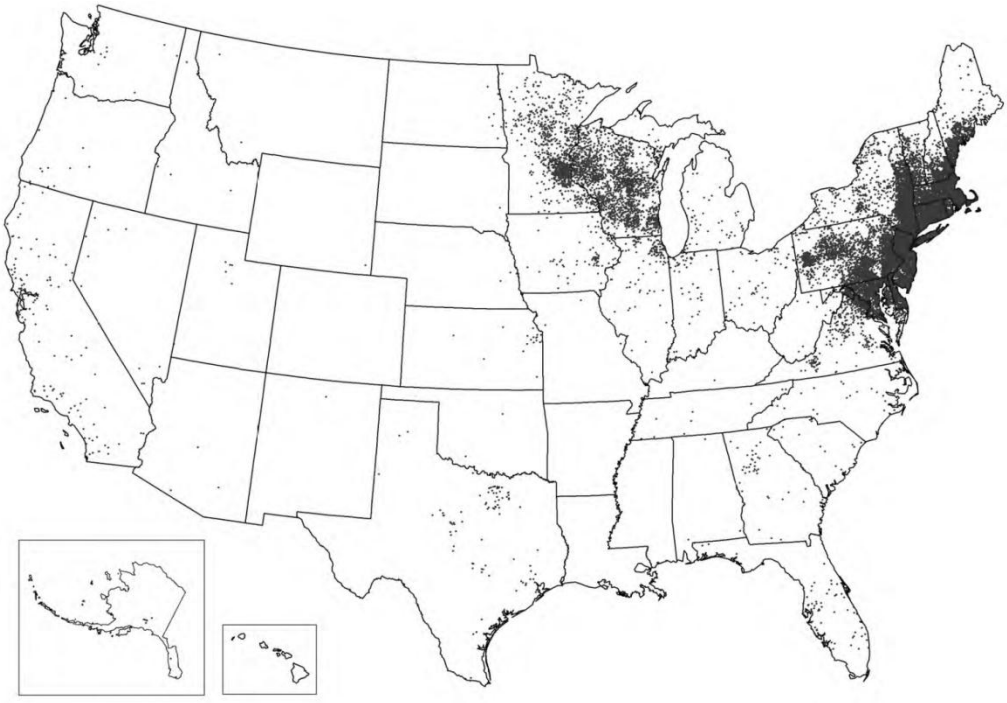

Figure 2. Reported Cases of Lyme Disease in the United States, 2009 (CDC Division of Vector-borne Infectious Diseases).

For each case of Lyme disease confirmed by the State Health Departments, one dot is placed randomly within the county of the patient's residence. Cases have been reported in nearly every state, but the county of residence is not necessarily the county in which the infection was acquired. The greatest number of cases was reported in the Northeastern and the upper Northeastern states.

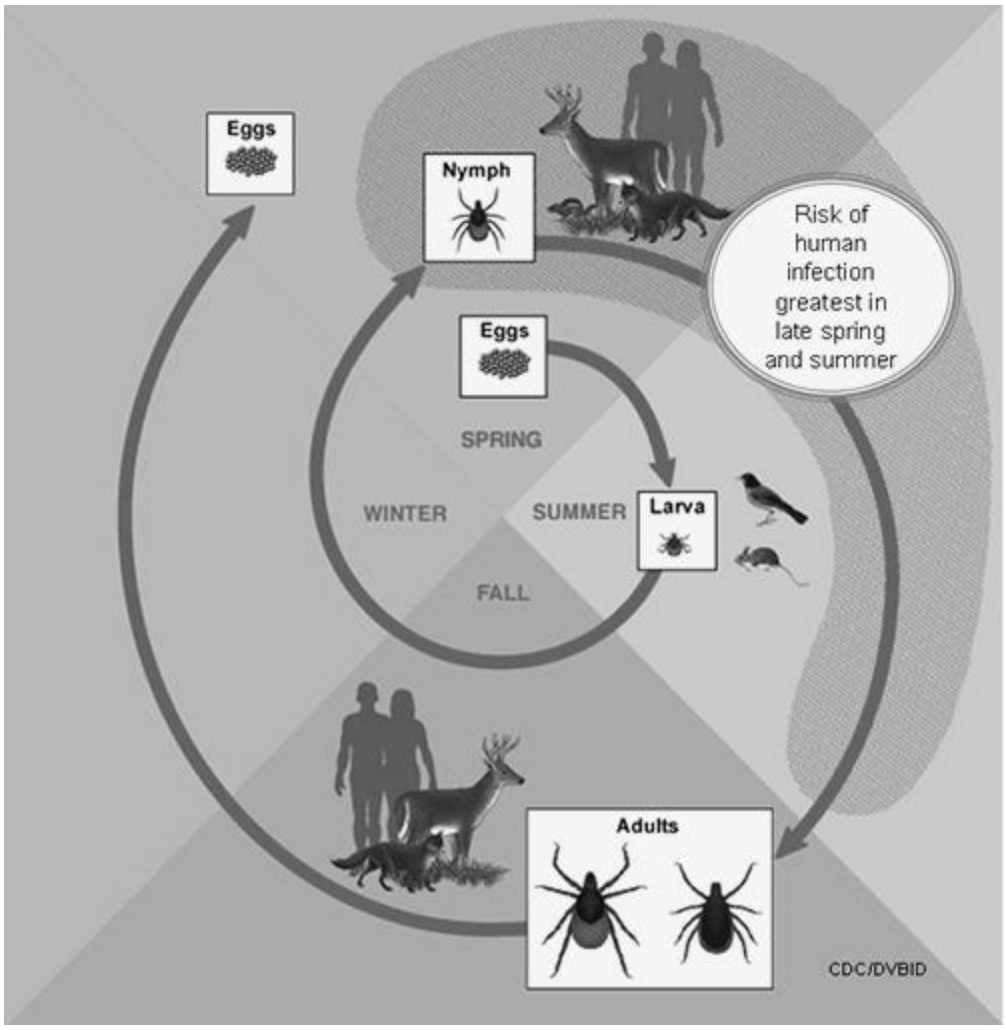

Figure 3. The enzootic life cycle of *Ixodes scapularis*, the tick that spreads Lyme disease (CDC Division of Vector-borne Infectious Diseases).

The *Ixodes* tick has four stages of life: the egg, larva, nymph and adult. The egg is laid during the spring and hatches into larva during the summer. The larva takes a blood meal from a *B. burgdorferi*-infected small mammal or bird and then molts into a nymph during the subsequent spring. The infected nymph feeds another time during the spring or summer during which the tick transmits the bacteria to an uninfected mammalian host thus completing the *B. burgdorferi* transmission cycle. The nymph molts into an adult and the adult mates, takes a blood meal from a large mammal, and then lays eggs in the following spring to complete the lifecycle of the tick.

Clinical Manifestations of Lyme disease

The clinical manifestations of Lyme disease vary depending on its progression. Early localized infection (Stage One) is within days to weeks following initial infection with *B. burgdorferi*. These patients may present fever, headache, fatigue and Erythema Chronicum Migrans. Erythema Chronicum Migrans is the red macule at the site of the tick bite that expands to become an annular rash that has central clearing giving the appearance of a bulls-eye. Following the early localized infection is the early disseminated infection (Stage Two), which occurs weeks or months after initial inoculation. Symptoms in this stage include severe neurologic and cardiac complications such as meningitis, encephalitis, Bells' palsy, and transient Atrio-Venticular block. The last stage of the disease is late or persistent infection (Stage Three) which begins months to years after initial infection. Intermittent and migratory arthritis, as well as neurologic and musculoskeletal problems such as encephalitis and altered memory and speech are manifestations of persistent infection (Warinner, 2001).

B. burgdorferi Structure and Genome

B. burgdorferi belongs to a group of bacteria phylogenetically distinct from other main bacterial groups, the spirochetes (Woese et al., 1984). Characteristic of spirochetes *B. burgdorferi* is a long, thin helical shaped bacterium with endoflagella that give *B. burgdorferi* its characteristic helical shape (**Figure 4**). There are 7 to 11 flagella inserted near the poles of the spirochete that wind around the rod-shaped protoplasmic cylinder, and overlap in the center, giving the spiral shape structure as well as motility to *B. burgdorferi* (Barbour and Hayes, 1986; Charon and Goldstein, 2002; Charon et al., 2009; Motaleb et al., 2000; Sartakova et al., 2001). *B. burgdorferi* lacks lipopolysaccharide and has an inner and outer membrane (Takayama et al., 1987). The outer membrane, or cytoplasmic cylinder, surrounds both the periplasmic flagella and the protoplasmic

cylinder, which is composed of a peptidoglycan layer and an inner membrane enclosing the cytoplasmic contents (Barbour and Hayes, 1986; Johnson et al., 1984; Kudryashev et al., 2010; Rosa, 2005; Rosa, 1997).

The genetic composition of *B. burgdorferi* is complex and unusual, thus creating obstacles for genetic studies and genetic manipulation. The segmented genome is composed of a linear chromosome of approximately 910 kilobases (kb) along with eleven circular plasmids (cp) and twelve linear plasmids (lp) totaling approximately 610 kb (Casjens, 2000; Fraser et al., 1997). *B. burgdorferi* has the largest number of plasmids of any characterized genome. The plasmids are numbered according to their size in kb pairs. The sequenced *B. burgdorferi* strain B31 contains circular plasmids cp9, cp26, and nine homologous plasmids (cp32-1 to cp32-9); linear plasmids lp5, lp17, lp21, lp25, four homologous plasmids (lp28-1 to lp28-4), lp36, lp38, lp54, lp56; and the 910 kb linear chromosome (Fraser et al., 1997). The stability of these plasmids varies. Frequently, some plasmids are lost after only a few generations of *in vitro* growth while others are stable through continuous passage (Barbour, 1988; Byram et al., 2004; Grimm et al., 2003; Schwan et al., 1995; Xu et al., 1996).

Many of *B. burgdorferi*'s plasmids encode essential functions which are required for the spirochete to complete its natural infectious cycle (Labandeira-Rey et al., 2003; Labandeira-Rey and Skare, 2001; Lawrenz et al., 2002; Schwan et al., 1988; Xu et al., 1996). Two plasmids relatively unstable during *in vitro* culture growth, which are also essential for persistent infection within the mammalian host, are lp25 and lp28-1 (Labandeira-Rey et al., 2003; Purser and Norris, 2000). *B. burgdorferi* lacking lp25 cannot grow in wild-type mice, severe combined immunodeficient (SCID) mice, or within dialysis membrane chamber (DMC) implants suggesting a physiological defect. The lp25

plasmid contains the gene *pncA*, encoding for a nicotinamidase, which is likely to have a role for biosynthesis of NAD that is essential for *in vivo* growth, but not required for *in vitro* growth (Purser et al., 2003; Purser and Norris, 2000).

Similarly to lp25, the lp28-1 plasmid is also essential for the persistent infection in mice (Grimm et al., 2004a). In *B. burgdorferi*, the lp28-1 plasmid contributes to the spirochete's ability to persistently infect the host by encoding for VlsE (Vmp-like sequence E). Vmp stands for variable major protein and contributes to the antigenic variation of proteins in spirochetes (Barbour, 1993). VlsE is able to cause antigenic variation by undergoing an extensive genetic recombination mechanism at the *vls*-locus (Coutte et al., 2009; Zhang and Norris, 1998).

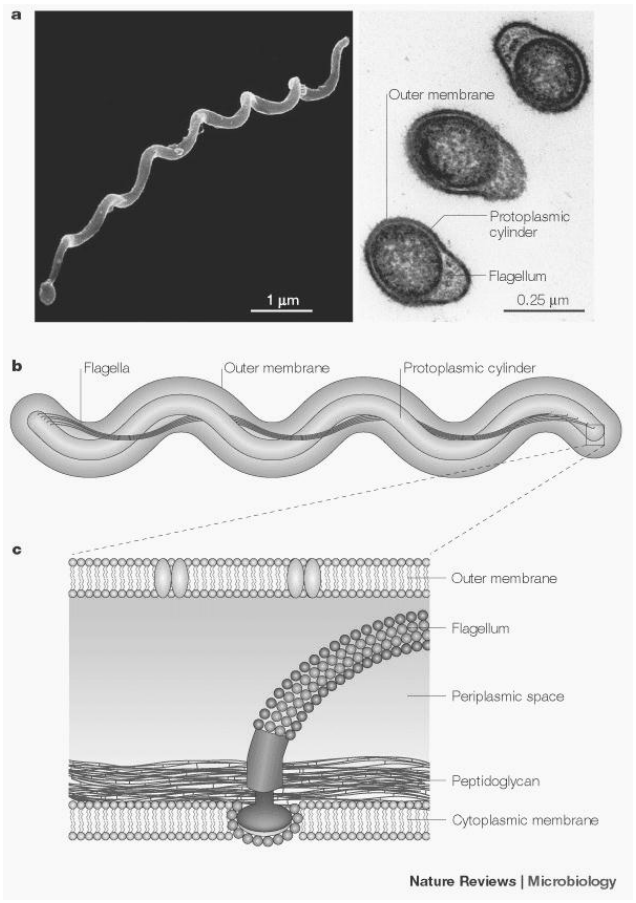

Figure 4. The spirochete *B. burgdorferi* and schematic of its structure (Rosa et al., 2005).

(A) The scanning electron micrograph (left) showing the morphology *of B. burgdorferi* and the cross-sectional view of the transmission electron micrograph (right) showing the helical shape imparted by the periplasmic flagella. (B) Schematic of the spirochete illustrating the bundles of 7 to 11 flagella wound around the protoplasmic cylinder, overlapping in the middle, with the insertion points near the poles of the cell. The flagella are enclosed within the periplasm by the outer membrane. (C) The protoplasmic cylinder is enclosed by the cytoplasmic membrane and a peptidoglycan layer. The endoflagella are inserted into the cytoplasmic membrane and extend through the cell wall into the periplasm, the space between the cytoplasmic and outer membranes.

Lipoprotein Role in Virulence

B. burgdorferi is a Gram-negative-like bacterium that contains an inner and outer membrane, but unlike Gram-negative bacteria, *B. burgdorferi* lacks lipopolysaccharide (LPS) (Takayama et al., 1987). Rather than LPS, *Borrelia* contains an abundance of lipoproteins on its outer surface (**Figure 5**). LPS and lipoproteins are both major components of the outer membrane of bacteria. In fact, *B. burgdorferi* has an unusually large amount of lipoproteins; putative lipoproteins comprise as much as 14.5% of the genes encoded on *B. burgdorferi's* plasmids, suggesting the lipoproteins play an important role in virulence (Casjens, 2000). Of all the open reading frames predicted in *B. burgdorferi*, lipoproteins account for 7.8% and this number is much higher than other bacterial genomes such as *Treponema pallidum*, 2.1%, or *Helicobacter pylori* containing 1.3% (Casjens, 2000; Fraser et al., 1997; Setubal et al., 2006).

Lipoproteins are membrane-anchored proteins found in *Borrelia* that are peripherally tethered to the lipid bilayer leaflets of the inner or outer membranes via the lipoprotein's acyl group of the terminal cysteine. The lipoprotein begins as a prolipoprotein precursor within the cytoplasm. Next, the prolipoprotein is translocated through the inner membrane via a sec-dependent transport mechanism, and the ABC transporter-like complex, LolCDE, releases the outer membrane-targeted lipoproteins from the inner membrane (Yakushi et al., 2000). After release from the inner membrane, while in the periplasm, the lipids are modified to form mature lipoproteins containing a lipid-modified N-terminus. Lipoprotein maturation is suggested to occur in three steps: 1) diacylglyceryltransferase transfers a diacylglyceride to the sulfur of the cysteine side chain as specified by the signal sequence LXYC on the unmodified prolipoprotein; 2) signal peptidase II cleaves at the amino side of the cysteine residue to form a prolipoprotein; and 3) transacylase adds a third fatty acid to the new amino terminus via

an amide linkage to form the mature lipoprotein (Fraser et al., 1997; Hayashi and Wu, 1990; Juncker et al., 2003). The periplasmic chaperone, LolA, forms a complex with the lipoprotein and crosses through the periplasm (Yokota et al., 1999). Next, this complex interacts with LolB, a receptor on the outer membrane, mediating the anchor of lipoproteins to the inner leaflet of the outer membrane (Yokota et al., 1999). The lipoprotein is flipped across the outer membrane through an unidentified outer membrane module.

The most abundant lipoproteins are outer surface lipoproteins (Osp). Of these Osps, the most well-known of these outer surface proteins are OspA, OspB, OspC, OspD, OspE and OspF (Bergstrom et al., 1989; Burgdorfer et al., 1983; Howe et al., 1985; Lam et al., 1994; Wilske et al., 1993; Zumstein et al., 1992). Other lipoproteins include decorin-binding proteins (Dbp), *Borrelia* glycosaminoglycan-binding proteins (Bgp), and the VlsE. Interestingly Borrelial lipoproteins and Gram-negative bacteria's LPS share similar roles: 1) they induce the host inflammatory response and are targets for bactericidal antibodies; 2) they contribute to the stability and structural integrity of the bacteria; and 3) they act as adhesions. Osps also have additional functions not shared by LPS: they are able use lipoproteins to 1) adapt to the various host environments; 2) evade phagocytosis by the host immune system; and 3) acquire nutrients (Liang et al., 2002).

Borrelial lipoproteins and Gram-negative bacteria's LPS activate the mammalian host's innate immune system. *Borrelia*'s activation of the host immune system is achieved via toll-like receptor II (TLR2) and toll-like receptor I (TLR1), whereas toll-like receptor IV (TLR4) recognizes the LPS of Gram-negative bacteria. TLR2 heterodimerizes with TLR1 to recognize triacyl-lipoproteins. The pattern recognition receptors of TLR2 recognize

Borrelial lipoproteins and activate the host's inflammatory mediators to elicit the inflammatory response at the site of infection (Hirschfeld et al., 1999).

Also similarly to LPS, Osps play a role in the structural integrity of the bacteria. Throughout its lifecycle, *B. burgdorferi* must transition to and survive within many chemically different environments within its tick and the mammalian hosts. To respond to and adapt to these various environments, the bacteria undergo drastic adaptive changes through differential gene expression to alter its surface lipoprotein expression profile (Brooks et al., 2003; de Silva and Fikrig, 1997; Indest et al., 2000; Liang et al., 2002; Pal et al., 2004b; Seshu and Skare, 2000). The absence of Osps lead to a weak bacterial membrane, however addition of Osps is able to restore the structural integrity of the lipoprotein (Xu et al., 2008).

Another characteristic shared with LPS is the lipoprotein's ability to function as an adhesion molecule used in bacterial transport and binding. Several lipoproteins have various adherence capabilities. Bgp is able to bind heparin sulphate and plays a role in mammalian infection (Parveen et al., 2003; Parveen and Leong, 2000). BBK32 is a lipoprotein that is able to bind fibronectin to promote *Borrelia* attachment to glycosaminoglycans and is important for dissemination (Probert and Johnson, 1998; Seshu et al., 2006). OspA is a lipoprotein that is able to attach to a protein within the tick gut, which is important for *Borrelia* colonization within the tick (Pal et al., 2000). Lipoproteins play a major role in *Borrelia* adhesion in a similar manner that LPS plays in adherence for Gram negative bacteria.

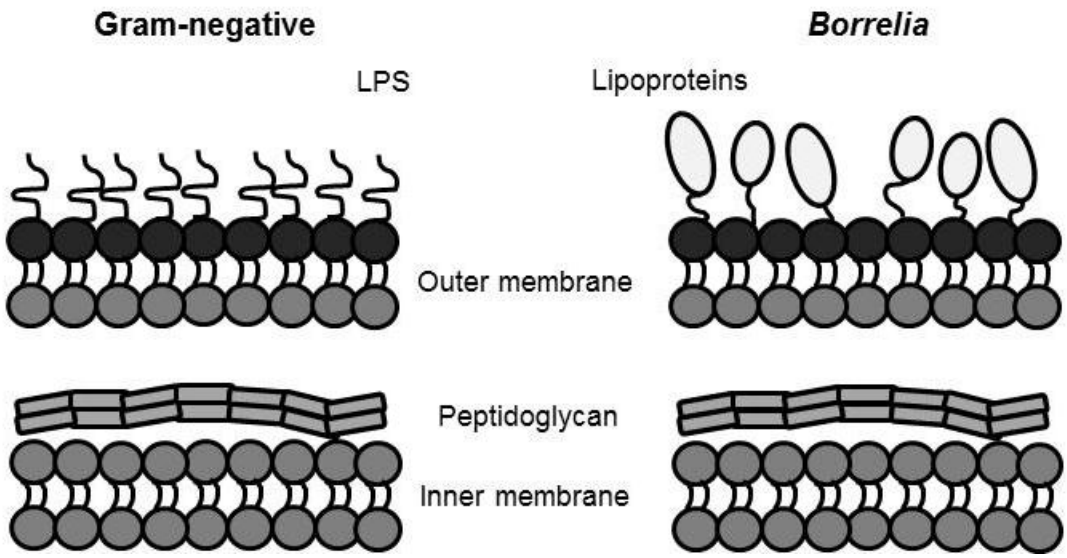

Figure 5. Membrane structure of Gram-negative bacteria and Borrelia.

Both Gram-negative bacteria and *Borrelia* contain an inner membrane and outer membrane with a peptidoglycan layer located within the periplasmic space. Gram-negative bacteria contain LPS on the outer surface whereas *Borrelia* lacks LPS. Rather, *Borrelia* contains an abundance of lipoproteins on its outer surface.

Functions of the lipoproteins OspA and OspC

Two major Osps which are highly regulated are OspA and OspC; both are located on the outer membrane of *B. burgdorferi*. OspA and OspC are reciprocally regulated throughout the lifecycle of *B. burgdorferi*. *B. burgdorferi* residing within the midgut of unfed ticks have OspA present, while OspC is absent. However, upon a blood meal, the bacteria migrate from the tick midgut to the tick salivary glands to be transmitted to the mammalian host. During this transition, OspA is down-regulated and OspC is expressed (de Silva et al., 1996; Kobryn and Chaconas, 2001; Schwan and Piesman, 2000; Schwan et al., 1995). Some environmental and host signals affecting OspA and OspC expression include pH, cell density, temperature, and the presence of blood or other nutritional factors (Carroll et al., 2000; Carroll et al., 2003; Ramamoorthy and Scholl-Meeker, 2001; Revel et al., 2002; Schwan et al., 1995; Tokarz et al., 2004; Yang et al., 2004). For example, during *in vitro* growth cultivation at 23°C, OspC is not expressed, but a temperature shift to 37°C results in OspC being highly expressed. OspA, however, is unaffected by temperature: at both 23°C and 37°C, OspA is constitutively expressed which makes studying the repression of *ospA* difficult.

The presence of OspC is essential for 1) the migration of *B. burgdorferi* from the tick vector to the mammalian host; and 2) the infection of the mammalian host (Gilbert et al., 2007; Grimm et al., 2004b; Pal et al., 2004b; Tilly et al., 2006). Studies have shown that *ospC* mutant *B. burgdorferi* was not able to establish an infection in either wild-type mice or SCID mice. However, the *ospC* mutant was able to be transmitted to and establish infection within the tick. Therefore, OspC is not required to establish an infection within the tick, but OspC is essential for the invasion and infection of mammalian host (Yang et al., 2003a). To help facilitate the invasion of *Borrelia* into the mammalian host, OspC specifically binds Salp15. Salp15 is a tick salivary protein that blocks the mammalian

host's $CD4^+$ T-cell activation. This suppresses the host's immune response at the site of the tick bite (Anguita et al., 2002; Ramamoorthi et al., 2005). In a study that compared *B. burgdorferi* without Salp15 present in the environment to *B. burgdorferi* with Salp15 present, it was found that *B. burgdorferi* with Salp15 present have an increased bacterial burden and are better protected against antibody-mediated killing. This indicates that Salp15 assists *B. burgdorferi* in evading the host immune system by blocking the host's $CD4^+$ T-cell activation thereby inhibiting the IgG antibody response (Anguita et al., 2002; Ramamoorthi et al., 2005).

OspC is required for the transmission and early infection of the mammalian host while OspA, on the other hand, is required for the colonization of *B. burgdorferi* within the tick (Yang et al., 2004). OspA specifically binds the tick receptor TROSPA found within the midgut of the tick to facilitate attachment of the spirochete to the vector midgut (Pal et al., 2004a). The receptor in the tick gut serves as a ligand for tethering spirochetes via OspA binding (Pal et al., 2000; Pal et al., 2004a). While in the midgut of an unfed tick, spirochetes express high levels of OspA; together OspA and the flagellin proteins account for one-third of the total protein in *B. burgdorferi* (Coleman and Benach, 1987). When the infected tick feeds on a vertebrate host, the bacterium multiplies within the tick midgut and OspA is repressed while OspC is up-regulated (Schwan et al., 1995). Little or no OspA is present in the *B. burgdorferi* transmitted to the mammalian host (Cassatt et al., 1998). The repression of *ospA* is essential during mammalian infection, because even low levels of OspA can elicit a humoral response by the host to cause clearance of the bacterium or cause great immunological pressure on the pathogen (Strother et al., 2007; Xu et al., 2008). While OspA is not essential for early infection in mice, OspA is necessary for tick infection and maintenance in the midgut (Yang et al., 2004). The converse is true about OspC: OspC is essential for transmission to and early infection in

the vertebrate host, but is not necessary for the infection and maintenance within the tick midgut (Grimm et al., 2004b; Pal et al., 2004b).

Reciprocal Production of OspA and OspC

As mentioned previously, *B. burgdorferi* drastically alters its surface lipoproteins as a strategy to adapt to its two diverse host environments: the tick and the mammalian host (Anguita et al., 2002; Haake, 2000; Philipp, 1998; Schwan, 2003). In particular, during tick feeding, the two virulence factors OspA and OspC are reciprocally regulated and this coordinated regulation is believed to be important for the transmission of the spirochete between the tick and the mammalian host (Akins et al., 1998; de Silva et al., 1996; Montgomery et al., 1996; Stevenson et al., 1995). The reciprocal regulation has been shown in populations of cells as well as within individual cells using flow cytometry. Individual spirochetes coordinate the increase of OspC with the down-regulation of OspA (Srivastava and de Silva, 2008).

Most bacterial species have several sigma factors which are able to bind to an RNA polymerase core to form an RNA polymerase holoenzyme. The addition of a sigma factor to the RNA polymerase allows for promoter recognition specificity during the initiation of transcription. Most bacteria have a general housekeeping sigma factor (σ^{70}) which transcribes the majority of genes as well as several alternative sigma factors. The alternative sigma factors are able to distinguish different promoter sequences to direct the RNA polymerase to initiate transcription of select groups of genes in response to various environmental or developmental signals. *B. burgdorferi* contains only three sigma factors: RpoD (σ^{70}), and the alternative sigma factors RpoN (σ^{54}) and RpoS (σ^{S}) (Fraser et al., 1997).

The Response regulator protein II (Rrp2), in combination with RpoN and RpoS, forms the Rrp2-RpoN-RpoS regulatory network. During mammalian acquisition of *Borrelia*, Rrp2-RpoN-RpoS plays a central role in controlling the repression of OspA and the production of OspC as well as other differentially expressed genes in *B. burgdorferi* like decorin-binding protein A (DbpA), which facilitates *Borrelia* adherence to mammalian extracellular matrix, and the lipoprotein BBK32 that binds to mammalian fibronectin to facilitate spirochete acquisition (**Figure 6**) (Boardman et al., 2008; Caimano et al., 2007; Hubner et al., 2001; Yang et al., 2003a). The Rrp2-RpoN-RpoS pathway becomes activated through a cognate sensor histadine kinase Hk2 (gene BB0764) or acetyl phosphate(Xu et al., 2010) sensing environmental signals and activating Rrp2 (gene BBE0763) via phosphorylation. Signals such as low pH, elevated temperature and CO_2 concentration, high cell density and the presence of host signals are known to activate the pathway upon tick feeding (Akins et al., 1998; Burtnick et al., 2007; Caimano et al., 2007; Carroll et al., 1999; Indest et al., 1997; Stevenson et al., 1995; Yang et al., 2003b). Rrp2 is an NtrC-like bacterial two-component response regulator that functions as an enhancer binding protein (EBP) dependent on RpoN to activate transcription. Together, RpoN and the phosphorylated Rrp2 control the transcription of RpoS, a second alternative sigma factor. RpoS then up-regulates many genes, including *ospC*, and represses others, like *ospA*, via an unknown mechanism (Boardman et al., 2008; Caimano et al., 2007; Fisher et al., 2005; Ouyang et al., 2008; Yang et al., 2003b; Yang et al., 2005).

A study using *B. burgdorferi* grown within DMC's implanted into rat peritoneal cavities (the "Host-adapted model") demonstrated that wild-type bacteria had high OspC and low OspA levels, a profile expected for *B. burgdorferi* grown within a mammal (Caimano, 2005). However, *rpoS* mutants grown in the Host-adapted model were not able to

repress *ospA* nor express *ospC* implying that *ospA* repression and *ospC* activation are connected at the molecular level through an unknown mechanism involving RpoS.

There is a second two-component response regulator predicted to be encoded by the *B. burgdorferi* genome, Response regulator protein 1 (Rrp1, gene BB0419). While Hk2 and the Rrp2-RpoN-RpoS pathway promote spirochete transmission from the tick to the mammal and early infection of the mammal, the two-component system of histadine kinase Hk1 (gene BB0420) and response regulator Rrp1 promotes spirochete survival within the fed tick midgut (Caimano et al., 2011). It is predicted that Hk1 activates Rrp1 through phosphorylation. Rrp1, a guanylatecyclase, is responsible for the synthesis of the second messenger bis-(3'-5')-cycle dimericguanosine monophosphate (c-di-GMP) which affects the production and activity of Borrelial virulence factors such as those responsible for glycerol transport (He et al., 2011; Rogers et al., 2009; Ryjenkov et al., 2005). Because *ospA* becomes upregulated during transmission from the mammal to the tick, an Rrp1 activation condition, the Rrp1 pathway may contribute to the activation of *ospA*.

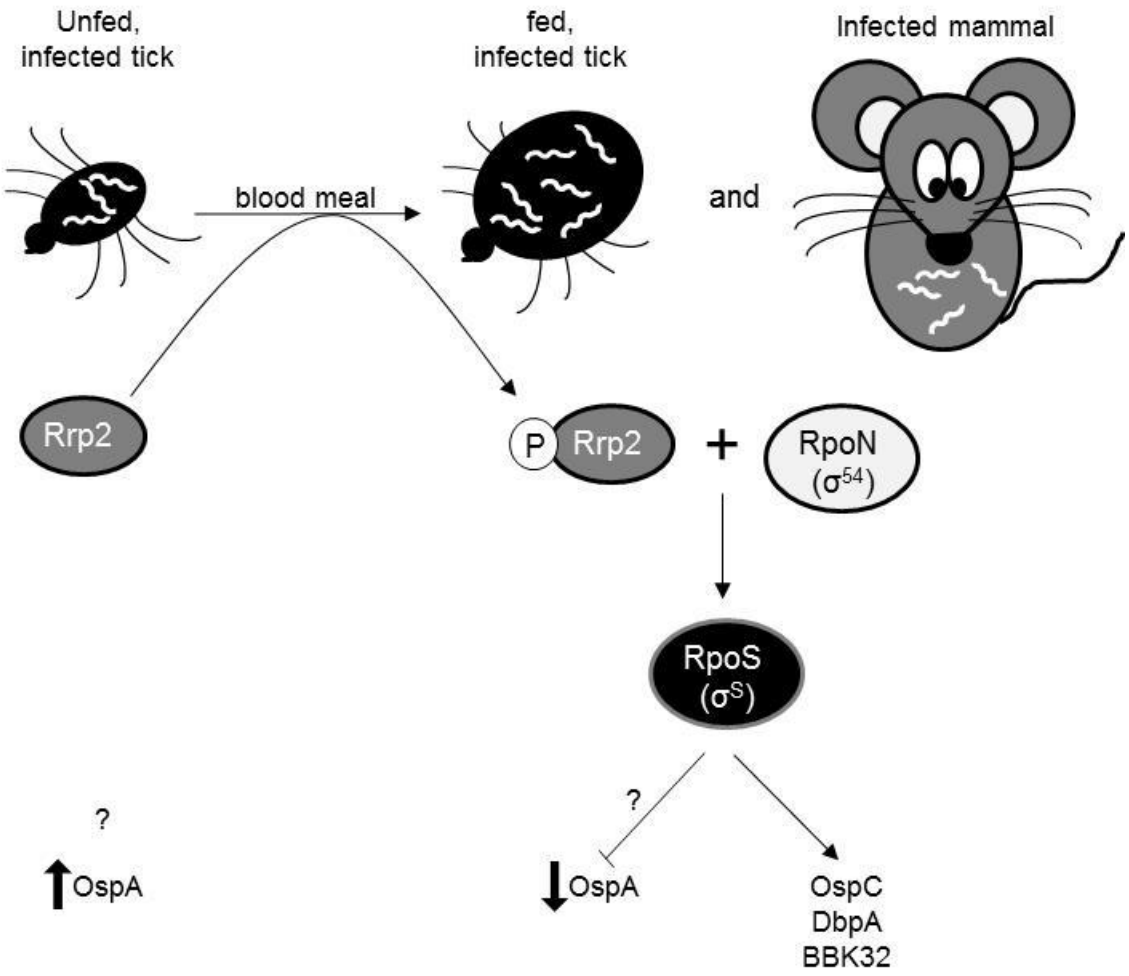

Figure 6. The Rrp2-RpoN-RpoS regulatory network, σ^{54}-σ^{S} sigma factor cascade, controls the inverse production of OspA and OspC.

An unfed, infected tick takes a blood meal from a mammal and engorges with blood. In the fed, infected tick as well as the infected mammal, the Rrp2-RpoN-RpoS pathway is activated in *B. burgdorferi* upon response regulator Rrp2 phosphorylation by histadine kinase Hk2. Rrp2 in conjunction with RpoN activates transcription of RpoS. RpoS activates outer surface lipoprotein C (OspC) and OspC-related genes like decorin-binding protein A (DbpA) and the fibronectin-binding protein (BBK32). RpoS represses OspA via an unknown mechanism. The Rrp2-RpoN-RpoS pathway is not activated in the

unfed, infected tick. The mechanism of OspA expression is also not known (←, positive activation; ⊢, negative activation).

Genetic Regulation of *ospA*

The signals and pathways by which *ospA* is expressed and the mechanism by which RpoS represses *ospA* have not been fully elucidated. OspA and OspB are encoded by a two-gene operon, the *ospAB* operon (genes BBA15 and BBA16), located on the lp54 plasmid (**Figure 7**) (Howe et al*.*, 1986). The RpoD promoter drives the expression of the *ospAB* operon (Sohaskey et al., 1999). RpoD is a major sigma factor within *B. burgdorferi* and is constitutively expressed. While the expression of *ospA* has not been fully elucidated, it is known that the σ^{70}-driven *ospAB* promoter contains three putative *cis*-elements: an inverted repeats (IR) region, a direct repeats (Rept) region, as well as a thymine rich (T-Rich) region (Sohaskey et al., 1999). Sohaskey et al*.* (1999) used the chloramphenicol acetyltransferase (CAT) reporter to show deletion of the T-Rich region of the *ospAB* promoter results in a decrease in reporter expression. Xu et al*.* (2010) demonstrated, by measuring *ospA* transcript of the *ospA* reporter within an *ospAB* mutant, that the Repeats and the T-Rich regions of the *ospAB* promoter are required for the full expression of *ospA*.

The alternative sigma factor RpoS, which is controlled through the Rrp2-RpoN-RpoS pathway, is required for *ospA* repression (Caimano et al., 2005; Yang et al., 2003a). It is postulated that RpoS represses *ospA* either indirectly or directly (**Figure 8**). A possible method by which RpoS could indirectly repress *ospA* is by controlling the expression of an unknown *ospA* repressor that binds to the *ospAB* promoter *cis*-element required for *ospA* repression. This would subsequently prevent the transcription of *ospA* by blocking access of the RpoD-containing holoenzyme to the *ospAB* promoter. Conversely, RpoS could bind directly to the *ospAB* promoter in the presence of an accessory factor that is specific to *in vivo* cultivation, the only condition in which *ospA* is repressed. RpoS could

then bind to the promoter blocking the RpoD-dependent transcription of *ospA* (Caimano et al., 2005).

In this study we seek to determine the *cis*-elements required for the repression and the activation of the *ospAB* promoter. A previous study by Sohaskey et al., used the transient CAT reporter system to evaluate which *cis*-elements were required for the activation of *ospA*. Their studies suggest that the T-Rich region is required for the full activation of *ospA*, however these studies were done using a transient, nonreplicating reporter. This reporter is transient because it does not replicate upon bacteria replication and is therefore an unstable reporter. Our goal is to develop a stable, replicating reporter system to more accurately determine which *cis*-elements play a role in the regulation of *ospA*. Another goal of these studies is to use our stable reporter system to identify the *trans*-factors required for the full activation and repression of *ospA*. And lastly, we want to develop an *in vitro* model to study *ospA* repression. Studying *ospA* repression has been proven tedious as the only way to acquire *Borrelia* with repressed *ospA* is to cultivate *Borrelia* in *in vivo* conditions. Cultivating *Borrelia in vivo* produces low yields of bacteria, so it is difficult to collect enough bacteria to use in experiments. The overall goal of our studies is to further elucidate the mechanisms by which *ospA* is regulated.

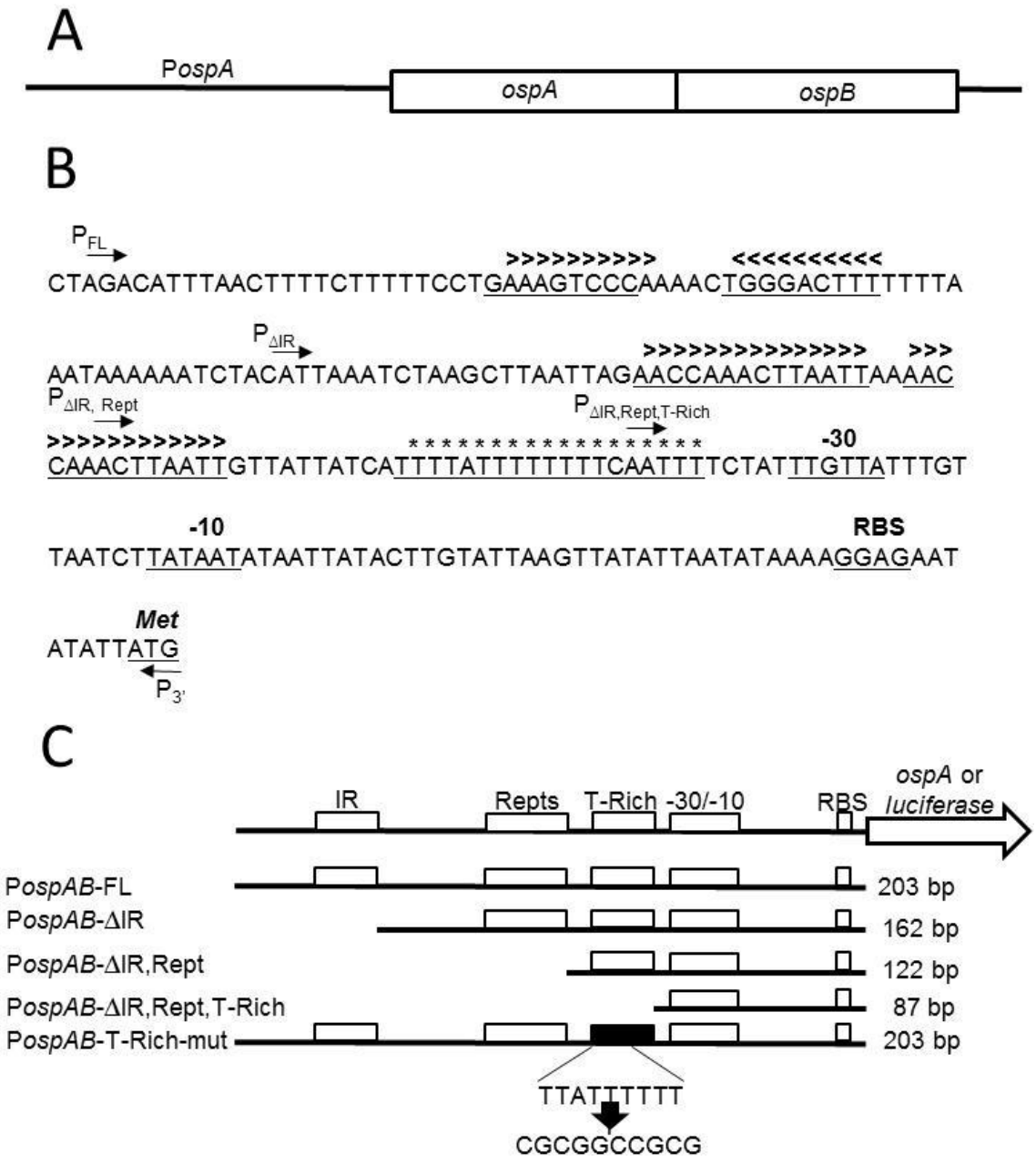

Figure 7. The *ospAB* operon the putative regulatory *cis*-elements in the upstream region of the *ospA* gene.

(A) Schematic of the *ospAB* operon located on the *B. burgdorferi* linear plasmid 54. (B) Inverted arrows (>> and <<) denote the inverted repeat element (IR); arrowheads (>>) denote the repeats element (Rept); asterisks (*) denote the T-Rich element (T-Rich). The -35 and -10 promoter elements, ribosomal-binding site (RBS), and the ATG start

codon (*Met*) are underlined. Arrows (→) indicate the 3' primer ($P_{3'}$) and 5' primers (P_{FL}; $P_{\Delta IR}$; $P_{\Delta IR,Rept}$; or $P_{\Delta IR,Rept,T\text{-}Rich}$) used to create the four *ospAB promoter* constructs shown below. (C) Schematic representation of the *ospAB promoter* deletion constructs controlling luciferase or *ospA* expression All four *ospAB promoter* constructs are denoted by the prefix P*ospAB*- followed by the name of the corresponding deletion (indicated at the left) with the size of the resulting promoter (indicated at the right) in base pairs (bp). The full-length promoter (P*ospAB*-FL) contains all three putative *cis*-elements; P*ospAB*-ΔIR contains the Repeats and the T-Rich regions; P*ospAB*-ΔIR,Rept contains only the T-Rich element; P*ospAB*-ΔIR,Rept,T-Rich promoter does not contain any of the putative *cis*-elements; and the P*ospAB*-T-Rich-mut is the full-length *ospAB* promoter with a mutated T-Rich region.

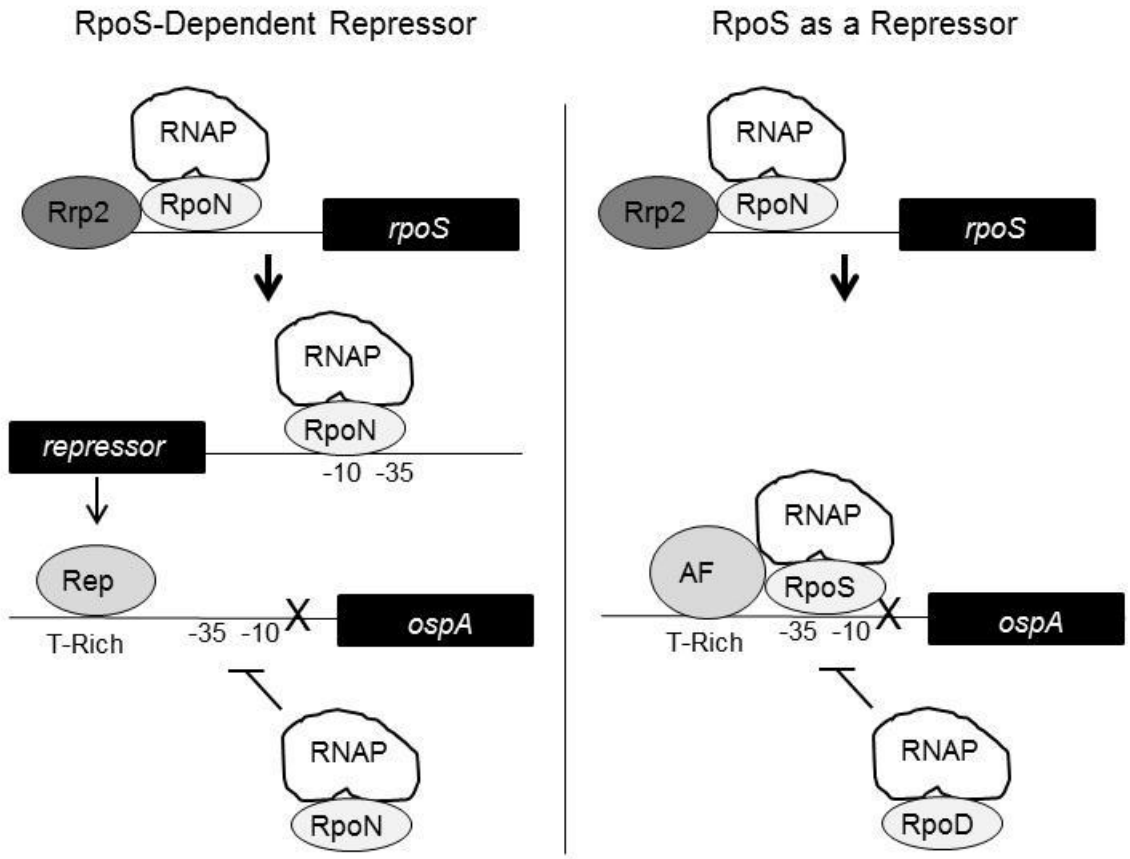

Figure 8. Proposed models for the RpoS-dependent *ospA* repression.

(Left panel) RpoS controls the expression of an unknown *ospA* repressor protein. The unknown *ospA* repressor protein binds to the *ospAB* promoter at the T-Rich *cis*-element, which in turn prevents transcription of *ospA* by the RpoD-containing holoenzyme. (Right panel) Association with an *in vivo*-specific accessory factor allows RpoS to bind directly to the *ospAB* promoter thereby blocking RpoD-dependent transcription initiation. Abbreviations: RNA polymerase (RNAP), repressor (Rep), accessory factor (AF).

HYPOTHESIS

Based on the previous literature concerning *ospA* regulation, I propose the following:

First, I hypothesize that either the IR, Repeats, or T-Rich regions on the *ospAB* promoter are key components required for the full activation or repression of *ospA*. The rationale behind this hypothesis is based on previous literature which states these three regions are highly conserved suggesting that they are of importance (Sohaskey et al., 1999).

Second, *ospA* is being repressed either directly or indirectly by RpoS. Previous literature has shown that RpoS is required for *ospA* repression (Caimano et al., 2005). This suggests that either RpoS is able to directly bind to the *ospAB* promoter to prevent RpoD from initiating transcription or RpoS is able to control the expression of an unknown *ospA* repressor.

Third, I hypothesize that *an in vitro* model can be created to study *ospA* repression. *ospA* is constitutively expressed *in vitro*. In *B. burgdorferi*, the abrogation of *ospAB* results in the constitutive expression of RpoS (He et al., 2008). Since RpoS mediates the repression of *ospA* either directly or indirectly, an *ospAB* mutant would have RpoS constitutively expressed thereby creating *ospA* repression conditions. In order to measure *ospA* repression within the *ospAB* mutant, a luciferase reporter can be placed under the control of an *ospAB* promoter to measure the *ospAB* promoter activity and thus allow for a novel method to study *ospA* repression *in vitro*.

CHAPTER TWO: MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are described in **Table 1**. The TOP10 *E. coli* strain (Invitrogen, Carlsbad, CA) was used as the cloning host. *B. burgdorferi* clones 13A and BbAH130 as well as the *ospAB* mutant strain were described previously (He et al., 2008; Hubner et al., 2001; Xu et al., 2007; Yang et al., 2004). The B31 13A strain was isolated by serially diluting a wild-type *B. burgdorferi* strain B31 5A13 to identify a single clone which lost lp25 and lp56 but retained the remaining 19 plasmids of the *B. burgdorferi* genome. This clone, 13A, is more easily transformable because it lost the lp25 and lp56 plasmids which contain restriction enzymes that negatively affect transformation efficiency (Lawrenz et al., 2002). BbAH130 is an infectious strain derived from plating a low passage 297 strain on Barbour-Stoenner-Kelly (BSK) agar medium. *B. burgdorferi* 297 is an infectious strain which was isolated from the cerebrospinal fluid of a patient with Lyme disease (Yang et al., 2004). The *ospAB* mutant strain (Δ*ospAB*) was previously generated by electroporating the suicide vector, pXT-OspA-Strep, into the *B. burgdorferi* strain BbAH130. The suicide vector underwent homologous recombination with the wild-type *Borrelia* genome to insert the streptomycin-resistance gene (*aadA*) into the native *ospA* gene thus inactivating the *ospAB* operon.

E. coli cultures were grown with appropriate antibiotics at 37°C with aeration in Miller Difco Luria Bertani (LB) broth (Becton, Dickinson and Company, Sparks, MD) or grown on LB plates consisting of LB broth with 2% agar. Kanamycin concentrations for both LB broth and LB agar were 50 µg ml^{-1} while ampicillin was 100 µg ml^{-1}. *Borrelia* were cultivated *in vitro* at 37°C with 5% CO_2 in BSK medium (Sigma, St Louis, MO) supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR).

Antibiotic concentrations used to select for mutants were 300 μg ml^{-1} for kanamycin and 100 μg ml^{-1} for streptomycin. Dark-field microscopy was used to enumerate spirochetes. Cultures were harvested at mid-logarithmic phase of growth (approximately 3×10^7 spirochetes ml^{-1}), unless otherwise noted. For the norepinephrine studies, cultures were grown to mid-log in 10 ml BSK, split equally into two 5ml cultures and then centrifuged at 4000 x g for 20 minutes. The pellets were suspended into either BSK only or BSK with the addition of norepinephrine bitartrate (Sigma) to a final concentration of 10μM. After suspension, cultures were grown for 48 hours before harvesting.

Table 1. Strains and plasmids used in this study.

Plasmid or Strain	Description	Source
Plasmids		
pBSV2	*B. burgdorferi/E. coli* shuttle vector with native *ospAB*	(He et al., 2008)
pOspA	*pBSV2* with full-length *ospAB* promoter controlling native *ospA*	(He et al., 2008)
pOspAB	*pBSV2* with full-length *ospAB* promoter controlling native *ospAB*	(He et al., 2008)
pOspAB-∆IR	pOspA carrying P*ospAB*-∆IR controlling OspA	This study
pOspAB-ΔIR,Rept	pOspA carrying P*ospAB*-∆IR,Rept controlling OspA	This study
pOspAB-ΔIR,Rept,T-Rich	pOspA carrying P*ospAB*-∆IR,Rept,T-Rich controlling OspA	This study
pOspAB-T-Rich-mut	pOspA carrying P*ospAB*-T-Rich-mut controlling OspA	This study
pJD48	*B. burgdorferi/E. coli* shuttle vector with a luciferase reporter under control of no promoter	(Blevins et al., 2007)
pLuc-P*flaB*	pJD48 carrying *flaB* promoter controlling luciferase	This study
pLuc-FL	pJD48 carrying P*ospAB*-FL controlling luciferase	This study
pLuc-∆IR	pJD48 carrying P*ospAB*-No-IR controlling luciferase	This study
pLuc-∆IR,Rept	pJD48 carrying P*ospAB*-No-IR,Rept controlling luciferase	This study

pLuc-ΔIR,Rept,T-Rich	pJD48 carrying P*ospAB*-No-IR,Rept,T-Rich controlling luciferase	This study
pLuc-T-Rich-mut	pJD48 carrying P*ospAB*-T-Rich-mut controlling luciferase	This study
***B. burgdorferi* Strains**		
13A	Clone derived from *B. burgdorferi* B31 5A13	(Xu et al., 2007)
AH130	Clone derived from *B. burgdorferi* RJ297	(Hubner et al., 2001)
Δ*ospAB*	*ospAB* mutant	(Yang et al., 2004)
AH130/pJD48	AH130 receiving pJD48	This study
AH130/pLuc-P*flaB*	AH130 receiving pLuc-P*flaB*	This study
AH130/pLuc-FL	AH130 receiving pLuc-FL	This study
AH130/pLuc-ΔIR	AH130 receiving pLuc-ΔIR	This study
AH130/pLuc-ΔIR,Rept	AH130 receiving pLuc-ΔIR,Rept	This study
AH130/pLuc-ΔIR,Rept,T-Rich	AH130 receiving pLuc-ΔIR,Rept,T-Rich	This study
AH130/pLuc-T-Rich-mut	AH130 receiving pLuc-T-Rich-mut	This study
13A/pJD48	13A receiving pJD48	This study
13A/pLuc-P*flaB*	13A receiving pLuc-P*flaB*	This study
13A/pLuc-FL	13A receiving pLuc-FL	This study
13A/pLuc-ΔIR	13A receiving pLuc-ΔIR	This study
13A/pLuc-ΔIR,Rept	13A receiving pLuc-ΔIR,Rept	This study
13A/pLuc-ΔIR,Rept,T-Rich	13A receiving pLuc-ΔIR,Rept,T-Rich	This study
13A/pLuc-T-Rich-mut	13A receiving pLuc-T-Rich-mut	This study

Δ*ospAB*/pOspAB	Δ*ospAB* receiving pOspAB	This study
Δ*ospAB*/pOspAB-Δ*IR*	Δ*ospAB* receiving pOspAB-ΔIR	This study
Δ*ospAB*/pOspAB-Δ*IR,Rept*	Δ*ospAB* receiving pOspAB-ΔIR,Rept	This study
Δ*ospAB*/pOspAB-Δ*IR,Rept,T-Rich*	Δ*ospAB* receiving pOspAB-ΔIR,Rept,T-Rich	This study
Δ*ospAB*/pJD48	Δ*ospAB* receiving pJD48	This study
Δ*ospAB/pLuc-PflaB*	Δ*ospAB* receiving pLuc-PflaB	This study
Δ*ospAB/pLuc-FL*	Δ*ospAB* receiving pLuc-FL	This study
Δ*ospAB*/pLuc-ΔIR	Δ*ospAB* receiving pLuc-ΔIR	This study
Δ*ospAB*/pLuc-ΔIR,Rept	Δ*ospAB* receiving pLuc-ΔIR,Rept	This study
Δ*ospAB*/pLuc-ΔIR,Rept,T-Rich	Δ*ospAB* receiving pLuc-ΔIR,Rept,T-Rich	This study
Δ*ospAB*/pLuc-T-Rich-mut	Δ*ospAB* receiving pLuc-T-Rich-mut	This study
Δ*ospAB*/*rrp2G239C*	Δ*ospAB* with *rrp2* point mutation	(He et al., 2008)
Δ*ospAB*/Δ*rpoN*	Δ*ospAB* with inactivated *rpoN*	(He et al., 2008)
Δ*ospAB*/Δ*rpoS*	Δ*ospAB* with inactivated *rpoS*	(He et al., 2008)

Generation of Native OspA Reporter Vectors

The plasmid pOspAB is a shuttle vector containing origins of replication for both *E. coli* (ColE1) and *B. burgdorferi* (cp9) as previously described (He et al., 2008; Yang et al., 2004). pOspAB contains the native *ospA* gene as a reporter under the control of the full-length *ospAB* promoter containing all three of the putative *cis*-elements. The plasmid also has a kanamycin resistance marker. To construct shuttle vectors with various deletions of the *ospAB* promoter controlling the *ospAB* reporter, multiple polymerase chain reactions (PCR) were performed using the primer sets indicated in **Table 2**. pOspAB, containing the full-length *ospAB* promoter, served as the cloning template. Each PCR reaction used Taq DNA Polymerase (New England BioLabs) and the same 3' primer, *ospAB*-Down-3'-*SacI*, but different 5' primers. The 5' primers were used in the reactions to create various length *ospAB* promoter fragments as noted in the ΔIR promoter removed the IR region from the full-length *ospAB* promoter; the ΔIR,Rept promoter removed the promoter segment encompassing the IR and the Repeat region of the *ospAB* promoter; and the ΔIR,Rept,T-Rich promoter removed the promoter segment encompassing each of the three putative *cis*-elements from the *ospAB* promoter.

During PCR amplification of the various promoters, the 3' primer *ospAB*-Down-3'-*SacI* introduced a *SacI* restriction site to the 3' end of the resulting fragments while each of the 5' primers introduced an *XbaI* restriction site at the 5' end (**Table 2**). The resulting PCR fragments (ΔIR, ΔIR,Rept, and ΔIR,Rept,T-Rich) were cloned into the pSC-A-amp/kan TA cloning vector (Stratagene, La Jolla, CA) to create the plasmids. Blue/white screening was used to select for transformants by spreading 40 µl of 40 µg ml^{-1}X-gal dissolved in dimethylformamide on top of the agar plates containing ampicillin. The three resulting plasmids and the parental plasmid pOspAB were digested with *SacI* and *XbaI* and then ligated together to create pOspAB-ΔIR, pOspAB-ΔIR,Rept, and pOspAB-

ΔIR,Rept,T-Rich (**Table 1**). These plasmids have various *ospAB* promoters site-directionally inserted upstream of the *ospAB* gene to control the transcription of *ospAB*. pOspAB has the full-length *ospAB* promoter containing all three of the putative *cis*-elements: the IR, Repeats and T-Rich regions. The pOspAB-ΔIR contains the Repeat and the T-Rich regions, but not the IR. The pOspAB-ΔIR,Rept contains the T-Rich region but lacks the IR and Repeats regions. pOspAB-ΔIR,Rept,T-Rich lacks all three of the putative *cis*-elements: IR, Repeats, and T-Rich.

Site-directed mutagenesis was performed by Genscript (Piscataway, NJ) to mutate the T-Rich region of the full-length *ospAB* promoter. The T-Rich region's 10 bp sequence "TTATTTTTT" of the plasmid pOspAB was mutated to the 10 bp sequence "CGCGGCCGCG" to create the plasmid pOspAB-T-Rich-mut. This plasmid contains *ospAB* expressed by the PospAB-T-Rich-mut promoter, which is the full-length *ospAB* promoter with a mutated T-Rich region.

To place the P*ospAB*-T-Rich-mut promoter controlling luciferase expression, PCR amplification of the plasmid pOspAB-T-Rich-mut using the primers *ospA*-FL-*BglI*I and *ospA*-ATG-3-*NdeI* was performed (**Table 2**). The resulting promoter fragment and the pJD48 plasmid were digested with *NdeI* and *BglII* and then ligated to create the plasmid pLuc-T-Rich-mut. This plasmid contains luciferase expressed by the full-length ospAB promoter with a mutated T-Rich region.

Table 2. Primers used in this study.

Primer name	Sequence (5' to 3')	Purpose
ospAB- No-IR-*XbaI*	CA**TCTAGA**CATTAAATCTAAGCTTAATTAGAAC	5' primer; PCR of the *ospAB* promoter for constructing pOspAB-ΔIR
ospAB- No-Repeat-*XbaI*	CA**TCTAGA**CCAAACTTAATTGAAGTTATTATC	5' primer; PCR of the *ospAB* promoter for constructing pOspAB-ΔIR,Rept
ospAB- No-T-Rich-*XbaI*	CA**TCTAGA**ATTTTCTATTTGTTATTTGTTAATC	5' primer; PCR of the *ospAB* promoter for constructing pOspAB-ΔIR,Rept,T-Rich
ospAB- Down-*3'-SacI*	CTT**GAGCTC**CTAAGAGACTTTTTCCAGAAGTAA(	3' primer; PCR of the *ospAB* promoter for constructing all of above listed plasmids.
ospA -FL-*BglII*	**AGATCT**AGACATTTAACTTTTC	5' primer; PCR of the *ospAB* promoter for constructing pLuc-FL
ospA -No-IR-*BglII*	CT**AGATCT**CATTAAATCTAAGCTTAATTAGAA	5' primer; PCR of the *ospAB* promoter for constructing pLuc-ΔIR
ospA- No-Repeat-*BglII*	CT**AGATCT**CCAAACTTAATTGAAGTTATTAT	5' primer; PCR of the *ospAB* promoter for constructing pLuc-ΔIR,Rept
ospA- No-T-Rich-*BglII*	CT**AGATCT**AATTTTCTATTTGTTATTTGTTAAT	5' primer; PCR of the *ospAB* promoter for constructing pLuc-ΔIR,Rept,T-Rich
ospA -ATG-3-*NdeI*	**CATATG**ATATTCTCCTTTTATATTAATATAACTT	3' primer; PCR of the *ospAB* promoter for constructing the four above listed plasmids.
FlaB-F	**AGATCT**TACCTTGGATTTTACCGTTAAGCGC	5' primer; PCR of the *flaB* promoter for constructing pLuc-P*flaB*
FlaB-R	**CATATG**ATATCATTCCTCCATGATAAAAT	3' primer; PCR of the *flaB* promoter for constructing pLuc-P*flaB*
Q-HX-OspA-F	TAGCAGCCTTGACGAGAAAAACAG	5' primer; PCR of the *ospA* gene for qRT-PCR
Q-HX-OspA-R	TTATCAGAAGTTCCTTTAAGCTCA	3' primer; PCR of the *ospA* gene for qRT-PCR
Q-HX-FlaB-F	ACCAGCATCACTTTCAGGGTCTCA	5' primer; PCR of the *flaB* gene for qRT-PCR
Q-HX-FlaB-R	CAGCAATAGCTTCATCTTGGTTTG	3' primer; PCR of the *flaB* gene for qRT-PCR
*Restriction sites for purpose of cloning are highlighted in **boldface** letters		

Generation of Luciferase Reporter Vectors

The previously described plasmid pJD48 was used to create the luciferase reporter vectors (Blevins et al., 2007). pJD48 is a shuttle vector containing a replication origin for *B. burgdorferi* (cp9) and for *E. coli* (ColE1). pJD48 contains an *NdeI* and *BglII* cloning site upstream of the luciferase gene which allows for placement of a promoter to control the expression of the luciferase. In addition to luciferase, pJD48 also contains a kanamycin-resistance marker. To create luciferase reporter constructs with varying length *ospAB* promoters, serial truncations of the *ospAB* promoter were created via PCR using four various 5' primers and using the same 3' primer (P*ospA*-ATG-3-*NdeI*). The 5' primer introduced a *BglII* restriction site to the 5' PCR fragment end while the 3' primer introduced an *NdeI* restriction site to the 3' end of the PCR fragment; pOspA, containing the full-length wild-type *ospAB* promoter, served as the PCR template. Primer pairs are listed in **Table 2**. The resulting PCR fragments (FL; ΔIR; ΔIR,Repts; and ΔIR,Repts,T-Rich) were cloned into the pSC-A-amp/kan TA cloning vector. The resulting plasmids and pJD48 were digested with *NdeI* and *BglII* and ligated together to create pLuc-FL, pLuc-ΔIR, pLuc-ΔIR,Repts, and pLuc-ΔIR,Repts,T-Rich (**Table 1**).

Genetic Manipulation of *Borrelia burgdorferi*

Borrelial electroporations were performed as previously described (Samuels, 1995; Yang et al., 2004). *B. burgdorferi* were recovered from -80°C frozen stock into 2mL of BSK medium. The culture was incubated at 34°C with 5% CO_2 until the culture cell density was approximately 3×10^5 spirochetes ml^{-1}. The culture was then transferred to 50ml BSK medium with appropriate antibiotics and grown until the cell density reached more than 3×10^7 spirochetes ml^{-1}. For preparation of competent cells, the *Borrelia* culture was centrifuged at 4,000 rpm for 20 minutes at 4°C and the supernatant was decanted. The cells were washed twice with 30 ml cold saline (0.9% sodium chloride dissolved in

double-distilled water) and washed an additional three times with 30 ml cold Electroporation Solution (EPS) which consists of 9.3% sucrose and 15% glycerol dissolved in double-distilled water. After the final wash, the final pellet was gently suspended in 50 µl of EPS and transferred to a 1.5 ml eppendorf tube. About 5-20 µg of plasmid DNA was added to the resuspension and the mixture was transferred to a pre-chilled electroporation 0.2 cm cuvette (Bio-Rad) and allowed to chill on ice for at least 1 minute. To electroporate, the cuvette was placed in the gene pulser (Gene pulser, Bio-Rad) and a single exponential decay pulse of 2.5 kV, capacitance of 25 µF, and resistance of 200 Ω was allowed producing a time constant of 4 to 6 ms. Immediately after electroporation, 1 ml of BSK was added to the cuvette and then this was transferred to 35 ml BSK without antibiotics. The culture was incubated overnight at 34°C with 5% CO_2. Following overnight incubation, appropriate antibiotics were added and 220 µl of the 35 ml overnight culture was aliquoted into each well of a 96 well plate. The color of the medium in the plate wells were monitored; a change of color from red to yellow indicated that cells were growing, which was verified by microscopic examination of a sample of the cells from the well. If cell growth is confirmed, 10 µl of the positive culture was transferred to 2 ml or 15 ml BSK with appropriate antibiotics and then further analyzed and assayed.

Quantitative RT-PCR

Cultures were pelleted by centrifugation and the RNA extracted using the RNeasy mini kit (Qiagen, Valencia, CA) per the manufacturer's instructions. Purified RNA was treated with DNase (Promega) and converted to cDNA using SuperScript III reverse transcriptase with random primers (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Platinum SYBR green qPCR SuperMix-UDG (Invitrogen) along with primers specific for *flaB* and *ospA* were used to perform qPCR in triplicate on

the cDNA samples (**Table 2**). An absolute quantification method using qPCR was performed according to the manufacturer's instructions (Stratagene) using the specific *flaB* primers and serial dilutions of the standard template, a cloning vector containing the *flaB* gene. Briefly, a standard curve was generated by plotting template quantity against the Ct values of the standards. The quantity of *ospA* transcript was determined by comparing the samples' Ct values to the standard curve plot. The standards and samples were performed in triplicate using an ABI 7000 Sequence Detection System.

Luciferase assays

Luciferase assays were performed as previously described using the commercial luciferase assay system (Promega Corp., Madison, WI) (Blevins et al., 2007). After enumerating the bacteria via dark-field microscopy, bacterial cultures were centrifuged at 4,000 rpm for 20 minutes at 4°C. The supernatant was decanted and the pellet was washed twice with PBS, with centrifugation at 4,000 rpm for 20 min at 4°C after each wash. The final pellet was transferred to a 1.5 ml Eppendorf tube and was suspended in 100 µl of the cell culture lysis reagent (CCLR), which was supplied by the manufacturer. The debris were pelleted by centrifugation in a microcentrifuge at 15,000 rpm for 1 min. Ten microliters of lysate was aliquoted into luminometer tubes (Promega) and 50 µl of luciferase assay reagent was added immediately prior to beginning measurements. Luciferase activity was measured for 10 sec using a Centro LB 960 luminometer (Berthold Technologies, Oak Ridge, TN). The average background luminescence was subtracted from the readings and the measurement reported as relative light units (RLU).

Host-Adapted Spirochete Model

The insertion of DMC's containing *B. burgdorferi* into the peritoneal cavities of rats was previously described (Akins et al., 1998). *Borrelia* were grown to mid-logarithmic and then diluted in BSK to 3×10^3 *Bb* ml^{-1}. Five milliliters of this dilution was put into a 10 kDa Spectra/Por dialysis membrane (Spectrum Medical Industries Inc., Los Angeles, CA) and inserted into the peritoneal cavity of four- to six-week-old Sprague-Dawley rats (Harlan, Indianapolis, IN) using strict aseptic technique. Fourteen days after implantation, the DMCs were harvested and its contents removed by syringe aspiration. Spirochetes were enumerated via dark-field microscopy and then prepared for the luciferase assay as described.

CHAPTER THREE: RESULTS

Section I: The T-Rich region is required for the full activation of the *ospAB* promoter.

Deletion of T-Rich element in wild-type *B. burgdorferi* with luciferase reporter results in decreased luciferase expression

The *ospAB* promoter contains three putative *cis*-elements: the IR region, the repeats region, and the T-Rich region (Sohaskey et al., 1999). To determine which of these three putative *cis*-elements are required for the full activation of the *ospAB* promoter, the various length *ospAB* promoters P*ospA*B-FL; P*ospAB*-ΔIR; P*ospAB*-ΔIR,Rept; and P*ospAB*-ΔIR,Rept,T-Rich were created by PCR using pOspAB as a template. The four promoters are as follows: 1) the P*ospAB*-FL promoter is the full-length *ospAB* promoter containing all three putative *cis*-elements; 2) the P*ospAB*-ΔIR promoter lacks the IR region while retaining the Rept and T-Rich regions; 3) the P*ospAB*-ΔIR,Rept promoter retains the T-Rich region but lacks the region encompassing the IR and the Rept elements; and 4) the P*ospAB*-ΔIR,Rept,T-Rich promoter deletes the *ospAB* promoter region encompassing all three of the putative *cis*-elements. These promoters were site-directionally cloned upstream of the luciferase open reading frame in the pJD48 shuttle vector to control the expression of the luciferase reporter, and then the plasmids were electroporated into wild-type *B. burgdorferi* 13A to create the strains pLuc-FL; pLuc-ΔIR; pLuc-ΔIR, Rept; and pLuc-ΔIR,Rept,T-Rich, respectively.

These four strains were grown *in vitro* at 37°C, a condition in which *ospA* is constitutively expressed, and then harvested and prepared for the luciferase assay. The effect of the absence of the putative *cis*-elements on the *ospAB* promoter activity was determined

using the luciferase assay. The luciferase assay results indicate that there was no significant difference in luciferase activity between the P*ospAB*-FL promoter and the P*ospAB*-ΔIR or the P*ospAB*-ΔIR,Rept promoters (**Figure 9**). However, there was a reduction in luciferase activity in the P*ospAB*-ΔIR,Rept,T-Rich promoter, showing that deletion of the T-Rich element results in a decrease of *ospAB* promoter activation.

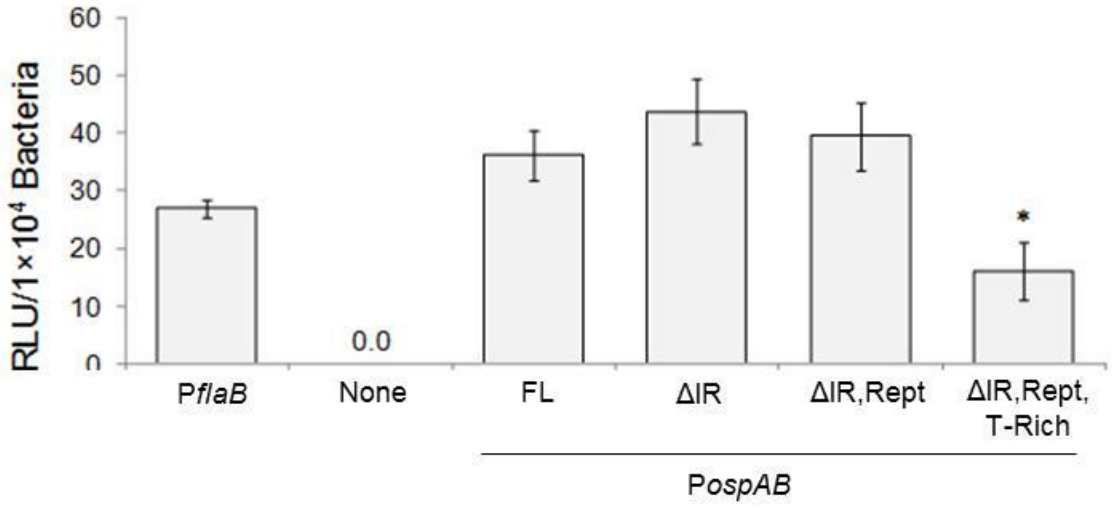

Figure 9. Influence of cis-elements on *ospAB* promoter activation.

The 13A strain electroporated with a shuttle vector containing the luciferase reporter under the control of either the constitutively expressed *flaB* promoter (P*flaB*), no promoter (none), the full-length *ospAB* promoter (P*ospAB*-FL), the *ospAB* promoter lacking the IR region (P*ospAB*-ΔIR), the *ospAB* promoter lacking the IR and Repeats regions (P*ospAB*-ΔIR,Rept) and the *ospAB* promoter lacking all three putative *cis*-elements (P*ospAB*-ΔIR,Rept,T-Rich). Strains were grown in BSK at 37°C and prepared for luciferase assay (*, $p<0.05$ using paired Student's t-test).

Deletion of T-Rich element in the *ospAB* mutant *B. burgdorferi* with *ospA* reporter results in decreased *ospA* transcript.

Using various *ospAB* promoters controlling the luciferase reporter expression within the wild-type *Borrelia* 13A, it was determined that deletion of the T-Rich region results in decreased activation of the *ospAB* promoter. To confirm this finding, we used a similar approach to measure *ospAB* promoter activity, but employed a native *ospAB* reporter rather than using the luciferase reporter.

The various length *ospAB* promoters P*ospAB*-FL; P*ospAB*-ΔIR; P*ospAB*-ΔIR,Rept; and P*ospAB*-ΔIR,Rept,T-Rich were site-directionally cloned upstream of the *ospAB* open reading frame in the pOspAB shuttle vector to control the expression of the *ospAB* reporter. The plasmids were electroporated into *ospAB* mutant *Borrelia* to create the strains pOspAB-FL; pOspAB-ΔIR; pOspAB-ΔIR, Rept; and pOspAB-ΔIR,Rept,T-Rich, respectively. The resulting strains were grown *in vitro* at 37°C, a condition in which *ospA* is highly expressed. The cultures were harvested and then prepared for analysis by qRT-PCR.

The *ospA* transcript number was measured relative to the flagellin (*flaB*) transcript which is constitutively expressed. The results show that deletion of the IR or deletion of the IR and Rept regions together did not result in a significant decrease of *ospA* transcript compared to the full-length promoter (**Figure 10**). However, deletion of the IR, Rept and T-Rich regions combined showed a decrease in the *ospA* transcript level compared to the full-length promoter suggesting that the T-Rich region is required for the full activation of *ospA*. This result is in agreement with our previous finding using the luciferase reporter.

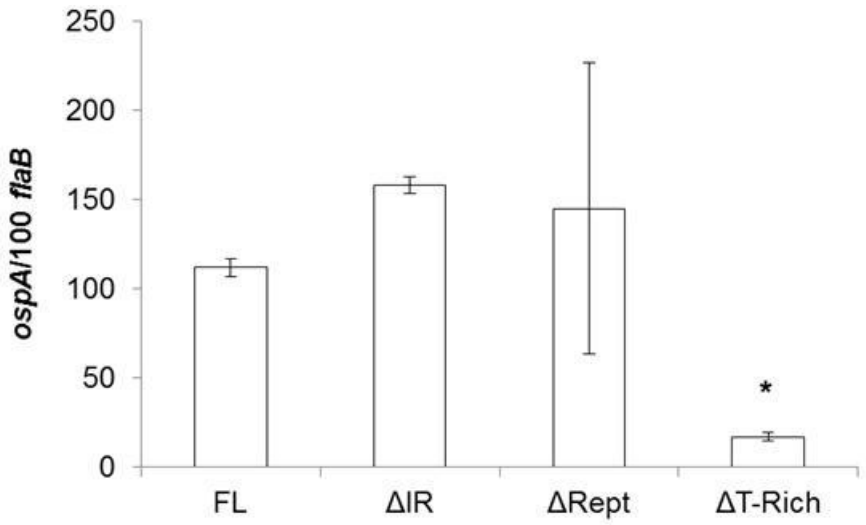

Figure 10. *ospA* transcript levels of the various *ospAB* promoters controlling expression of *ospAB* within the *ospAB* mutant *B. burgdorferi*.

The *B. burgdorferi ospAB* mutant with a shuttle vector containing the *ospAB* reporter under the control of either the full-length *ospAB* promoter (P*ospAB*-FL), the *ospAB* promoter lacking the IR region (P*ospAB*-ΔIR), the *ospAB* promoter lacking the IR and Repeats regions (P*ospAB*-ΔIR,Rept) and the *ospAB* promoter lacking all three putative *cis*-elements (P*ospAB*-ΔIR,Rept,T-Rich). Strains were grown in BSK at 37°C and prepared for qRT-PCR. Transcript relative to copies of flagellin, *flaB* (*, $p<0.05$ using paired Student's t-test).

Mutation of the T-Rich element in wild-type *B. burgdorferi* with luciferase reporter results in decreased luciferase expression

Next, we wanted to further dissect the *ospAB* promoter. The promoters used to show that the T-Rich region is required for the full activation have multiple elements missing and not just the T-Rich region. To ensure that the effect we have seen is dependent solely on the T-Rich region and not a combination of the T-Rich element and the IR or Rept elements, we used site-directed mutagenesis to mutate only the T-Rich region.

Site-directed mutagenesis was used to replace the 10 bp sequence "TTATTTTTTT" of the T-Rich region with a different 10 bp sequence "CGCGGCCGCG" to create the promoter P*ospAB*-T-Rich-mut. This promoter was site-directionally cloned upstream of the luciferase open reading frame in the pJD48 shuttle vector and then transformed into the 13A *B. burgdorferi* strain and grown *in vitro* at 37°C. The luciferase expression level of the P*ospAB*-T-Rich-mut promoter was compared to the P*ospAB*-FL. The P*ospAB*-T-Rich-mut promoter had significantly reduced levels of luciferase (**Figure 11**). The reduction in the *ospAB* promoter expression caused by the mutagenesis of the T-Rich region indicates that the T-Rich region alone is responsible for the full expression of *ospA*. Together, these studies show that the T-Rich region is required for the full activation of *ospA*.

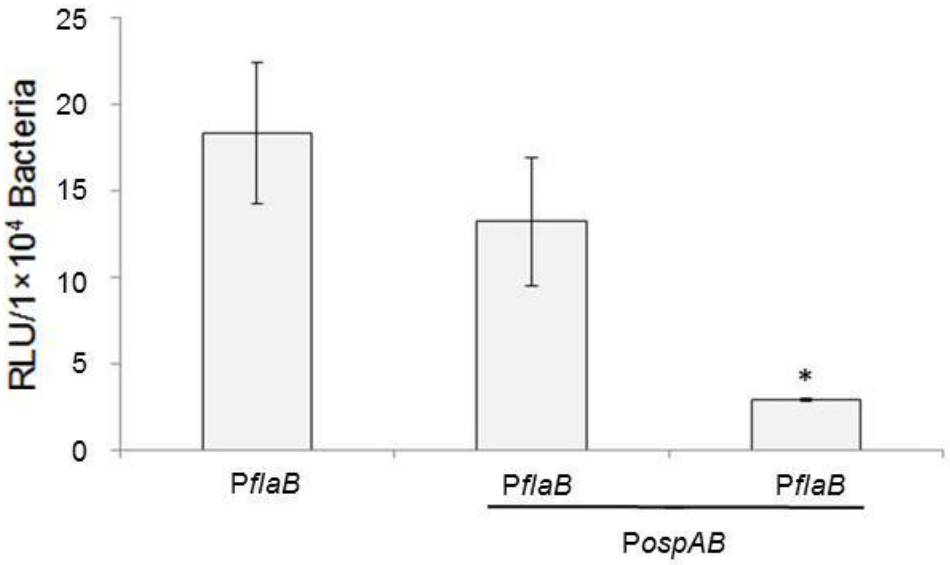

Figure 11. Influence of the T-Rich *cis*-element on *ospAB* promoter activation.

The 13A strain electroporated with a shuttle vector containing the luciferase reporter under the control of either the constitutively expressed *flaB* promoter (P*flaB*), the full-length *ospAB* promoter (P*ospAB*-FL), and the P*ospAB*-FL promoter with a mutated T-Rich region (P*ospAB*-T-Rich-mut). Strains were grown in BSK at 37°C and prepared for luciferase assay (*, $p<0.05$ using paired Student's t-test).

Section II: Establishment of an *in vitro* model of *ospA* repression

Abrogation of *ospAB* results in constitutive activation of *ospC*

OspA and OspB are major surface lipoproteins in *B. burgdorferi*. During mammalian infection, OspC is produced while OspA is repressed. However, the inverse regulation is true during the infection of ticks: OspA is produced while OspC is not. A previous study by Yang et al. (2004) abrogated OspAB by disrupting the *ospAB* operon. Their studies demonstrated that the *ospAB* mutant was able to infect mice but was not able to colonize or replicate in ticks (Yang et al., 2004). In addition to this finding, we observed that the deletion of *ospAB* within *B. burgdorferi* results in the increased, constitutive expression of *ospC* (He et al., 2009)

To determine the influence of OspA and OspB on the expression of OspC, the protein profiles of the *ospAB* mutant and the wild-type parental strain BbAH130 were compared. The *ospAB* mutant and BbAH130 were grown *in vitro* at either 23°C, a condition in which OspC is not produced, or 37°C, a condition where OspC is produced. The cultures were harvested and the lysates were subjected to SDS-PAGE before the proteins were stained by Coomassie blue (**Figure 12**). As expected, at 23°C, the wild-type BbAH130 did not produce OspC; at low temperatures which mimic the tick environment, OspA is highly expressed whereas OspC is not. At both 23°C and 37°C, the *ospAB* mutant did not produce OspA, as expected, due to the disruption of the *ospAB* operon. However, the *ospAB* mutant exhibited OspC production at both 23°C and 37°C. OspC is normally expressed at 37°C, but not at 23°C. These results show that OspC is constitutively expressed in the *ospAB* mutant regardless of temperature.

In addition to determining the effect of *ospAB* abrogation on OspC, the effect of *ospAB* abrogation on other differentially expressed lipoproteins was also evaluated. Decorin-binding protein (DbpA), fibronectin-binding protein (BBK32), and multicopy lipoprotein-8 (Mlp8) are all lipoproteins which are known to be expressed at 37°C, but not at 23°C (He et al., 2007; Hubner et al., 2001; Yang et al., 2007). To determine if expression of these temperature-induced lipoproteins was affected by the abrogation of *ospAB*, immunoblot assays were performed on the wild-type BbAH130 and *ospAB* mutant whole cell lysates from cultures grown at 23°C. Antibodies specific to the differentially expressed lipoproteins DbpA, BBK32, Mlp-8 as well as OspC were used. FlaB, the constitutively expressed flagellin protein, served as the protein loading standard. The lipoproteins DbpA, BBK32 and Mlp-8 were not present or in low presence in the wild-type BbAH130, however, they were abundantly produced in the *ospAB* mutant (**Figure 13**). These results demonstrate that in addition to *ospC* being constitutively expressed in the *ospAB* mutant grown at 23°C, *dbpA*, *bbk32* and mlp-8 are constitutively expressed or upregulated in the *ospAB* mutant.

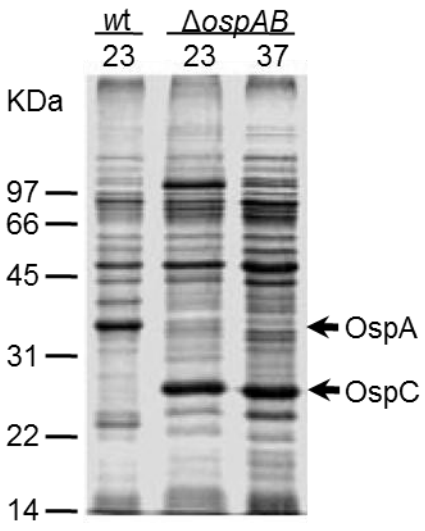

Figure 12. Abrogation of *ospAB* results in constitutive activation of ospC (He et al., 2009).

The wild-type clone BbAH130 (wt) and the isogenic *ospAB* mutant (Δ*ospAB*) were grown in BSK medium at either 23°C (23) or 37°C (37). Cultures were harvested at mid-logarithmic phase, and cell lysates were subjected to SDS-PAGE before the protein was stained with Coomassie blue. The molecular mass markers are indicated at the left of the figure in kilodaltons (kDa). Protein bands corresponding to OspA and OspC are labeled on the right.

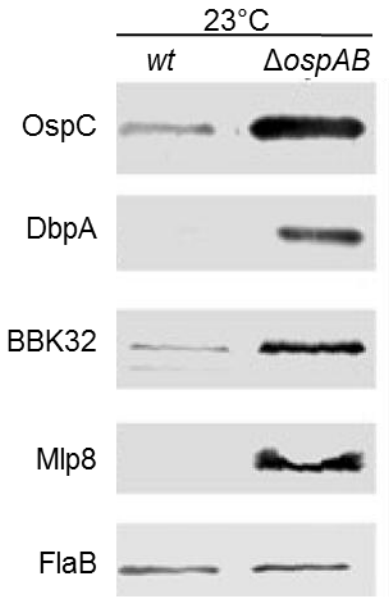

Figure 13. Abrogation of *ospAB* influences the production of lipoproteins (He et al., 2009).

The wild-type clone BbAH130 (wt) and the isogenic *ospAB* mutant (Δ*ospAB*) were grown in BSK medium at 23°C. Cultures were harvested at mid-logarithmic phase and whole cell lysates were probed with antibodies directed against the specific lipoprotein (indicated on left). FlaB is constitutively expressed and serves as the control for equal lysate amounts.

Mutation or inactivation of *rpoS*, *rpoN* or *rrp2* abolishes constitutive expression of *ospC* in the *ospAB* mutant

In *B. burgdorferi*, the Rrp2-RpoN-RpoS pathway mediates the temperature-induced expression of *ospC*, *dbpA*, and *bbk32* (**Figure 6**) (Eggers et al., 2004; Gilbert et al., 2007; He et al., 2007; Hubner et al., 2001; Yang et al., 2003a). Since abrogation of *ospAB* resulted in constitutive expression of *ospC*, *dbpA* and *bbk32*, and expression of these three lipoproteins is mediated through the Rrp2-RpoN-RpoS pathway, we hypothesized that the Rrp2-RpoN-RpoS regulatory pathway is constitutively activated in the *ospAB* mutant.

To determine if the Rrp2-RpoN-RpoS pathway is responsible for the constitutive expression of *ospC* within the *ospAB* mutant, mutations in the *rrp2*, *rpoN*, or *rpoS* genes were generated in the *ospAB* mutant. The *ospAB* mutant and the double mutant cultures were cultivated in BSK at 35°C, harvested at mid-log and the whole cell lysates subjected to SDS-PAGE before staining with Coomassie blue. The *rrp2* point mutation, the inactivated *rpoN* and the inactivated *rpoS* in the *ospAB* mutant resulted in decreased constitutively expressed OspC production compared to the wild-type or the *ospAB* mutant (**Figure 14**). These results indicate that *ospC* is constitutively expressed due to the Rrp2-RpoN-RpoS pathway being constitutively expressed upon abrogation of *ospAB*.

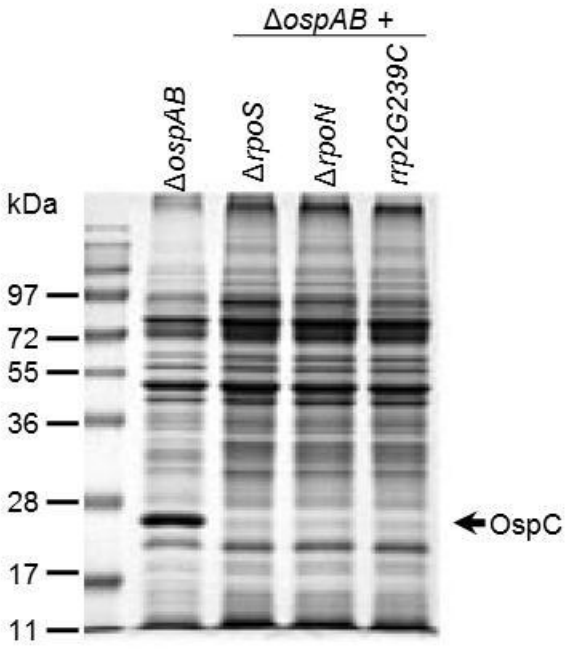

Figure 14. Constitutive expression of OspC in the *ospAB* mutant is abolished upon mutation or inactivation of *rpoS, rpoN or rrp2* (He et al., 2008).

The *ospAB* single mutant (Δ*ospAB*), and the *ospAB* double mutants of *rpoS* (Δ*rpoS*), *rpoN* (Δ*rpoN*), and *rrp2* (*rrp2G239C*) were cultured in BSK at 35°C, harvested at mid-log and the whole cell lysates subjected to SDS-PAGE before staining with Coomassie blue. The molecular mass markers are indicated at the left of the figure in kilodaltons (kDa).

Complementation of the *ospAB* mutant with a wild-type copy of *ospAB* or *ospA* alone restores repression of *ospC* at 23°C

The constitutive activation of the Rrp2-RpoN-RpoS pathway in the *ospAB* mutant could be due to spurious mutation locking the pathway in an active state rather than the constitutive expression being due to the abrogation of *ospAB*. To ensure that the constitutive activation of the Rrp2-RpoN-RpoS pathway was due to the loss of *ospAB* expression, the *ospAB* mutant was complemented with the shuttle vector pOspAB carrying a wild-type copy of native *ospAB*. The wild-type, *ospAB* mutant and the *ospAB* complement were cultivated in BSK at 23°C and whole-cell lysates were subjected to SDS-PAGE before staining with Coomassie blue. The *ospAB* mutant complemented with *ospAB* was not able to constitutively produce OspC at 23°C like the *ospAB* mutant (**Figure 15**). This suggests that the abrogation of *ospAB* is responsible for the constitutive activation of the Rrp2-RpoN-RpoS pathway.

In addition to complementing the *ospAB* mutant with native *ospAB,* the *ospAB* mutant was complemented with only *ospA* driven by the native *ospAB* promoter (pOspA) to determine if the absence of *ospA* was mainly responsible for the constitutive OspC production phenotype. The shuttle vector alone without *ospAB* or *ospA*, pBSV2, was used as a control to ensure the effects seen in pOspAB or pOspA were due to the shuttle vector, but rather to reintroducing *ospAB* and *ospA*. Complementation with *ospA* alone restored the temperature-dependent repression of *ospC* expression (**Figure 15**). Together, these data demonstrate that the abrogation of *ospA*, rather than spurious mutation, was responsible for the constitutive activation of the Rrp2-RpoN-RpoS regulatory pathway.

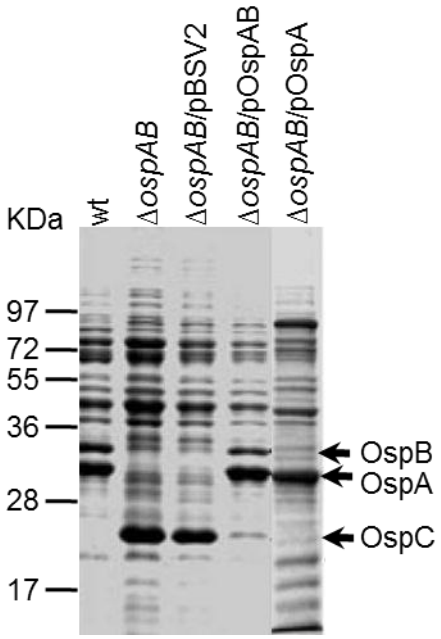

Figure 15. Complementing the *ospAB* mutant with *ospAB* or *ospA* alone restores ospC repression at 23°C (He et al., 2008).

The wild-type strain (wt), the *ospAB* mutant (Δ*ospAB*), the *ospAB* mutant complemented with the shuttle vector alone (Δ*ospAB*/pBSV2), and the *ospAB* mutant complemented with the shuttle vector carrying the native copy of *ospAB* (Δ*ospAB*/pOspAB) or *ospA* (Δ*ospAB*/pOspA) were cultivated at 23°C. Whole-cell lysates were subjected to SDS-PAGE before staining with Coomassie blue. The labeled bands on right correspond to OspA, OspB and OspC. The molecular mass markers are indicated at the left of the figure in kilodaltons (kDa).

Proposed model for the positive feedback circuit between the activation of the Rrp2-RpoN-RpoS regulatory pathway and the reduction of OspA

The constitutive production of OspC via the Rrp2-RpoN-RpoS regulatory pathway, which is constitutively activated upon abrogation of *ospAB*, suggests a positive feedback circuit (**Figure 16**). Feedback loops have been reported in other bacteria (Guespin-Michel and Kaufman, 2001), and such a feedback loop could be beneficial to *B. burgdorferi*'s transmission and mammalian infection. Given our results, we hypothesize that the Rrp2-RpoN-RpoS regulatory pathway is activated during tick feeding by an unknown signal. The activation of the Rrp2-RpoN-RpoS pathway results in the expression of an unknown transcriptional repressor regulated by RpoS, which is able to repress *ospA* expression. Upon repression of *ospA*, OspA production is decreased and there is a reduction in the surface-associated OspA. This reduction further activates the Rrp2-RpoN-RpoS pathway to achieve and maintain the maximal level of Rrp2-RpoN-RpoS activation to encourage the expression of OspC leading to *B. burgdorferi*'s migration from the tick midgut to establish infection in mammals.

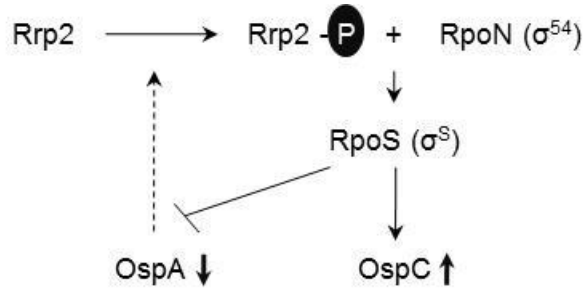

Figure 16. Proposed model for the positive feedback circuit between the activation of Rrp2-RpoN-RpoS regulatory pathway and the reduction of OspA.

The activation of the Rrp2-RpoN-RpoS is hypothesized to be activated upon tick feeding by an unknown signal. The activation of this pathway results in expression of an unknown transcriptional repressor which is able to repress *ospA*. OspA production is decreased upon the repression of *ospA* causing a reduction in the surface-associated OspA. The reduction further activates the Rrp2-RpoN-RpoS pathway to achieve and maintain the maximal level of Rrp2-RpoN-RpoS activation, which in turn activates expression of *ospC* (←, positive activation; ⊢, negative activation).

The proposal of an *in vitro* model of *ospA* repression

The expression of *ospC* is temperature-dependent during *in vitro* cultivation in BSK. Expression of OspA, however, is not temperature-dependent; OspA is abundantly expressed during *in vitro* cultivation at both 23°C and 37°C. Since OspA is not temperature-dependent like OspC, there has been a lack of an *in vitro* model for studying the regulation of *ospA*. Studying the downregulation of *ospA* can only be done *in vivo*, which is very difficult, therefore very little is known about the mechanism underlying the downregulation of *ospA*.

While the complete mechanism of *ospA* downregulation has not been fully elucidated, Caimano et al. discovered that the alternative sigma factor RpoS is required for the repression of *ospA* (2005). RpoS likely represses *ospA* by either 1) binding directly to the *ospAB* promoter; or 2) by controlling the expression of an unknown *ospA* repressor.

He and coworkers have shown that *rpoS* is constitutively activated in an *ospAB* mutant (He et al., 2008). Thus, the *ospAB* mutant can serve as a system to study the repression of the *ospAB* promoter *in vitro*. To develop this system, the pLuc-FL plasmid (a shuttle vector containing the luciferase reporter under the control of the full-length *ospAB* promoter) was electroporated into the *ospAB* mutant. In this construct, the *ospAB* mutant constitutively expresses *rpoS* and subsequently RpoS represses the full-length *ospAB* promoter (probably either directly or indirectly). The full-length *ospAB* promoter controls the expression of a luciferase reporter to measure the level of *ospA* repression by RpoS (**Figure 17A**).

The pLuc-FL plasmid was transformed into both the wild-type parental BbAH130 and the *ospAB* mutant. These strains were grown in BSK at 37°C and analyzed by the luciferase

assay. The *ospAB* mutant had reduced luciferase activity compared to the wild-type BbAH130 (**Figure 17B**). The reduced luciferase activity in the *ospAB* mutant indicates that RpoS was able to repress the *ospAB* promoter. This *ospAB* mutant containing the luciferase reporter under the control of the *ospAB* promoter can be used as a tool to study the repression of *ospA in vitro*. This is a novel *in vitro* model of *ospA* repression.

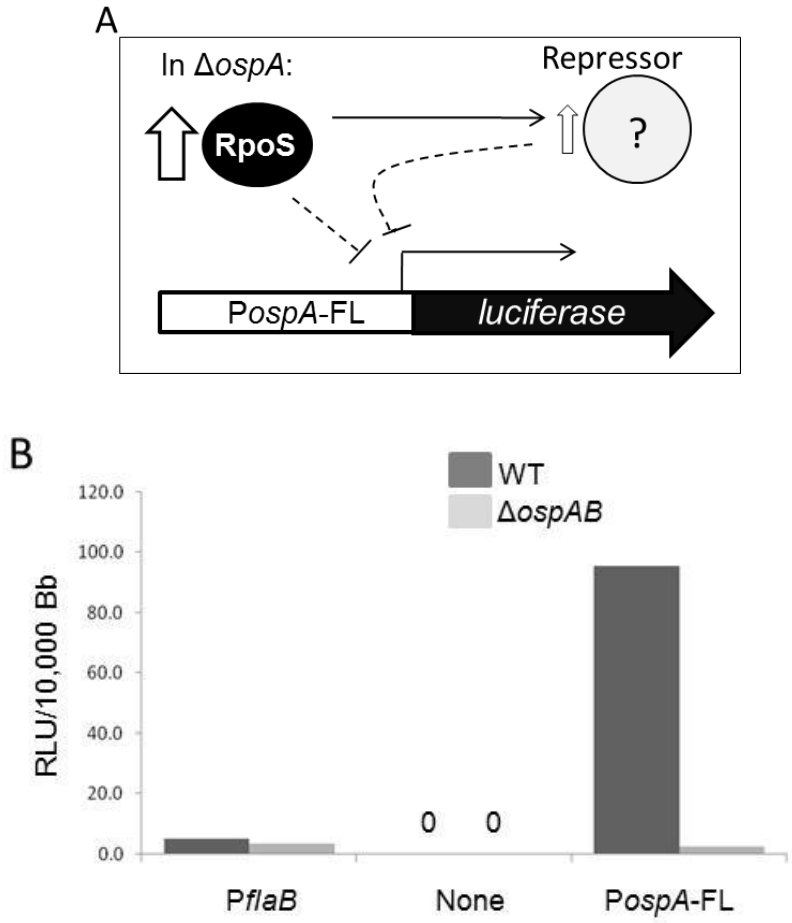

Figure 17. Establishment of an *in vitro* model of *ospA* repression.

(**A**) Schematic of proposed *in vitro* model of *ospA* repression. In *ospAB* mutant *B. burgdorferi* (Δ*ospAB*), RpoS expression is constitutive. RpoS is required for the repression of *ospA* via an unknown mechanism: either RpoS directly represses *ospA* or RpoS influences the expression of an unknown *ospA* repressor. The luciferase reporter expressed by the full-length *ospAB* promoter within the *ospAB* mutant can used to measure the level of *ospAB* promoter repression due to the overexpression of the *ospA* repressor. (**B**) BbAH130 (wt) and the *ospAB* mutant (Δ*ospAB*), both containing the shuttle vector with the full-length *ospAB* promoter controlling luciferase expression, were grown at 37°C. The constitutive *flaB* promoter and a promoterless promoter (none) controlling luciferase expression were used as controls.

Deletion of the T-Rich element results in increased luciferase expression using the *in vitro* model of *ospA* repression

We hypothesize that in *ospA* repression conditions, RpoS controls an unknown repressor (or repressors) which bind to the *ospAB* promoter at one or more of the three putative *cis*-elements (**Figure 16**). To determine which of the three putative *cis*-element(s) are required for the full repression of *ospA,* we employed the *in vitro* model of *ospA* repression where *rpoS* is constitutively expressed (**Figure 17**). Using this model, the *ospAB* mutant, which constitutively expresses the putative *ospA* repressor, was electroporated with a shuttle vector containing the luciferase reporter under the control of either 1) the full-length *ospAB* promoter, P*ospAB*-FL; 2) the P*ospAB*-ΔIR promoter which is lacking the IR element; 3) the P*ospAB*-ΔIR,Rept promoter lacking the region encompassing the IR and Rept elements; or 4) the P*ospAB*-ΔIR,Rept,T-Rich promoter which lacks all three putative *cis*-elements. These strains were grown at 37°C in BSK medium and then harvested and prepared for the luciferase assay. The P*ospAB*-ΔIR,Rept,T-Rich strain had a significant increase in luciferase expression compared to the full-length, P*ospAB*-ΔIR, and P*ospAB*-ΔIR,Rept promoters (**Figure 18A**). This data suggests that the T-Rich region is required for full repression of *ospA* and that RpoS either directly binds to the T-Rich region or RpoS controls a repressor which binds to the T-Rich region.

To determine if the T-Rich region alone is responsible for the full repression of *ospA*, we used our full-length *ospAB* promoter with the mutated T-Rich region (P*ospAB*-T-Rich-mut) controlling luciferase expression and placed the construct in the *ospAB* mutant *B. burgdorferi* and grown *in vitro* at 37°C. The luciferase expression levels of the P*ospAB*-T-Rich-mut promoter was compared to the P*ospAB*-FL. The P*ospAB*-T-Rich-mut promoter had significantly increased levels of luciferase (**Figure 18B**). The reduced

ospA repression caused by the mutagenesis of the T-Rich region indicates that the T-Rich region alone is responsible for the full repression of *ospA*.

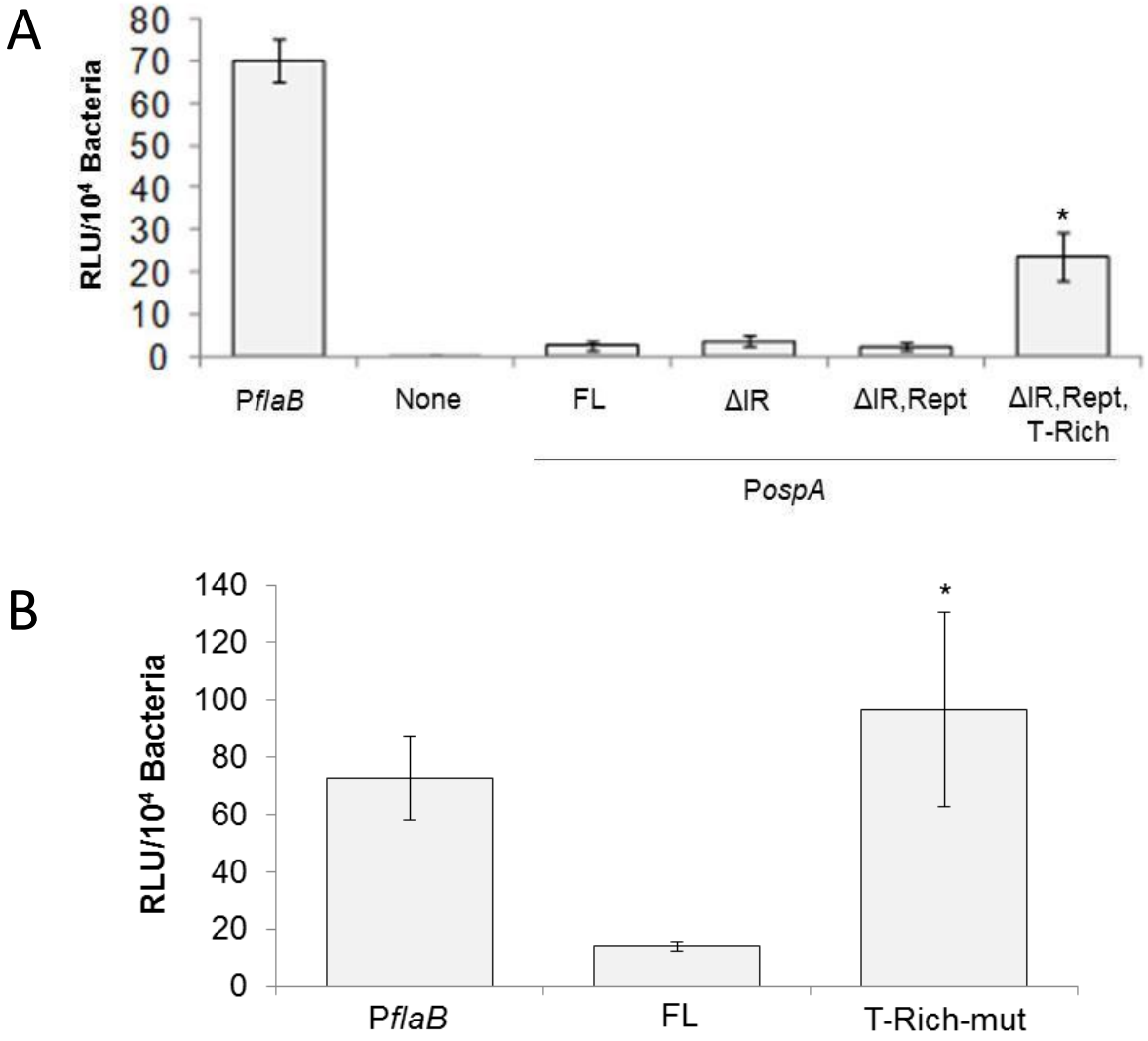

Figure 18. Influence of *cis*-elements on luciferase repression using the *in vitro* model of *ospA* repression.

(A) Luciferase assay of the *ospA* mutant transformed with a shuttle vector containing the luciferase reporter under the control of either constitutively expressed *flaB* promoter (P*flaB*), promoterless (none), the full-length *ospAB* promoter (P*ospAB*-FL), the *ospAB* promoter lacking the IR region (ΔIR), the *ospAB* promoter lacking the IR and Repeats regions (ΔIR,Rept) and the *ospAB* promoter lacking all three putative *cis*-elements (ΔIR,Rept,T-Rich). Strains were grown in BSK at 37°C and prepared for luciferase assay. (B) The *ospAB* mutant strain electroporated with a shuttle vector containing the

luciferase reporter under the control of either the constitutively expressed *flaB* promoter (P*flaB*), the full-length *ospAB* promoter (P*ospAB*-FL), and the P*ospAB*-FL promoter with a mutated T-Rich region (P*ospAB*-T-Rich-mut). Strains were grown in BSK at 37°C and prepared for luciferase assay (*, $p<0.05$ using paired Student's t-test).

Addition of norepinephrine results in a decrease of repression using the *in vitro* model of *ospA* repression

During mammalian infection, *ospA* is continually repressed. However, when an infected mammal is bitten by a naïve tick, *ospA* must be expressed in order for the *B. burgdorferi* to attach to colonize the tick midgut through OspA. The mechanism by which *ospA* is able to switch from repression to expression within the mammalian environment is not clear, although it is speculated that catecholamines from the host are able to signal the release of the repression of *ospA* (Scheckelhoff et al., 2007). Catecholamines, such as norepinephrine and epinephrine, are released by the mammalian host upon the presence of a stressor- such as a tick bite. To determine if the catecholamine norepinephrine is able to release *ospA* repression, *B. burgdorferi* were grown in the *in vitro* model of *ospA* repression in BSK containing either BSK only or BSK with the addition of norepinephrine. The full-length *ospAB* promoter (P*ospAB*-FL) and the *ospAB* promoter without any of the three putative *cis*-elements (P*ospAB*-ΔIR,Rept,T-Rich), both controlling the luciferase reporter, were cultivated.

The addition of norepinephrine to the *in vitro* model of *ospA* repression resulted in an increase of *ospA* expression, indicating release of *ospA* repression in both the *ospAB* promoter containing all three putative *ospA cis*-elements (P*ospAB*-FL) as well as the *ospAB* promoter lacking all three putative *ospA cis*-elements (P*ospAB*-ΔIR,Rept,T-Rich) (**Figure 15**). The release of repression in the promoter lacking all three putative *cis*-elements suggests that norepinephrine releases the repression of *ospA* through a mechanism independent of any of the three putative *cis*-elements.

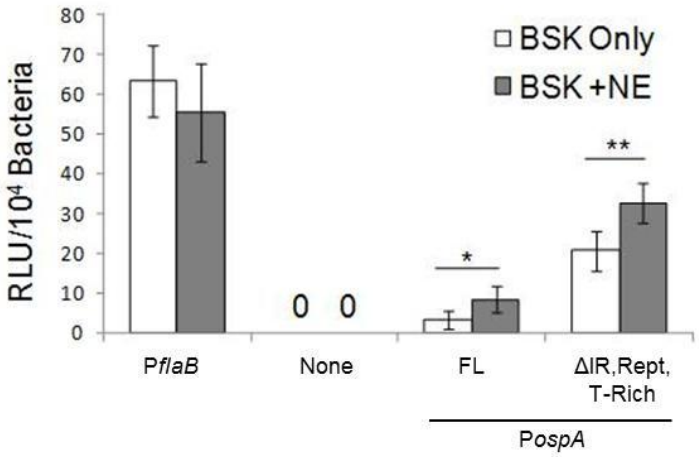

Figure 15. Effect of norepinephrine on luciferase expression driven by various *ospAB* promoters. Luciferase assay of the *ospAB* mutant *B. burgdorferi strain* (Δ*ospAB*) transformed with the various pLuc shuttle vectors containing either the constitutively activated promoter (P*fla*B), promoterless (None), full-length *ospAB* promoter (P*ospAB*-FL) or truncated *ospAB* promoter (P*ospAB*-ΔIR,Rept,T-Rich) were treated at 37°C with 10 µM norepinephrine (BSK+NE) or without norepinephrine (BSK Only) for 48 hours and harvested at late logarithmic phase (*, $p < 0.05$; **, $p<.01$ using ANOVA).

Section III. Host-adapted model for studying *ospA* repression

B. burgdorferi cultivated in dialysis membrane chambers exhibits reciprocal regulation: repression of OspA and expression of OspC

Studying the repression of *ospA* in the past has been difficult. During *in vitro* cultivation, *ospA* is constitutively expressed making the study of repression impossible; however, during *in vivo* conditions, *ospA* is constitutively repressed. The repression of *ospA* can be reproduced using the host-adapted spirochete model. The host-adapted spirochete model was developed by Akins et al. (1998) in order to obtain sufficient amounts of spirochetes from the vertebrate host environment for further assays and evaluations. In this method, *B. burgdorferi* is cultivated within DMC's of 10 kDa pore size to retain the bacteria within the chamber. The chamber is surgically implanted into the peritoneal cavity of a rat and incubated for two weeks before harvesting the DMC's to recover the *Borrelia*. *Borrelia* cultured in this manner display a similar antigenic composition to that of bacteria during mammalian infection. Specifically, *ospA* repression can be observed in *B. burgdorferi* cultured in this manner.

To establish the host-adapted model, the wild-type infectious strain BbAH130 used in these studies was cultivated within DMC's of Sprague-Dawley rats. The culture was incubated within the peritoneum for two weeks before harvest. OspA was expressed in the *in vitro* grown *B. burgdorferi*, as expected, because *ospA* is constitutively expressed *in vitro*. However, there was no OspA expressed in the host-adapted spirochete model culture (**Figure 19**). The repression of *ospA* within the host-adapted spirochete model shows that this model is an excellent tool that mimics the *in vivo* repression of *ospA*.

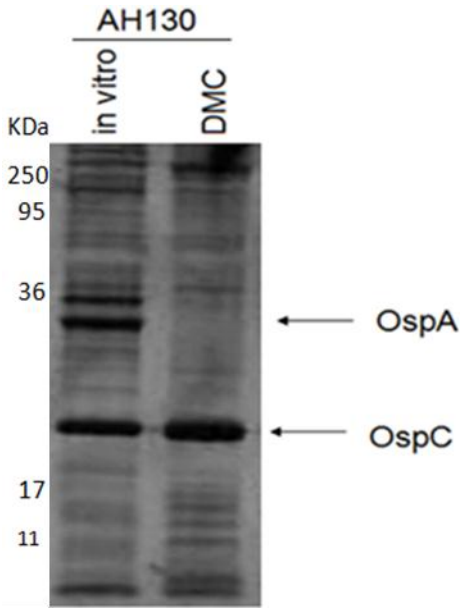

Figure 19. Establishment of the host-adapted model.

BbAH130 (AH130) was either cultivated in BSK at 37°C (*in vitro*) or within DMC's (10 kDa pore size) inside the peritoneal cavity of a Sprague-Dawley rat for two weeks. Whole cell lysates were subjected to SDS-PAGE before Coomassie blue staining. Protein bands corresponding to OspA and OspC are indicated on right. Protein size is indicated on left in kilodaltons (kDa).

Deletion of the T-Rich element results in increased luciferase expression using the host-adapted model

Using the *in vitro* model of *ospA* repression, it was determined that the T-Rich element was required for the full repression of *ospA*. To confirm the *in vitro* model of *ospA* repression, wild-type BbAH130 was electroporated with the shuttle vectors expressing the luciferase reporter under the control of either the full-length *ospAB* promoter or the P*ospAB*-ΔIR,Rept,T-Rich promoters. These constructs were cultivated in the host-adapted model for two weeks and then harvested and prepared for luciferase assay. The P*ospAB*-ΔIR,Rept,T-Rich had a higher amount of luciferase activity than the full-length *ospAB* promoter (**Figure 20**). This suggests that the P*ospAB*-ΔIR,Rept,T-Rich promoter is lacking a *cis*-element essential for the repression of *ospA* within the host-adapted model. This result agrees with previous findings from the *in vitro* model of *ospA* repression.

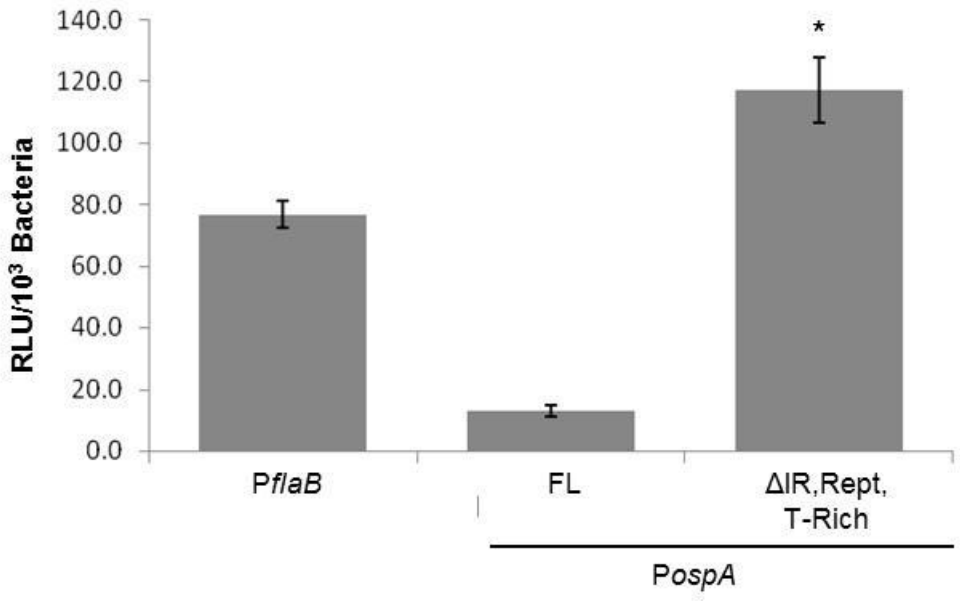

Figure 20. Influence of host signals on luciferase expression of *B. burgdorferi* cultivated in the host-adapted model.

Luciferase assay of the wild-type *B. burgdorferi* strain BbAH130 transformed with shuttle vectors expressing luciferase under the control of either the full length *ospAB* promoter (FL) or the ΔIR,Rept,T-Rich *ospAB* promoter. The constitutively expressed *flaB* promoter (P*flaB*) was used as a control (*, $p<0.05$ using paired Student's t-test).

BB0219, a manganese transporter, is not essential for *B. burgdorferi* growth within the host-adapted model

B. burgdorferi is transmitted to mammals through the *Ixodes* tick vector. *Borrelia* must adapt to the unique environments of its two hosts to be able to establish infection. To establish infection, the bacterium needs to acquire essential nutrients and transition metals, like iron and magnesium, from its hosts. A metal transporter, BmtA (BB0219) has been recently identified as being important for transporting manganese and detoxifying reactive oxygen species (Ouyang et al., 2008).

A *bmtA* mutant was created by disrupting BB0219. The phenotype of the *bmtA* mutant displayed a decreased uptake in magnesium, an inability to establish an infection within mice and the need of *bmtA* for reactive oxygen species protection. We sought to determine if the *bmtA* mutant's inability to establish an infection is due to a defect in the mutant's growth. We cultivated the *bmtA* mutant within DMC's implanted within the peritoneal cavity of a rat. Upon harvest, *B. burgdorferi* was enumerated and it was found that both the wild-type and the *bmtA* mutant had comparable growth within the DMC indicating that BmtA is not essential for growth within the host-adapted model (**Table 3**). Rather than BmtA affecting growth, BmtA must play a role in establishing infection in some other manner.

Table 3. Growth of wild-type, bmtA mutant and complement *Borrelia* within the DMC.

Growth Condition	WT	*bmtA* mutant	Complement
In vitro	++++	+++	++++
Mice	++++		++++
DMC	++++	++++	++++

Growth represented as “+”.

CHAPTER FOUR: DISCUSSION

The T-Rich Region is required for the full activation of the *ospAB* promoter

Previous studies on *ospA* regulation had used nonreplicating, transient reporters to measure the *ospAB* promoter efficacy. These studies failed to use reporters which are able to replicate in *B. burgdorferi*, due to their lack of a *B. burgdorferi* replication origin. Therefore these previous studies were not as accurate as would be if using a stable reporter construct. Unlike the previous studies, to ensure a more accurate measurement and evaluation of *ospAB* promoter activity, we 1) used shuttle vectors that could replicate within *B. burgdorferi*; and 2) used the luciferase reporter which is generally 100-fold more sensitive than the previously used CAT reporter.

In our studies, we the luciferase reporter which was codon-adapted to *B. burgdorferi (Blevins* et al*., 2007)*. *B. burgdorferi* has a GC content of only 28.6%, so the luciferase gene was codon-adapted to ensure that the luciferase gene was accurately representing the transcriptional activity rather than having a codon bias (Fraser et al., 1997). To determine which of the three putative *cis*-elements was required for the full activation of *ospA*, the luciferase reporter was placed under control of the full-length *ospAB* promoter and its activity was compared to several truncated *ospAB* promoters which deleted various regions containing *cis*-elements. We had observed that the deletion of the *ospAB* promoter region spanning from the IR to the Repeat elements did not cause a significant decrease in *ospAB* promoter expression. However, when the *ospAB* promoter region spanning the IR, Rept and T-Rich regions was deleted, there was a significant decrease in *ospAB* promoter expression. This suggested that the T-Rich region was required for the full activation of *ospA*.

The same series of *ospAB* promoters used to control luciferase expression in a wild-type *Borrelia* strain were placed to control the native *ospA* reporter within an *ospAB* mutant strain to evaluate which putative *cis*-element was required for the full activation of *ospA*. The native *ospA* reporter demonstrated similar results to the luciferase reporter results: deletion of the IR and Rept region together did not cause a significant decrease in *ospA*, however, deletion of the IR, Rept, and T-Rich regions together resulted in a decrease in *ospA*. These *ospA* activation studies using a luciferase reporter and *ospA* reporter both suggested that the T-Rich region was required for the full activation of the *ospAB* promoter. However, we needed to ensure that the T-Rich region was solely responsible for the decrease in *ospAB* promoter activation, rather than the T-Rich region in conjunction with either the IR or Rept regions. To do this, site-directed mutagenesis was performed to mutate a 10 bp region of the T-Rich region of the full-length *ospAB* promoter to create P*ospAB*-T-Rich-mut. Like the other promoters tested, the P*ospAB*-T-Rich-mut promoter was placed controlling luciferase expression and evaluated using the luciferase assay. The P*ospAB*-T-Rich-mut promoter showed reduced luciferase expression compared to the full-length *ospAB* promoter indicating that the T-Rich region alone was responsible for the reduction in *ospAB* promoter activity observed in these studies. Our *ospA* expression findings were consistent with previous data in the literature demonstrating that the T-Rich region is required for the full activation of the *ospAB* promoter within a transient reporter system (Sohaskey et al., 1999). Our studies, which used stable reporters, showed that the T-Rich region is an important regulatory element required for the full activation of *ospA*.

A Novel Approach to Studying *ospA* repression

Studying *ospA* repression in *Borrelia* has proven to be a very difficult task as *ospA* is only expressed during *in vivo* cultivation and thus collecting enough bacterial sample

volume to do experiments has proven to be very laborious. Therefore, there is very little known about the repression of *ospA* other than that it is mediated by RpoS in an unknown manner (Caimano et al., 2005). To create an easier way to study *ospA* repression, we developed an *in vitro* model to study *ospA* repression. This was achieved by using the luciferase reporter under the control of the *ospAB* promoter within an *ospAB* mutant strain, which has RpoS constitutively expressed and thus mediating the repression of *ospA*. This advance in the field should make it easier for researchers to study *ospA* repression.

The T-Rich Region is required for the full repression of the *ospAB* promoter

Our *in vitro* model to study *ospA* repression was used to determine which *cis*-elements are required for the full repression of *ospA*. The luciferase reporter was placed under control of the full-length *ospAB* promoter and other truncated *ospAB* promoters so the *cis*-elements required for *ospA* repression could be determined. Deletion of the IR or IR Rept regions did not cause a significant change in *ospAB* promoter activity compared to the full-length *ospAB* promoter. However, deletion of the IR, Rept, and T-Rich regions resulted in an increase in promoter activity showing that the truncated promoter was not able to be fully repressed. This indicated that the T-Rich region was important for the full repression of *ospA*. To ensure that the decreased repression was due solely to the T-Rich region and not the T-Rich region in conjunction with the IR or Rept regions, we compared the P*ospAB*-T-Rich-mut promoter, which has the full-length *ospAB* promoter with a mutated T-Rich region, to the full-length *ospAB* promoter. Our results had shown a decrease in repression which indicates that it is solely the T-Rich region responsible for the decrease in *ospA* repression. Our *in vitro* model of *ospA* repression demonstrated that the T-Rich region of the *ospAB* promoter was required for the full repression of the *ospAB* promoter.

To further study *ospA* repression, we explored the effects of norepinephrine on *ospA* repression. A previous study demonstrated that norepinephrine is able to de-repress *ospA* (Scheckelhoff et al., 2007). De-repressing *ospA* is required for the tick to acquire *Borrelia* from an infected mammal. In an infected mouse, *ospA* is repressed, however, upon tick feeding, *B. burgdorferi* must de-repress *ospA* in order to colonize the tick gut where OspA binds specifically to the tick's TROSPA receptor. The previous study demonstrates that when a tick bites the mammal, the host releases norepinephrine and this contributes to de-repressing *ospA*. Given this, we wanted to determine if norepinephrine would affect *ospA* repression in our *in vitro* repression model.

Using our *in vitro* repression model, we cultured the *ospAB* mutant containing either the full-length *ospAB* promoter (P*ospAB*-FL) or the *ospAB* promoter lacking the IR,Rept, and T-Rich region (P*ospAB*-ΔIR,Rept,T-Rich) in BSK media only or BSK media containing norepinephrine. For both the P*ospAB*-FL and the P*ospAB*-ΔIR,Rept,T-Rich promoter, there was an increase in luciferase expression upon the addition of norepinephrine indicating that there was a de-repression of *ospA* for both promoters. From this study, we concluded that norepinephrine is able to de-repress *ospA*, which is agreement with the previous studies. Further advancing this, we were able to conclude that the de-repression of *ospA* is independent of the T-Rich region. There is likely another regulatory element between the T-Rich region and the *ospA* gene that plays a role in the de-repression of *ospA* via the addition of norepinephrine.

One last approach we used to study the repression of *ospA* was the host-adapted animal model in which *Borrelia* are cultivated within DMC's implanted into the peritoneal cavity of a rat (Akins et al., 1998). We first established the host-adapted model within our lab. We observed that growing wild-type *Borrelia* within the DMC's resulted in the

repression of *ospA*. Since *ospA* was able to be repressed, we were able to use the host-adapted model to confirm our *ospA* repression finding we observed *in vitro*. In the DMC's, we grew the wild-type *Borrelia* strain with luciferase reporter expression under the control of either the full-length *ospAB* promoter or the P*ospAB*-ΔIR,Rept-T-Rich promoter. There was an increase in luciferase expression in the P*ospAB*-ΔIR,Rept-T-Rich promoter compared to the full-length *ospAB* promoter which mirrors our results from the *in vitro* studies that the T-Rich region is required for the full repression of *ospA*. One caveat of the host-adapted model is that, unlike *in vitro* cultivation, there is no antibiotic selection in order for the plasmids to be retained and replicating within *Borrelia*. However, it is probable that since both strains used the same parental vector and are identical except at the region of the *ospAB* promoter, that the plasmid would be lost at the same rate for both strains.

Previous studies in *ospA* repression had already shown that RpoS is required for the full repression of *ospA*. Our studies further dissected the mechanism of *ospA* repression by demonstrating that the T-Rich region of the *ospAB* promoter is an important *cis*-element required for the full repression of *ospA*. For *ospA* repression, Rrp2 becomes phosphorylated upon tick feeding. Then, Rrp2 in conjunction with RpoN activate RpoS. RpoS then represses *ospA* either by 1) associating with an *in vivo*-specific accessory factor that allows RpoS to directly bind to the T-Rich *cis*-element of the *ospAB* promoter; or 2) by controlling the expression of an unknown *ospA* repressor protein that binds to the T-Rich *cis*-element of the *ospAB* promoter to block RpoS-dependent transcription initiation. For *ospA* activation, on the other hand, RpoD is able to bind to the T-Rich region of the *ospAB* promoter to drive the transcription initiation of *ospA*.

CHAPTER FIVE: FUTURE DIRECTIONS

Potential role of BosR as a repressor of *ospA*

In *B. burgdorferi*, BosR (BB0647) has been found to encode a novel DNA-binding protein in the Fur/Per family of transcriptional regulators. BosR is required for the induction of RpoS (Hyde et al., 2010; Ouyang et al., 2009). RpoS represses *ospA* via an unidentified mechanism (Caimano et al., 2005). A recent study has discovered that BosR binds specifically to the *rpoS* gene at three distinct sites. By making specific mutations in the *rpoS* gene and measuring the binding capability of BosR, a novel direct repeat and inverted repeat sequence (TAAATTAAAT), which is critical for BosR binding, was identified (Ouyang et al., 2011). This direct repeat sequence which BosR specifically binds is similar to areas upstream of the T-Rich element found on the *ospAB* promoter. Since BosR regulates RpoS, and RpoS is required for the repression of *ospA*, it is plausible that BosR could play a key role in *ospA* repression through binding to a putative BosR binding site located on the *ospAB* promoter.

In an *rpoS* mutant, *ospA* is not able to be repressed (Caimano et al., 2005). Thus if BosR directly represses *ospA*, then inducing BosR in an *rpoS* mutant should restore *ospA* repression. These experiments can be performed in two different *ospA* repression conditions: 1) in the host-adapted model, or 2) in the *ospAB* mutant. For the host-adapted model study, either wild-type, *rpoS* mutant, or an *rpoS* mutant complemented with *bosR* can be electroporated with a shuttle vector containing the full-length *ospAB* promoter controlling luciferase expression. The wild-type *Borrelia* would display low luciferase expression because this condition supports the repression of *ospA*. The *rpoS* mutant would display high luciferase expression because previous studies have shown that *ospA* is not able to be repressed in *rpoS* mutants (Caimano et al., 2005). This is

likely due to the absence of a repressor controlled through RpoS. The *rpoS* mutant complemented with *bosR* would have low luciferase expression if BosR is able to specifically bind to the *ospAB* promoter to cause repression. If complementation with *bosR* results in high luciferase expression, this would indicate that BosR is not specifically binding to the *ospAB* promoter to cause repression and that repression of *ospA* is due to a trans-factor(s) controlled by RpoS.

A second method of determining if BosR is able to directly repress *ospA* would be by using the *in vitro* model of *ospA* repression. In this method, the same shuttle vector which contains luciferase under the control of the full length *ospAB* promoter would be electroporated into either 1) the BbAH130 wild-type strain; 2) the *ospAB* mutant strain; 3) the *ospAB*, *rpoS* double mutant; or 4) the *ospAB*, *rpoS* double mutant complemented with *bosR*. The strains would be cultivated at 37°C and analyzed by luciferase assay. The wild-type strain would expect to have high luciferase activity because *ospA* is highly expressed during *in vitro* cultivation. The *ospAB* mutant would be expected to have no luciferase activity because this is the *in vitro* model of *ospA* repression where the abrogation of *ospAB* causes *ospA* repression conditions. The *ospAB*, *rpoS* double mutant would show high luciferase activity because RpoS is required for the repression of *ospA*. Lastly, the *ospAB* mutant strain complemented with *bosR* would exhibit no luciferase activity if BosR is able to bind to the *cis*-elements of the *ospAB* promoter to cause repression. However, if BosR is not able to specifically bind to the *ospAB* promoter to repress its activity, then the strain would show high levels of *ospA* because *ospA* cannot be repressed in the absence of RpoS.

Identification of putative *trans*-factors via a knowledge-based approach

Previous studies have shown that *B. burgdorferi rpoS* mutants are not able to downregulate OspA even during *in vivo* cultivation when *ospA* is normally repressed. The precise mechanism by which RpoS controls *ospA* repression is not known. We hypothesized that an *ospA* repressor is regulated under the control of RpoS and that a knowledge-based approach can be used to help identify putative DNA-binding proteins controlled by RpoS.

Microarray data from Caimano et al. (2005) and Boardman et al (2008) identified genes being differentially regulated by RpoS within the host-adapted model. From this microarray data, a list was compiled which identified genes that appeared to be the most highly regulated by RpoS. From this list of RpoS-regulated genes, the genes with the highest transcript fold-change were next evaluated for their probability of binding to DNA. Their amino acid sequence obtained from Entrez Gene (NCBI) was entered on an online server (www.netasa.org/dbs-pred) to predict the probability of the protein binding to DNA. After determining the DNA-binding probability, we narrowed down the list to the top five gene candidates based on their: 1) fold change in the transcript levels between the RpoS mutant and the wild-type as determined from the microarray data; and 2) DNA-binding capability as predicted by the online server. The top five gene candidates are 1) BBL29; 2) BBD01; 3) BB0449; 4) BBJ01; and 5) BBJ02.

These putative DNA-binding proteins were cloned into a shuttle vector that placed their expression under the control of a constitutive promoter. Next, the shuttle vectors were electroporated into a wild-type *B. burgdorferi* strain. These strains were grown *in vitro* at 23°C, a condition in which *ospA* is expressed, to determine if the any of the five repressor candidates were able to repress *ospA*. If the gene is an *ospA* repressor, then

the overexpression of the gene in the WT strain would result in lower or no *ospA*. There was no difference in any of the five expressed genes indicating that genes did not have an effect on *ospA* repression and therefore are not *ospA* repressors.

In the future, other genes can be selected using out knowledge-based approach to determine if they are able to repress *ospA*. If the overexpressed gene does appear to repress *ospA* when overexpressed, an EMSA would need to be done to determine if the protein of interest is able to act as a trans-factor and bind to the *ospAB* promoter. Lastly to confirm that the gene is indeed an *ospA* repressor, the gene would need to be knocked out in *B. burgdorferi* and grown within a DMC. If *ospA* is not able to be repressed in this condition, then the gene would prove to be needed to repress *ospA*.

Mass spectrometry identification of the putative *trans*-factors acquired using biotinylated *ospAB* promoter oligonucleotide

The T-Rich region of the *ospAB* promoter has been identified as an important regulatory *cis*-element in both the expression and repression of *ospA*. The *ospAB* promoter can be used to isolate the trans-factor(s) binding to the *ospAB* promoter *cis*-elements by employing sequence-specific DNA affinity chromatography. In a method adapted from (Babb et al.), chemically synthesized biotin-labeled complementary oligodeoxynucleotides that encompassed the full-length *ospAB* promoter were purchased (2006). The full-length *ospAB* promoter was used because it contains the T-Rich region, which we found was crucial for the full activation of *ospA*, and thus the activator is likely binding to this region. Next, the biotin-conjugated oligodeoxynucleotides were affixed to streptavidin magnetic beads and protein extracts from either wild-type *B. burgdorferi* (for *ospA* expression conditions since wild-type has

high *ospA* expression) or *ospAB* mutant (for the *ospA* repression conditions since the *ospAB* mutant has constitutive expression of an unknown *ospA* repressor) cultures were added. The supernatant was removed and the beads coated with biotin-labeled *ospAB* promoter were gently washed. Next, the proteins bound to the *cis*-element oligonucleotide fragments were eluted from the magnetic beads and separated by SDS-PAGE and visualized. Protein bands were extracted and analyzed by matrix-assisted laser desorption ionization-time of flight (mass spectrometry) performed by the Proteomics Core Facility at Indiana University School of Medicine (Indianapolis, IN). The proteins were identified as shown in **Table 4**.

Of particular interest is gene BBJ02 (**Table 4**). This gene was identified in our knowledge-based approach as being a putative *ospA* repressor due to its regulation by RpoS and its DNA binding probability. BBJ02 was constitutively expressed in an *ospA* activation condition to determine if it was able to act as an *ospA* repressor; it did not.

In the future, the isolation of the activator need to be redone. To improve these studies, the bacterial lysis and washing steps need to be optimized to ensure less non-specific binding. Also, the full-length *ospAB* promoter with the mutated T-Rich region (P*ospAB*-T-Rich-mut) should be used as a control because it contains the same elements as the full-length promoter except the T-Rich region. Since the activator is likely binding to the T-Rich region, the P*ospAB*-T-Rich-mut should not have the activator present in the proteins eluded from the promoter whereas the P*ospAB*-FL should have the activator present.

Table 4. Proteins identified by mass spectroscopy to bind to the *ospAB* promoter.

The name of the protein or gene (protein/gene) on the left and its putative role in *B. burgdorferi* (comments). The number of peptides is noted (peptides) and its number of distinct sequence (# of distinct sequences).

protein/gene	Comments	Peptides	# of distinct sequences
ospA	outersurface protein	**33**	
bbk32	p35 antigen	**72**	**23**
ospC	outersurface protein	**157**	**21**
BBK01	membrane protein	**39**	**21**
FlaB	Flagellin	**22**	**11**
BBG01	lipoprotein	17	10
BBI06	pfs protein (phosphorylate super	11	8
DbpA	adhesion protein	**24**	**8**
BB0603	membrane associate protein p66	9	7
BB0323	lipoprotein	7	7
dbpB	adhesion protein	17	7
BBM27	lipoprotein	10	6
Bol26	surface protein	8	6
BB0238		6	6
WI91-23	REV protein	9	5
ospB	outersurface protein	8	5
BB0689	outersurface protein	6	5
BBI39		**4**	**3**
bb0337	phosphopyruvate hydratase	3	3
bb0476	translation elongation factor	2	2
bba66	surface antigen p35	2	2
BBJ02		2	2
bb0034	outermembrane protein P13	2	2

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CURRICULUM VITAE

Tara Lynn Oman

Education

Ph.D. in Microbiology & Immunology, *Indiana University*, Indianapolis, IN, 2012
Dissertation Title: "The Regulation of Outer Surface Protein A in the Lyme Disease Spirochete *Borrelia burgdorferi*"
Dissertation Advisor: Professor Xiaofeng Yang

B.S. in Biology, *Indiana University*, Bloomington, IN, 2006
Course highlights: Microbiology, Molecular Biology, Cellular Biology, Genetics, Statistical Techniques, Organic Chemistry I & II, Calculus I & II, Summer Flowering Plants, Spanish

Teaching and Mentoring Experience

Guest Lecturer, *Indiana University School of Medicine*, Indianapolis, IN
- Served as the mycology lecturer for the undergraduate level course "Microbiology for Nursing Majors" (J210)
- Created PowerPoint presentations, lectured, answered students questions and created questions for exams (3 semesters: Spring 2010 - Spring 2011; 150 students)

Graduate Teaching Assistant, *Indiana University School of Medicine*, Indianapolis, IN,
- Taught laboratory sessions, improved experiments, created lecture notes, graded reports and created exam questions for the undergraduate level course "Microbiology for Nursing Majors" (Fall 2009; 32 students)

Undergraduate Tutor, *Indiana University*, Bloomington, IN, 2004 - 2006
- Tutored undergraduate and high school students in basic chemistry, human genetics, biology and finite math

Research Experience

Graduate Research Assistant, *Indiana University*, Indianapolis, IN, 2007 - 2012
- Investigated the regulation mechanism and function of the outer surface protein A (OspA) in the Lyme disease causative agent *Borrelia burgdorferi*
- Genetically manipulated *Borrelia* to create mutants to determine the *cis*-elements involved in *ospA* regulation
- Combined molecular and bioinformatic techniques to identify the *trans*-factor involved in *ospA* regulation
- Created the first *in vitro* model for studying *ospA* repression within *Borrelia*

Undergraduate Research Assistant, *Indiana University*, Bloomington, IN, 2005 - 2006
- Studied signal transduction of the Epidermal Growth Factor Receptor (EGFR) in *Drosophila melanogaster* and the effects of insulin production on cellular development
- Designed experiments, collected and analyzed data, genotyped drosophila, maintained insect stocks, prepared solutions and medias

Undergraduate Lab Technician, *Indiana University*, Bloomington, IN, 2004
- Studied solicitation pheromones in the burrower bug *Sehirus cinctus*
- Kept insect husbandry records, preparatory set-up for experiments, harvested clover seeds for insect feed

Awards and Honors

Fellowships
- Immunology and Infectious Disease Training Grant Fellowship, *National Institutes of Health and Indiana University School of Medicine* 2010 - 2012

Other distinctions
- IU School of Medicine's Harold Raidt Graduate Student Teaching Award 2011
- Travel Award, *16th Annual Midwest Microbial Pathogenesis Meeting, Purdue University, IN* 2010
- Indiana University College of Arts and Sciences Dean's List 2003 - 2006
- Founders Day High Scholastic Achievement Honors 2003 - 2006
- National Society of Collegiate Scholars 2004 - 2005
- Phi Eta Sigma National Honor Society 2004
- Alpha Lambda Delta National Honor Society 2004

Professional Activities

- Graduate Student Representative, Indiana University School of Medicine Faculty Community Relations Committee, Indianapolis, IN 2007 - 2012
- Class representative for Organic Chemistry I & II*, Indiana University Department of Chemistry*, Bloomington, IN 2005-2006
- Class representative for Principles of Chemistry & Biochemistry , *Indiana University Department of Chemistry*, Bloomington, IN 2004 - 2005

Conferences and Presentations

- 17th Annual Midwest Microbial Pathogenesis Conference, St. Louis, MO (2010)
- Gordon Research Conference: The Biology of Spirochetes, Ventura, CA (2010)
- Charon Symposium: Spirochetes, Microbial Motility and Chemotaxis, Morgantown, WV (2009)
- 16th Annual Midwest Microbial Pathogenesis Conference, West Lafayette, IN (2009)
- 14th Annual Midwest microbial Pathogenesis Meeting, Chicago, IL (2007)

Publications

- Tara Oman, Ming He, Frank Yang. "The T-Rich region of the *ospAB* promoter is required for both repression and expression of *ospA*." *Journal of Bacteriology*, (in preparation)

- Zhiming Ouyang, Ming He, Tara Oman, X. Frank Yang, and Michael V. Norgard. “A manganese transporter, BB0219 (BmtA), is required for virulence by the Lyme disease spirochete, *Borrelia burgdorferi*.” *Proceedings of the National Academy of Sciences*, **2009**, 106(9):3449-54.

- Ming He, Tara Oman, Haijun Xu, Jon Blevins, Michael V. Norgard, and X. Frank Yang. “Abrogation of *ospAB* constitutively activates the Rrp2-RpoN-RpoS pathway (SigmaN-sigmaS cascade) in *Borrelia burgdorferi*.” Molecular Microbiology, **2008**, 70(6):1453-64