2014

Outer Surface Lipoprotein Layer Homeostasis and Gene Regulation in Borrelia burgdorferi

Poonam Dadhwal
Louisiana State University and Agricultural and Mechanical College

OUTER SURFACE LIPOPROTEIN LAYER HOMEOSTASIS AND GENE REGULATION IN *BORRELIA BURGDORFERI*

A Dissertation

Submitted to the Graduate Faculty of
The Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Pathobiological Sciences
School of Veterinary Medicine

by
Poonam Dadhwal
BVSc & A.H., GADVASU, 2008
December 2014

I dedicate my dissertation to my parents,

Mr. Pawan Jit Singh Dadhwal and Mrs. Neelam Kumari Dadhwal

A special gratitude to my sister Hanu Dadhwal, brother, Karan Jit Singh Dadhwal,

and my husband Rahul Sharma

ACKNOWLEDGMENTS

I am grateful to LSU, and my graduate advisor Dr. Fang Ting Liang for giving me the opportunity to do research and obtain a Ph.D. It could not have been possible to complete this research project without Dr Liang's consistent support, guidance and motivation. I appreciate the support and help extended by all the committee members, Dr. Philip H. Elzer, Dr. Kevin Macaluso, Dr. Rhett W. Stout, and Dr. Evanna L. Gleason for their time, valuable input and advice. I am also grateful for all the support extended by Dr. Yanlin Shi. I admire Dr. Yihe Ge molecular biology skills and thankful for being a great teacher on the bench and helping me understand the minor details of cloning, gene expression and western blots. I would like to acknowledge the support and help extended by Kristy McShan, Sunita Seemanapalli, Dr. Qilong Xu and Brian Leydet. I am also extremely grateful to Dr. Xin Li from The Ohio State University for extending my DMC experiments to more meaningful conclusions and a nice publication.

I am also thankful to friends and organizations who made me feel Baton Rouge a home away from home. I would also like to offer my gratitude to my parents for continuous support and motivation, this endeavor was not possible to accomplish without their blessings.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The outer surface lipoprotein (osp) layer forms an interface between the internal and the external environment of the Lyme disease spirochete, *Borrelia burgdorferi*. The homeostatic maintenance of the osp layer effectuates adaptation of *B. burgdorferi* as it gets transmitted from the tick vector to a mammalian host and vice-versa. However, the regulation of the outer surface lipoproteins (osps) is still a conundrum for borrelia scientists. Part of this dissertation inquires about the homeostatic maintenance of the osp layer. We found that the deletion of the dominantly expressed tick phase osp, OspA, induces expression of two other osps. OspD, and BBJ41. Also, increased expression of OspC was seen in borrelia mutants lacking OspA, OspD, and BBJ41. These results suggest constant osp layer maintenance, irrespective of the presence or the absence of the dominant Osps, like OspA and OspC. Furthermore, our conclusive electron microscopic study demonstrates that the overall density of the osp layer remains identical in wild type and mutant *B. burgdorferi,* lacking either several osps or the dominantly expressed OspA.

OspA is abundantly expressed on the borrelial surface as it persists in an unfed tick. A blood meal causes rapid downregulation of OspA as *B.burgdorferi* prepares to infect the mammalian host. The downregulation of OspA is speculated to be regulated by an unknown repressor protein. The remaining part of this dissertation pertains to the investigation of this unknown repressor protein for *ospA.* The borrelia oxidative stress regulator protein, BosR, has been attributed with an indirect role in OspA downregulation. However, due to its homolgy with a family of transcriptional repressors, BosR is more likely to cause direct repression of OspA. Therefore, we investigated the direct interaction of BosR and the *ospA* regulatory region. The DNA binding experiments demonstrated that borrelia oxidative stress regulator, BosR, binds directly to the *cisI* and *cisII* regulatory regions of *ospA* promoter. Thus, conclusively, BosR acts as a repressor protein which causes OspA downregulation in *B. burgdorferi*.

CHAPTER 1
BACKGROUND AND OBJECTIVES

Literature review

The Lyme disease agent, *Borrelia burgdorferi* is a microaerophilic, gram negative motile bacteria, which belongs to an ancient phylum: spirochaetes, genus: *Borrelia*, class: Spirochaetes, order: Spirochaetales, and family: Spirochaetaceae (Paster and Dewhirst 2000, Brisson et al. 2012).

The term spirochete emanates from the helical/serpentine morpholgy of the bacteria. In nature the spirochetes may be free dwelling or exist within a host, a very few spirochetes act as a pathogen (Paster and Dewhirst 2000). *Borrelia burgdorferi* is one of the pathogenic genus under spirochetes causing tick borne disease called the Lyme disease (Paster and Dewhirst 2000).

In 1982, the first isolate of *Borrelia burgdorferi* was obtained (Burgdorfer et al. 1982). Since then many borrelia isolates have been obtained throughout the world. The genetic analysis of the different isolates have resulted in formation of the *Borrelia burgdorferi sensu lato* complex. This complex includes up to 18 Borrelia species (Stanek and Reiter 2011), only three of these are known to be true human pathogens, namely: *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii*. While *B. garinii* and *B. afzelii* are predominant Lyme disease agents in Europe, *B. burgdorferi sensu stricto* causes Lyme disease in the North America (Baranton et al. 1992). Figure 1.1 taken from (Steere, Coburn, and Glickstein 2004) below shows distribution of other borrelia isolates.

The genospecies of *Borrelia burgdorferi* and their tick vectors and locations

	Principal tick vector	Location
Three pathogenic species		
Borrelia burgdorferi	*Ixodes scapularis*	Northeastern and north central US
	Ixodes pacificus	Western US
	Ixodes ricinus	Europe
Borrelia garinii	*Ixodes ricinus*	Europe
	Ixodes persulcatus	Asia
Borrelia afzelii	*Ixodes ricinus*	Europe
	Ixodes persulcatus	Asia
Eight minimally pathogenic or nonpathogenic species		
Borrelia andersonii	*Ixodes dentatus*	Eastern US
Borrelia bissettii	*Ixodes spinipalpis*	Western US
	Ixodes pacificus	
Borrelia valaisiana	*Ixodes ricinus*	Europe and Asia
Borrelia lusitaniae	*Ixodes ricinus*	Europe
Borrelia japonica	*Ixodes ovatus*	Japan
Borrelia tanukii	*Ixodes tanukii*	Japan
Borrelia turdae	*Ixodes turdus*	Japan
Borrelia sinica	*Ixodes persulcatus*	China

Figure 1.1. Genospecies of *Borrelia burgdorferi* and their tick vectors and location: Taken from (Steere, Coburn, and Glickstein 2004) depicts worldwide distribution of several members of *Borrelia burgdorferi* sensu lato complex along with principal tick vectors and location

Lyme disease recognition in the United States

The first Lyme disease cases were reported in the form of juvenile rheumatoid arthritis amongst a group of children from Lyme village, Connecticut in year 1976 (Steere et al. 1977, Steere 1989). In the years that followed, the doctors and scientists proved that Lyme disease was transmitted by bite from an infected *Ixodes* tick (Steere 1989) and caused by a spirochete called *Borrelia burgdorferi* (Burgdorfer et al. 1982, Steere 1989).

Even after three decades of its discovery, today Lyme disease stands as most commonly reported vector borne disease (Steere 2006). According to CDC *"Lyme disease is the most commonly reported vector borne illness in the United States in 2012, it was the 7th most common*

Nationally Notifiable disease" (http://www.cdc.gov/lyme/stats/index.html). As shown in figure 1.2 Lyme disease continues its trend of newly reported cases in past 10 years, no significant control of the disease has been achieved.

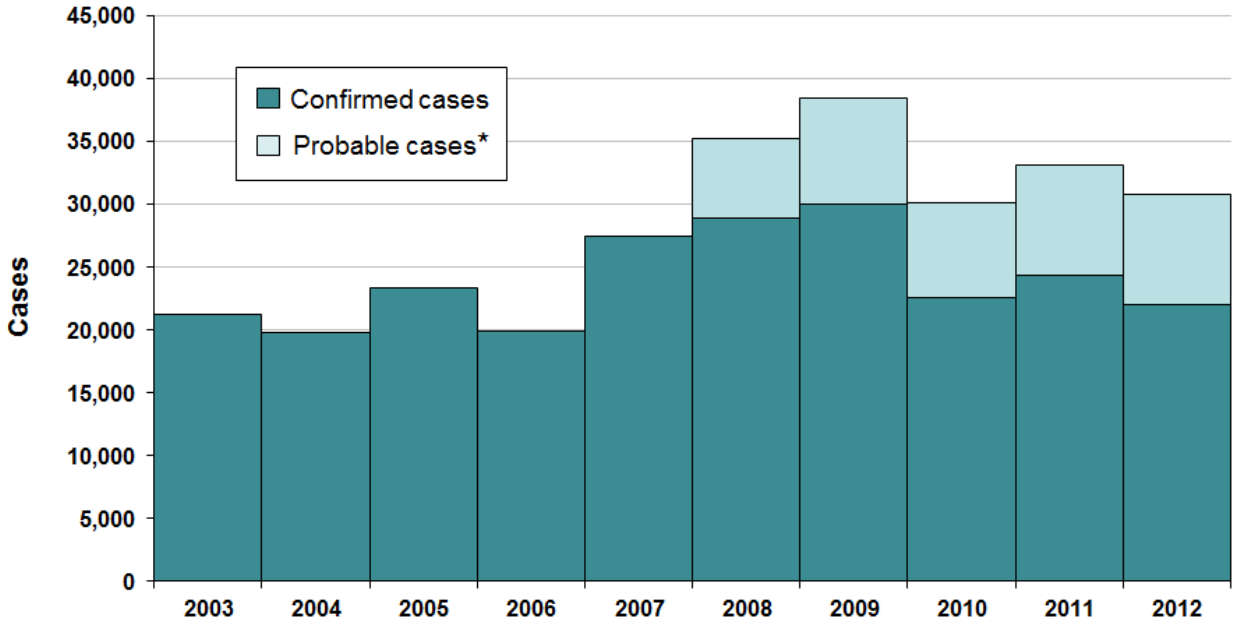

Figure 1.2. Reported cases of Lyme disease by year, United States, 2003-2012:
(Centers for Disease Control and Prevention National Center for Emerging and Zoonotic Infectious Diseases Division of Vector-Borne Diseases, last reviewed September, 2013)
http://www.cdc.gov/lyme/stats/chartstables/casesbyyear.html.
The number of confirmed cases ranged from a low of 19,804 in 2004 to high of 29,959 in 2009.
*National Surveillance case definition revised in 2008 to include probable cases; (detail:http://www.cdc.gov/osels/ph_surveillance/nndss/casedef/lyme_disease_2008.htm)

The comparison of reported cases in figure 1.4 and figure 1.5 show visible increase in distribution of the reported cases since year 2001 to 2012, the total number of cases reported in the years 2001- 2011 varied from 17029 - 24364, (Centers for Disease Control and Prevention, revised september,2012, http://www.cdc.gov/lyme/stats/maps/interactiveMaps.html).

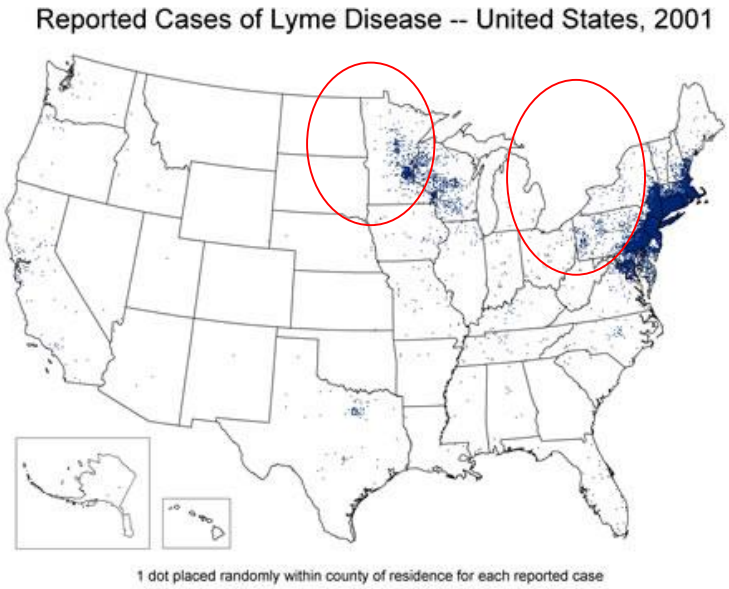

Figure 1.3. Map showing Lyme disease reported case distribution in year 2001 (Centers for Disease Control and Prevention, http://www.cdc.gov/lyme/stats/maps/interactiveMaps.html)

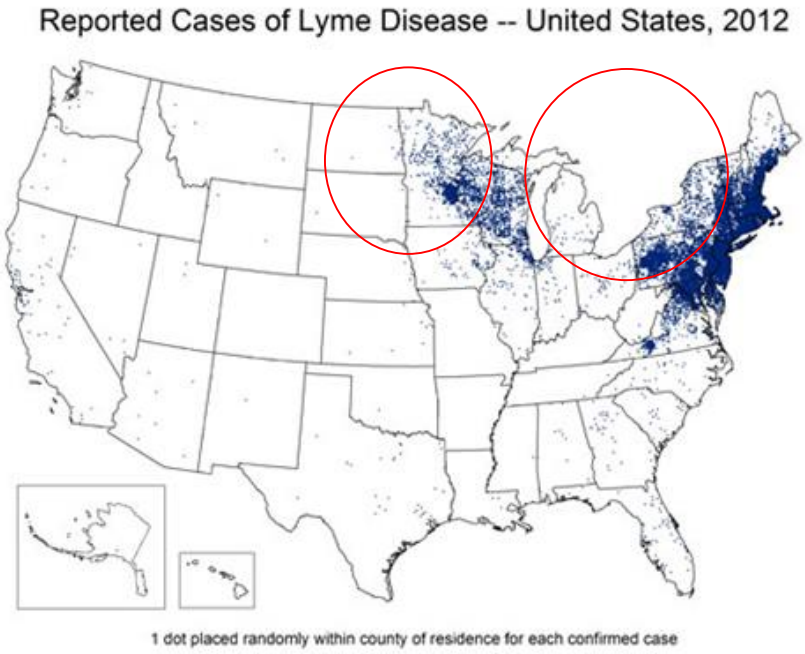

Figure 1.4. Map showing Lyme disease reported case distribution in year 2012 (Centers for Disease Control and Prevention, http://www.cdc.gov/lyme/stats/maps/map2012.html)

Clinical symptoms

In order to transmit the Lyme borrelia and initiate appearance of the cutaneous manifestation, the *Ixodes* tick needs to complete at least 36-48 hours on its host (Peavey and Lane 1995). One of the studies indicated even lower tick attachment time (24hrs) may be required in order to transmit Lyme borrelia (Piesman et al. 1987). While up to 90% people show erythema migrans at the site of tick bite (Gerber et al. 1996) (Krause et al. 1996), more than half of the infected people fail to detect or recall the tick bite, also the nymph stage of tick is hard to see and be recognized (Asbrink and Hovmark 1988). The lesion of erythema migrans initiates as a small red macule from the tick bite site and within a week of bite, migrates outwardly leading to formation of typical "Bull's eye rash" figure1.6 (Nadelman et al. 1996). However, erythema migrans cannot be considered as a diagnostic lesion for Lyme disease (Asbrink and Hovmark 1988), as some times rash may lack the central clearance, appear similar to other tick bites (Kirkland et al. 1997), or appear as a migrating lesion away from the unnoticed initial tick bite site (Nadelman et al. 1996).

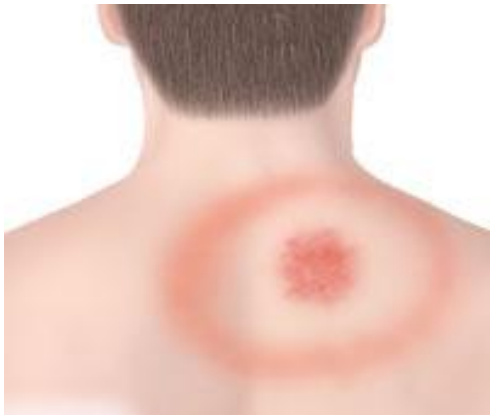

Figure 1.5. Erythema migrans leision with central clearing (bull's eye rash): Picture from Centre for Disease Control and Prevention webpage, last revised on April, 2013 (http://www.cdc.gov/lyme/signs_symptoms/index.html)

Early classification for clinical symptoms of Lyme disease was given by Steere et al. in 1983. Under this classification the course of Lyme disease was divided into three stages: Stage one of Erythema migrans, stage two including meningitis or Bell's palsy, and stage three showing arthritis. As there was overlap of symptoms under different stages of above early classification the clinical manifestations of Lyme disease was reclassified by Asbrink & Hovmark in 1988. The new classification was based on classification for Syphilis caused by spirochete called *Treponema pallidum*. Under this classification, clinical manifestation for Lyme disease has been divided into three broad stages (Nadelman and Wormser 1998, Steere 1989, Yoshinari, Steere, and Cossermelli 1989):

1. Stage one: The early localized stage (Asbrink and Hovmark 1988), occurs within 3-30 days of the infectious Ixodes tick bite. Main symptoms seen are erythema migrans, malaise, headache, fever, myalgia, and fever (viral fever like signs) (Steere et al. 1983).

2. Stage two: The early disseminated stage (Asbrink and Hovmark 1988), ensues within days to weeks. This stage is characterized by borrelia lymphocytoma, meningo-polyneuritis, and multiple systemic effects like: Atrioventricular block (Steere et al. 1980), multiple erythema migrans, myalgia, arthralgia, facial or Bell's palsy (loss of muscle tone on one or both sides of the face, figure 1.6), and severe headaches and neck stiffness due to meningitis (inflammation of the spinal cord).(Ho, Melanson, and Desai 2012)

3. Stage three: The late stage (Asbrink and Hovmark 1988) or stage of persistent infection, known to occur months to years of initial tick bite. This stage includes acrodermatitis chronica atrophicans, monoarticular or oligoarticular arthritis, figure 1.7 (Steere et al. 1977) or chronic neurologic manifestations like encephalomyelytis and peripheral neuropathy. (Steere 2001)

Figure 1.6. Loss of facial muscle tone on one or both sides: Bell's palsy. Can be seen in early disseminated stage (picture from Centre for Disease Control and Prevention, CDC- Division of Vector-borne Infectious Diseases http://www.cdc.gov/lyme/signs_symptoms/index.html).

Figure 1.7. Lyme arthritis, painful swelling of either single or both knee joints can ensue in late disseminated stage. Picture from CDC- Division of Vector-borne Infectious Diseases

Diagnosis of Lyme disease

In endemic regions , the clinical signs like erythema migrans (Bull's eye rash) have preliminary diagnostic value, however, the culturing of *Borrelia burgdorferi* from these leisions enables definitive diagnosis (Tugwell et al. 1997). The diagnostic cultures can be obtained from erythema migrans, acrodermatitis, and occasionally from plasma and cerebrospinal fluid samples. However, the patients in late stage of the disease may be diagnosed based on Polymerase Chain Reaction (PCR) for detection of borrelia in joint fluid (Nocton et al. 1994). In

suspected cases for extra cutaneous Lyme disease tissues having borrelia may be inaccessible for culturing and hence serum based diagnosis is of greater value, these tests include Enzyme Linked Immunosorbent Assay (ELISA), immunoblot or western blot test, and indirect immunofluorescence assay (Lebech 2002, Tugwell et al. 1997).

The Center for Disease Control and Prevention has recommended two step (Figure 1.8) diagnosis of Lyme disease using blood sample for detection of antibodies against *Borrelia burgdorferi*. Under the first step testing, either the Enzyme Immunoassay (EIA) or the Immunofluorescence Assay (IFA) test is performed. If the first test is negative then no further test is conducted. In case of an indeterminate or positive result, second test, the Western blot or immunoblot is performed. If both the tests are positive only then the sample is considered true positive. However, serum based tests are not considered reliable by many scientists working with the Lyme disease (Steere et al. 1993, Hofmann 1996), as antibodies may be absent in patients with early disease symptoms, while, antibodies may persist even after years after clinical recovery (Gajovic et al. 2010), further patients treated do not show presence of antibodies. Due to shortcomings in serum based diagnosis a recent improved ELISA test has been recommended , in this test recombinant chimeric *Borrelia burgdorferi* proteins are used for detection (Gomes-Solecki et al. 2000).

The search and standardization for an adequate diagnosis for Lyme disease continues and someday CDC two tier test recommendation may be changed, covering for distinct Lyme disease diagnostic requirements (Aguero-Rosenfeld et al. 2005, Wormser et al. 2013). But at this time the following Two–Tier decision system (Figure 1.8) recommended by CDC continues as diagnostic guide tool.

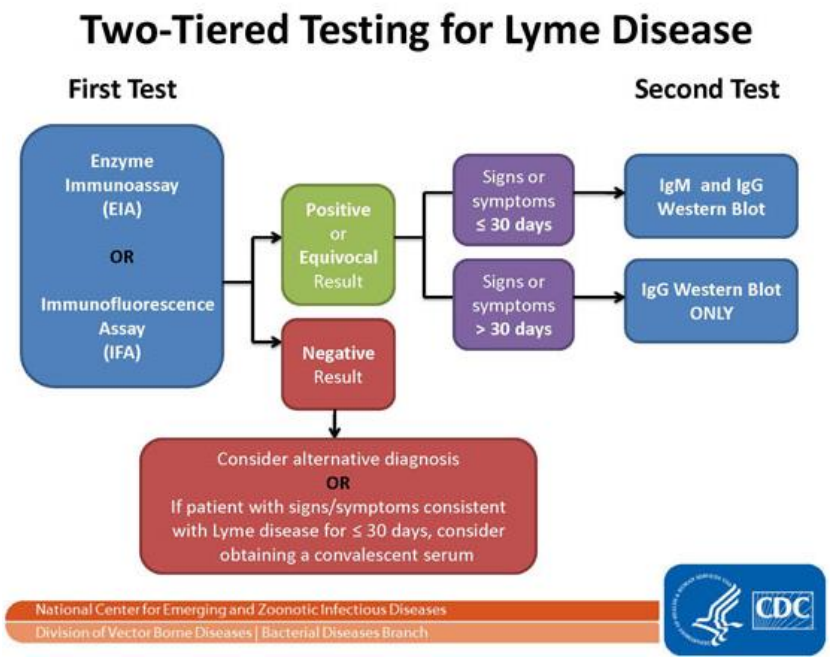

Figure 1.8. Two-tier testing decision Tree from Centre for Disease Control and Prevention (http://www.cdc.gov/lyme/diagnosistesting/LabTest/TwoStep/index.html)

Treatment of Lyme disease

In the early Lyme disease stage the serum testing is of limited utility , as there is no IgG in circulation during first 14 days of infection (Dressler et al. 1993). If an engorged *Ixodes* nymphal tick is found attached then a single 200mg dose of doxycycline is curative (Nadelman et al. 2001). In apparent erythema migrans the antibiotic treatment is continued for 10-20 days using doxycycline, both in adults and children above 8 years of age. Amoxicillin is considered best choice in children younger than 8 years (Wormser et al. 2003). The patients treated with antibiotics during the initial phase of the disease tend to have reinfection with Lyme disease (Bennet and Berglund 2002). Intravenous clindamycin, oral atovaquone, or oral quinine, and azithromycin is considered effective in people having coinfection with other vector-borne agents like Babesia or Anaplasma (Brown et al. 1995). In neurologic symptoms of Lyme disease,

intravenous antibiotic therapy with ceftriaxone is considered effective (Oschmann et al. 1998). Lyme arthritis can be treated with oral antibiotics doxycycline or amoxicillin, however treated patients can still develop either neuronal Lyme disease, or persistent arthritis with no effect of antibiotics (Steere et al. 1994).

Lyme disease transmission

Lyme disease is a zoonotic disease transmitted amongst ticks of *Ixodes ricinus* complex and a mammalian host (Anderson 1989). *Borrelia burgdorferi* infects the mammalian host as the *Ixodes* tick vector takes up the blood meal (Steere, Broderick, and Malawista 1978, Wallis et al. 1978). Though Lyme disease is considered as a zoonosis, humans are incidental hosts and once infected act as dead end host (Baum 2008).

The first *B. burgdorferi* isolate was found in an ixodid tick from Shelter island at New York in 1981 (Burgdorfer, Barbour et al. 1982). The ixodes ticks thrive well in the temperate climate of the northern hemisphere, this is also true for *Borrelia burgdorferi* surviving amongst various mammalian reservoir hosts (Radolf et al. 2012). The distribution of ticks vary with geographical location and climatic conditions, while *Ixodes scapularis* is main vector for northeastern and upper mid-western United States, *Ixodes pacificus* is common in the far western United States, *Ixodes persulcatus* is found in Asia and *Ixodes ricinus* in Eurasia (Lane, Piesman, and Burgdorfer 1991).The change in land use patterns in the late 20th century, particularly the deforestation followed by reforestation in northeastern and mid-western parts of United States has provided optimum conditions for Lyme disease vector to propagate (Barbour 1998, Chen et al. 2005).

Ixodes scapularis, commonly known as the black legged tick was first described in 1979 by Spielman et al. (Spielman et al. 1979). The different life stages of *I. scapularis*, include eggs,

larvae, nymphs, and dimorphic adults (Figure 1.10). The detailed two year life cycle for Lyme disease vector has been shown in figure 1.9. The trans-stadial transmission of *Borrelia burgdorferi* from larval stage to nymphal stage and a blood meal at each developmental stage, on same reservoir host species ensure the horizontal transmission of infection from infected to uninfected developmental stage (Burgdorfer 1984b, Drouin, Glickstein, and Steere 2004, Levine, Wilson, and Spielman 1985).

The questing activity of nymphs coincides with the high outdoor human activities, further the small size of nymphs help them attach undetected, due to these advantages, the tick vector nymphs are critical to cause high incidence of Lyme disease in humans, especially during late spring and early summer (Lane, Piesman, and Burgdorfer 1991).

The developmental stages of tick vector have a broad host range including small mammals and birds (Anderson 1989, 1988). In its current cycle within the north eastern United States, *Peromyscus leucopus* (the white footed mouse) acts as the main reservoir host for the nymphal and larval stages of ticks (Anderson and Magnarelli 1980, Anderson et al. 1985). The adult ticks are known to feed on larger animals especially white tailed deer (*Odocoileus virginianus*) (Bosler et al. 1984, Carey, Krinsky, and Main 1980, Spielman et al. 1979).

Adaptation of *Borrelia burgdorferi*

In an unfed tick vector *B. burgdorferi* (Figure 1.11) remains dormant within the midgut. In response to blood meal the spirochetes move from hemocoel to the salivary gland (Ribeiro et al. 1987). It is during the blood meal that borrelia infects the mammalian host through tick saliva (Burgdorfer 1984a, Schwan and Piesman 2000). During its journey from tick midgut to the mammalian host environment *B. burgdorferi* undergoes several adaptive changes (Schwan and

Piesman 2000, Schwan et al. 1995). Most eminent of these adaptations is the rapid change in the outer surface lipoprotein expression (Schwan and Piesman 2000, Schwan et al. 1995).

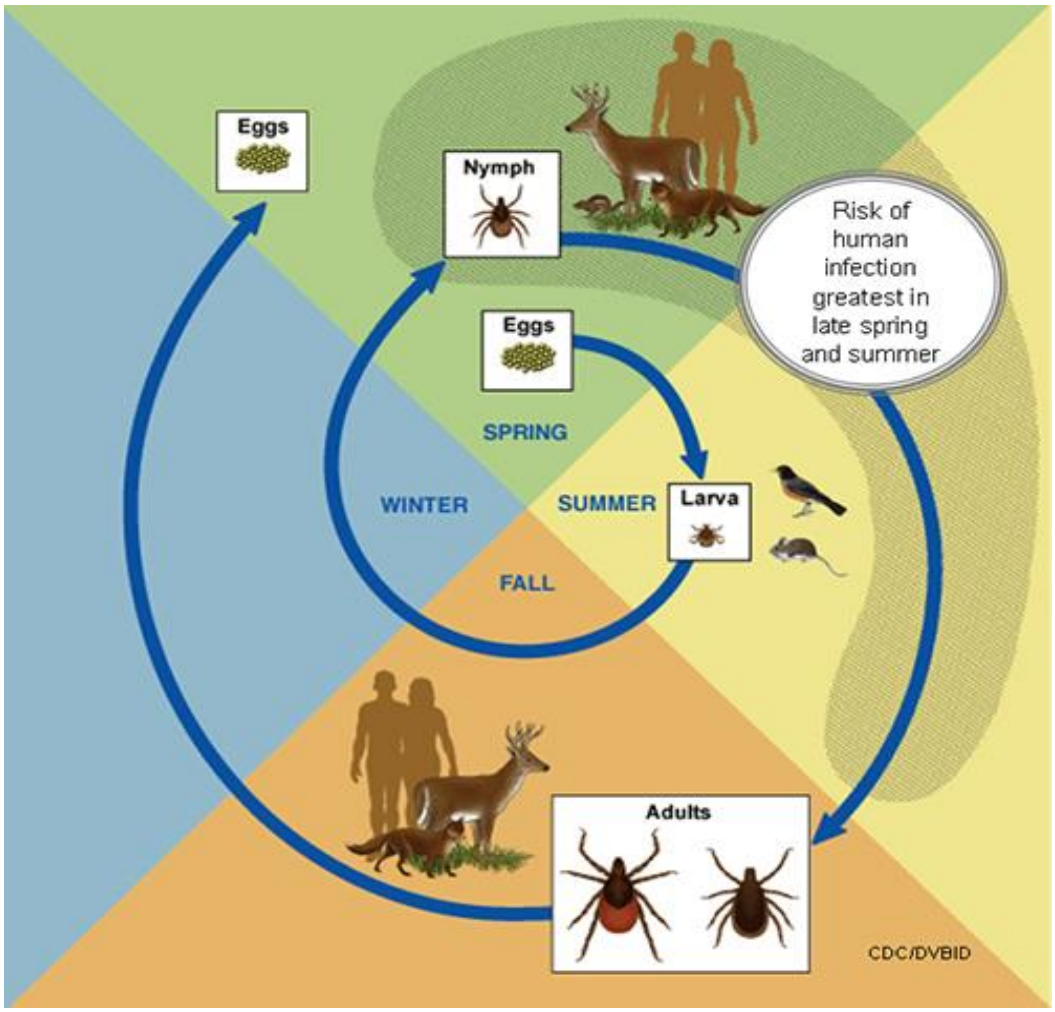

Figure 1.9. Two year cycle of Lyme disease vector: at least one blood meal is taken at every stage, the adult female tick lay eggs in the spring after feeding on a large mammalian host, in summer eggs hatch to larvae which feed on small mammals and birds and molt into nymphs during next spring, these nymphs again feed on a mammalian host during late spring or early summer and molt into adults the following fall. It is during the nymph feeding season that humans become incidental host as the peak outdoor activities coincide with the questing nymphs. CDC- Division of Vector-borne Infectious Diseases, last modified Nov 15, 2011. (http://www.cdc.gov/lyme/transmission/blacklegged.html)

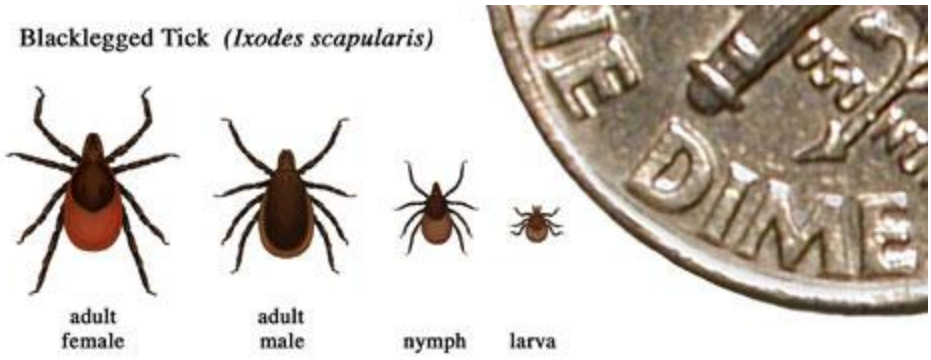

Figure 1.10. Comparative morphology of the different life stages of the Lyme disease vector Ixodes Scapularis (CDC- Division of Vector-borne Infectious Diseases, last modified January 11, 2013, http://www.cdc.gov/lyme/transmission/index.html)

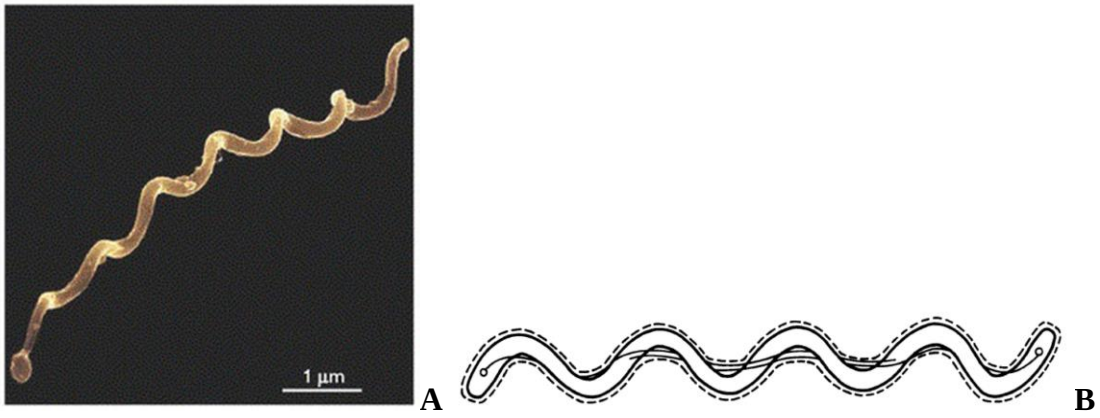

Figure1.11. Morphology of *Borrelia burgdorferi* (A) Shows spiral morphology of *Borrelia burgdorferi.*(Rosa, Tilly, and Stewart 2005) *Borreliae* are 10-30µm long and .2-.3µm wide and have 7-11 periplasmic flagella imparting spiral shape (Burgdorfer et al. 1982) (B) "Schematic representation of a spirochete" from (Canale-Parola 1977) shows outer envelope in broken line , protoplasmic cylinder in solid line which follows along outer envelope, and thin solid line indicates the axial fibrils.

Unlike other double membraned gram negative bacteria, that express lipopolysaccharide on their outer-membrane, borrelia express proteins covalently linked to lipids known as outer surface lipoproteins (osps) (Barbour and Hayes 1986, Bergstrom, Bundoc, and Barbour 1989, Brandt et al. 1990). The outer surface lipoproteins form an interface between borrelia's internal and external environment (Figure1.12).

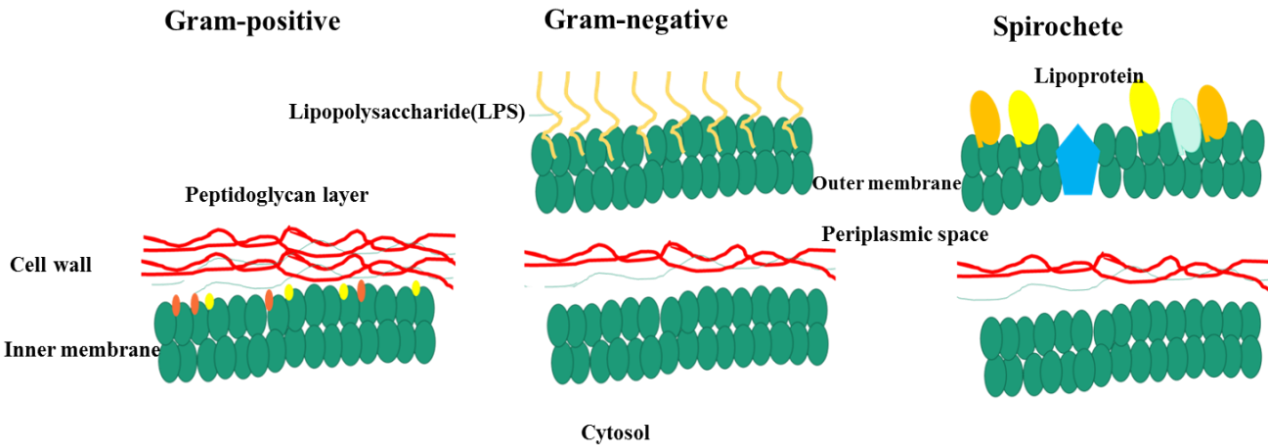

Figure1.12. Comparative membrane structures of Gram-positive, Gram-negative and *B. burgdorferi*. The outer membrane of borrelia is markedly distinct from Gram-positive and other Gram-negative bacteria. The borrelial outer membrane is fluid, fragile, and lacks Lipopolysaccharide, instead it is adorned with several lipoproteins anchored to the outer membrane by process called lipidation.

As the external environment alternates from tick midgut to mammalian host, the osps play critical role in survival of borrelia (Brandt et al. 1990, Bergstrom, Bundoc, and Barbour 1989). As shown in figure 1.13, in an unfed tick, *Borrelia burgdorferi* remains low in number and expresses outer surface protein A (OspA) as the major surface lipoprotein (Fingerle et al. 1995). In response to the blood meal there is rapid multiplication of borrelia, along with increase in number there is rapid downregulation of OspA (Schwan et al. 1995). As the borrelia migrates from hemocoel to the tick salivary gland it expresses Outer surface protein C as major surface protein (De Silva and Fikrig 1997b). This reciprocal expression of OspA and OspC enables the mobilization of borrelia from the tick midgut to the salivary gland, and finally into the mammalian host (Schwan et al. 1995, de Silva and Fikrig 1997a).

Viability determination of *B. burgdorferi*

The Lyme disease agent, *B. burgdorferi* can be cultured readily from the EM skin lesions (Berger et al. 1992, Mitchell et al. 1993) and often from the concomitant blood samples in BSK-H medium, thus indicating the presence of viable organisms in the specimen.

One of the most commonly used methods for differentiating alive and dead spirochetes is enumeration of the motile spirochetes in culture medium inoculated with the infected specimen under the dark field microscope. The spirochetes which lie still under the dark field microscope are considered dead and not taken into account while enumeration.

The culture based classical methods for detecting the spirochete viability and enumerations have limited sensitivity, and are time consuming. Several molecular methods targeting DNA and RNA have also been developed for the detection of total as well as viable spirochetes offering higher speed, sensitivity, and specificity. However, most attempts to culture the causative organism form low burden sites such as synovial fluid (SF) have been negative (Steere et al. 1984, Bradley, Johnson, and Goodman 1994) in contrast, Polymerase Chain Reaction (PCR) results are often positive at these sites. The presence of viable spirochetes have been quantified by real-time PCR and confirmed by in-vitro culture after 7 months of infection and up to four weeks after completion of penicillin treatment (Pahl et al. 1999). PCR targeting spirochete DNA have been reported to present some discrepancy with culture results, which could be because of the very low spirochete load or the organisms are injured, or dead. Therefore, detection of *B. burgdorferi* DNA may not be a reliable test of active joint infection in Lyme disease. Quantification of *B. burgdorferi* messenger RNA (mRNA), and ribosomal RNA (rRNA) has been a reliable tool for differentiation and enumeration of viable spirochetes form the total load identified by DNA based assays. Quantitative PCR and RT-PCR based assays are being increasingly used in Lyme

disease patients for determination of active infection in patients with persistent arthritis after antibiotic therapy.

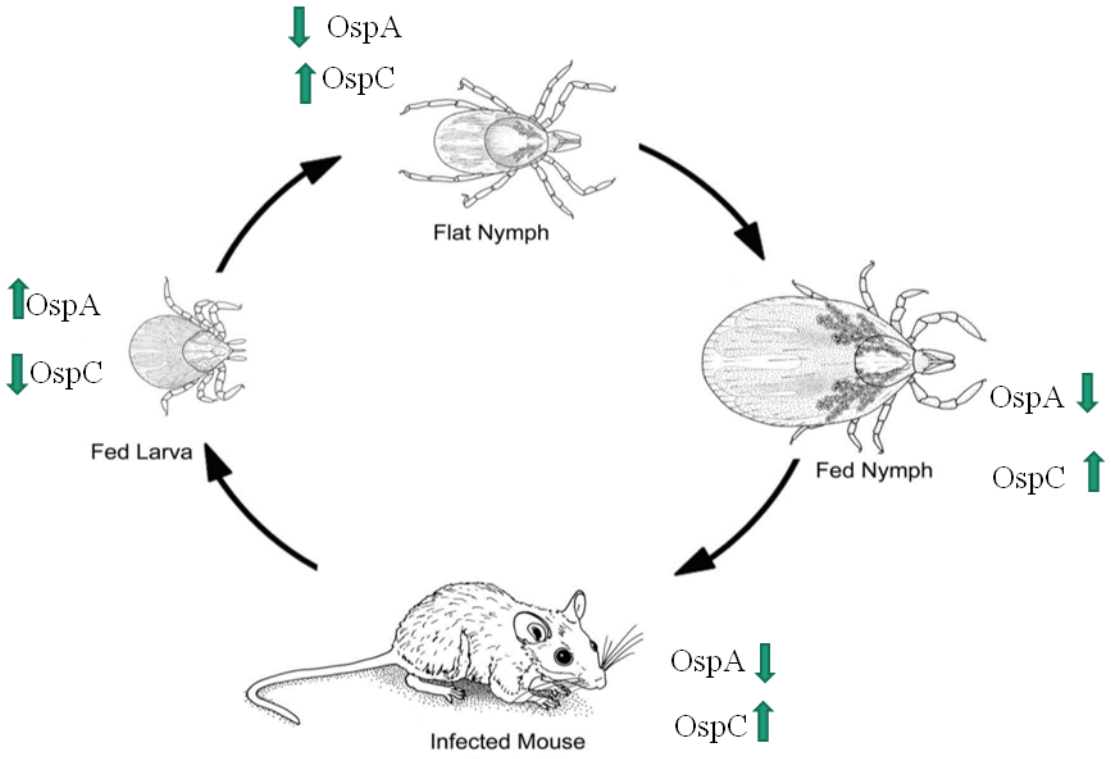

Figure 1.13. OspA and OspC crossregulation during the infectious cylce of *B. burgdorferi*. OspA is abundantly expressed in tick vector and is rapidly downregulated in response to blood meal in order to evade immune detection. OspC expression is increased as *B. burgdorferi* prepares to infect a mammalian host. Figure from (Mulay et al. 2009)

Animal model of Lyme disease

In general, the animal model for Lyme disease should display disease pathology similar to that of human patients. Thus the animals which can be infected with the Lyme disease agent in laboratory and show persistent infection with *B. burgdorferi* along with one or more arthritic joints, and involvement of multiple organs with disease symptoms in heart, joint, and skin can be considered as the Lyme disease animal model.

The first animal model for Lyme disease was introduced by Schaible et al. in 1989 as they found inbred mouse strains (C57BL/6 and BALB/c) inoculated with live *B. burgdorferi* B31 show antigen specific T-cell response similar to pathomorphology seen in Lyme disease patients (Schaible et al. 1989). Same group also showed that the mice having severe combined immundeficiency syndrome (SCID), thus lacking or having abnormal B-cell and T-cell function, show progressive polyarthritis and carditis and hence serve even more useful model then immunocompetent mice strains for studying Lyme disease (Schaible et al. 1990).

In addition to mice, many other animal species were proved to be useful in studying Lyme disease. In the year 1984, Kornblatt et al. showed that rabbits could contract Lyme disease experimentally and show initial signs similar to that in humans (Kornblatt, Steere, and Brownstein 1984). The same group showed that dogs can show Lyme arthritis similar to humans (Kornblatt, Urband, and Steere 1985). Mursic et al. found that gerbils show multiorgan involvement in response to experimental inoculation of *B. burgdorferi*, and hence can serve as suitable Lyme disease model to study the pathogenesis and the extent of organ damage (Preac Mursic et al. 1990). Furthermore, guinea pig was also found to be useful as a animal model for Lyme disease as it showed Lyme disease symptoms upon inoculation (Sonnesyn et al. 1993).

While most of the rodent and lagomorphs showed Lyme disease infection, none of the animals mentioned above show central nervous system (CNS) signs of Lyme disease. Rhesus monkeys are the only experimental animals that could develop neuroborreliosis and hence serve as another unique Lyme disease animal model (Philipp and Johnson 1994).

Furthermore, the animals which show infection upon laboratory inoculation, can also be used for cultivation of the pathogen in the mammalian host adapted form. The term host adapted form represents the antigenic and structural changes taken up by the spirochetes as they transgress

from ticks to the mammalian host. The isolated borreliae from infected tissue samples undergo changes in antigenic expression as they multiply and adapt to the laboratory culture conditions. Thus in order to enable study of spirochetes in the mammalian host adapted form it was essential to develop a new animal model which could provide mammalian stimuli and allow pathogen to grow in host adapted stage. Such a novel model was developed by Akins et al. in year 1998, this model involved implantation of lyme disease spirochetes enclosed within the dialysis membrane chambers (DMCs) into the peritoneal cavities of sprague dawley rats (Akins et al. 1998). This model provided researchers with an opportunity to cultivate sufficient amount of the mammalian host adapted Lyme disease spirochetes for the laboratory experimental analysis. Another added advantage of this model was possible cultivation of noninfectious borrelia mutant variants in the mammalian host environment, as DMC protects spirochetes from the host immune system. The spirochetes in DMC adapt in response to the physiological stimuli provided in the peritoneum of rats and express virulence determinants which are essential for the disease pathogenesis (Akins et al. 1998). The detailed use and procedure for the DMC technique has been provided in the materials and methods section of chapter 2. Hence, the role of Lyme disease animal model is extended from serving as a disease simulator to cultivator of *B. burgdorferi* in host adapted form.

Molecular regulation of osp in *Borrelia burgdorferi*

The deliberate expression and repression of osps in *B. burgdorferi* is enabled by an intricate molecular regulatory system. Though the genome of *B. burgdorferi* has been sequenced completely by Fraser et al. in 1997, the underlying molecular regulation pathways for coordinated osp expression is still not completely understood. *B. burgdorferi* has unique genomic features (Figure1.14), including a linear chromosome of about one megabasepair, 12 linear and 9 circular plasmids, and each plasmid exists as a single copy per chromosome (Fraser et al. 1997,

Casjens 2000, Stewart et al. 2005). While there are no genes for cellular biosynthetic reactions in borrelial genome, the function of a large number of predicted coding sequence is yet to be understood (Casjens 2000). The outer surface proteins are encoded in the borrelial plasmids, thus the plasmids basically carry virulence related genes in *B. burgdorferi* (Stewart et al. 2005).

The molecular regulatory machinery in borrelia differs considerably from other bacteria, as very few homologous regulatory proteins are present (Samuels 2011). *B. burgdorferi* has three RNA polymerase sigma factors: RpoD, RpoN, and RpoS. While RpoD is a major sigma factor, regulating most of the house keeping genes, RpoN and RpoS are two alternate sigma factors. RpoN and RpoS form a regulatory pathway in which transcription of *rpoS* is controlled by RpoN (Ouyang, Blevins, and Norgard 2008). RpoN binds to the consensus promoter region of *rpoS* and enables further RpoS regulated gene expression (Ouyang, Blevins, and Norgard 2008). The RpoN mediated *rpoS* expression has been found to be dependent on enhancer binding protein and two component regulatory system response regulator: Rrp2 (Ouyang, Blevins, and Norgard 2008). Thus the RpoN-RpoS pathway can be called Rrp2-RpoN-RpoS pathway. Further studies found that Rrp2 is activated by acetyl phosphate a phosphate donor (Xu et al. 2010).

The RpoN-RpoS regulatory pathway has been found to up-regulate several virulence factors, most of these are essential for borrelial adaptation within the mammalian host (Yang et al. 2005). The known RpoS regulated crucial virulence factors include outer surface lipoproteins: OspC, Decorin binding protein A (DbpA) and Decorin binding protein B (DbpB) (Yang et al. 2005). The *rpoS* mutant fails to downregulate OspA in the mammalian host like conditions. Hence, RpoS is essential to cause repression of OspA in mammalian host (Caimano et al. 2005).

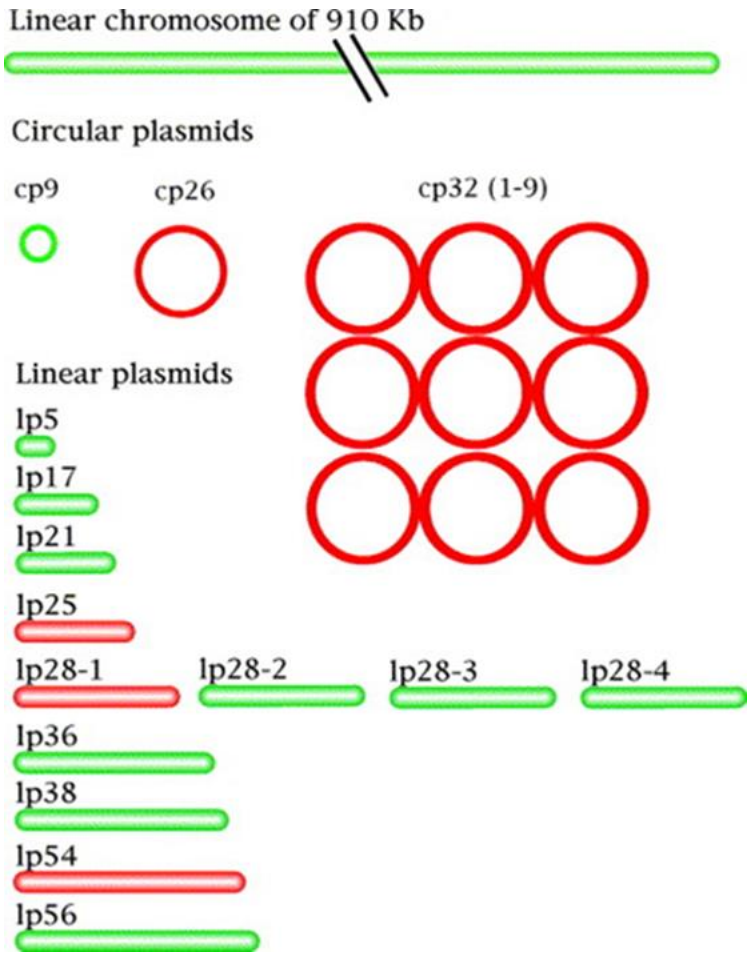

Figure 1.14. Segmented Genome of *B. burgdorferi* carries numerous plasmids, more than reported for any other bacteria. The borrelia plasmids are both linear (lp) and circular (cp), named according to their approximate size in kilobase pairs. Above picture was adapted from a review by Patricia A.Rosa (Stewart et al. 2005). *B. burgdorferi* B31 strain is known to carry 22-23 plasmids (Stewart et al. 2005, Fraser et al. 1997, Hinnebusch and Barbour 1992). The plasmids depicted in red required either for maintaining infectivity or persistence of *B. burgdorferi* in tick vector.

Unlike other bacteria, RpoS in *B. burgdorferi* is critical for expression of virulence genes rather than only in general stress response (Caimano et al. 2007). Also, *B. burgdorferi* lacking *rpoS* fail to produce infection in mouse (Xu et al. 2012). Once the tick vector takes up blood meal, RpoS is generated and is continuously expressed throughout the borrelial adaptation within the mammalian host (Banik et al. 2011). Thus in conclusion, RpoS is required by borrelia for

initiating and maintaining infection in the mammalian host (Banik et al. 2011). However, recent studies by contemporary research groups have found another regulator called BosR, required for transcription of *rpoS B. burgdorferi* (Ohnishi, Piesman, and de Silva 2001, Hyde et al. 2009, Ouyang et al. 2009).

Due to its preconcieved role in oxidative stress response, the borrelial protein called BB0647 was renamed as *B. burgdorferi* oxidative stress regulator or BosR (Hyde et al. 2009). However, the role of BosR as a borrelial oxidative stress response protein has not been established yet. BosR mutant successfully colonize the tick vector but fails to establish infection in lab mice. Therefore, BosR is considered essential for causing infection in a mammalian host (Hyde et al. 2009). BosR is regarded as the master regulator of RpoN-RpoS regulatory pathway acting like a DNA binding protein which binds directly on the *rpoS* gene (Samuels and Radolf 2009, Ouyang, Deka, and Norgard 2011). Thus, BosR acts as a positive transcription activator and plays additional regulatory role (Ouyang, Deka, and Norgard 2011).

Our study shows that *bosR* mutants fail to repress OspA and OspD in mammalian host environment. OspA and OspD both are outer surface lipoproteins in borrelia primarily expressed within the tick vector and not required for the infection in the mammalian host (Yang, Pal et al. 2004, Xin Li et al 2007). Though RpoD is known to regulate expression of *ospA* within the tick vector, the mechanism causing its repression in response to blood meal is still not known. Thus the major regulatory role of BosR within the mammalian host is undisputed but remains unknown (Samuels and Radolf 2009, Ouyang, Deka, and Norgard 2011, Samuels 2011).

Statement of the problem

Lyme disease still remains the most commonly reported vector born disease in USA. Even though the genome of *Borrelia burgdorferi* has been completely sequenced (Fraser et al. 1997), successful strategies to check the transmission are yet to be developed.

The knowledge accumulated since discovery of Lyme disease is enough to yield a cohesive picture of cellular and molecular events within the enzootic cycle of *B. burgdorferi*. As the lipoproteins form an interface between inside and outside environment for borrelia, the delicate balance of outer surface proteins play a central role in its survival (De Silva and Fikrig 1997b, Schwan et al. 1995, Xu, McShan, and Liang 2008). The outer surface lipoproteins play a collective role in adaptation of *B. burgdorferi* within the Ixodes tick vector and a mammalian host (Xu, McShan, and Liang 2008), however, the survival strategies adopted by the Lyme disease spirochetes are not completely understood.

The sophisticated regulation of the different surface lipoproteins by borrelia relies on complex regulatory mechanisms. Though, borrelia has limited metabolic pathways as it evolved to parasitize nutrients from its hosts, the molecular regulatory mechanisms are impressively complex and hard to unravel (Fraser et al. 1997). The knowledge of the complex regulatory pathways involving limited regulatory factors is still incomplete and is an open field for research. The studies revealing new mechanisms of borrelial gene control are crucial to develop new strategies to interrupt the spirochete's life cycle and curb the emerging Lyme disease.

The outer surface lipoprotein A is abundantly expressed on the surface of *B. burgdorferi* as it lays dormant within tick midgut, however there is rapid downregulation of this protein in response to blood meal (Schwan et al. 1995). The vigorous downregulation of OspA is

coordinated with upregulation of OspC (Schwan et al. 1995, Schwan and Piesman 2000). This reciprocal expression of OspC and OspA is responsible for exit and transmission of borrelia from the invertebrate tick vector to a mammalian host (Schwan et al. 1995). The 2010 publication by Xu et al. reported a regulatory structure consisting of two sequences flanking the *ospA* promoter: *cisI* and *cisII*, these two sequences provide *B. burgdorferi* with molecular mechanism to quickly downregulate or upregulate OspA in response to changes in external environment. However, the factors binding these sequences in response to specific environmental stimuli has not been identified yet. As the downregulation of OspA is important for survival of *B. burgdorferi* within the mammalian host, identification of underlying molecular mechanism may yield new strategies to prevent transmission of Lyme disease (Strother et al. 2007, de Silva et al. 1997).

The homeostasis on the outer membrane layer of *B. burgdorferi* is exemplified by several studies (Battisti et al. 2008, He et al. 2008, Pal et al. 2004). Crossregulation of OspA and OspC is one classic well studied homeostatic expression, as it is central to establishment of the Lyme disease and maintenance of the spirochete in enzootic life cycle (Schwan and Piesman 2000, Schwan et al. 1995).

Goal, objectives, and hypothesis

Goal

The overall goal of this dissertation is to add to the current knowledge of gene regulation in the Lyme disease agent and highlight the homeostatic maintenance of the surface lipoproteins on the outer membrane layer of Lyme disease spirochete *B. burgdorferi*.

Objectives

The specific objectives and hypothesis of the study are listed below:

1. To confirm the repression of OspA in *bosR* mutant and investigate if this repression is directly mediated by BosR mediated or indirectly mediated by RpoS.
2. To find out specific region of *ospA* operon involved in BosR mediated repression.
3. To study osp expression in *ospA* mutant and identify the other surface lipoproteins that are involved in maintaining the homeostasis of the outer surface lipoprotein layer in *B. burgdorferi*.

Hypothesis

The hypothesis underlying the above mentioned objectives can be stated as following:

1. BosR is the repressor protein responsible for downregulation of OspA by direct action on *ospA* operon.
2. The outer surface lipoprotein layer is in a state of homeostasis and loss of a dominant osp like OspA shall cause upregulation of other osps to maintain the lipoprotein layer on borrelial outer surface.

References

Aguero-Rosenfeld, M. E., G. Wang, I. Schwartz, and G. P. Wormser. 2005. "Diagnosis of lyme borreliosis." *Clin Microbiol Rev*. 18 (3):484-509. doi: 10.1128/CMR.18.3.484-509.2005.

Akins, D. R., K. W. Bourell, M. J. Caimano, M. V. Norgard, and J. D. Radolf. 1998. "A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state." *J Clin Invest*. 101 (10):2240-50.

Anderson, J. F. 1988. "Mammalian and avian reservoirs for *Borrelia burgdorferi*." *Ann N Y Acad Sci*. 539:180-91.

Anderson, J. F. 1989. "Epizootiology of Borrelia in Ixodes tick vectors and reservoir hosts." *Rev Infect Dis*. 11 Suppl 6:S1451-9.

Anderson, J. F., R. C. Johnson, L. A. Magnarelli, and F. W. Hyde. 1985. "Identification of endemic foci of Lyme disease: isolation of *Borrelia burgdorferi* from feral rodents and ticks (Dermacentor variabilis)." *J Clin Microbiol*. 22 (1):36-8.

Anderson, J. F., and L. A. Magnarelli. 1980. "Vertebrate host relationships and distribution of ixodid ticks (Acari: Ixodidae) in Connecticut, USA." *J Med Entomol*. 17 (4):314-23.

Asbrink, E., and A. Hovmark. 1988. "Early and late cutaneous manifestations in Ixodes-borne borreliosis (erythema migrans borreliosis, Lyme borreliosis)." *Ann N Y Acad Sci*. 539:4-15.

Banik, S., D. Terekhova, R. Iyer, C. J. Pappas, M. J. Caimano, J. D. Radolf, and I. Schwartz. 2011. "BB0844, an RpoS-regulated protein, is dispensable for *Borrelia burgdorferi* infectivity and maintenance in the mouse-tick infectious cycle." *Infect Immun*. 79 (3):1208-17.

Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J. C. Piffaretti, M. Assous, and P. A. Grimont. 1992. "Delineation of *Borrelia burgdorferi* sensu stricto, Borrelia garinii sp. nov., and group VS461 associated with Lyme borreliosis." *Int J Syst Bacteriol*. 42 (3):378-83.

Barbour, A. G. 1998. "Fall and rise of Lyme disease and other Ixodes tick-borne infections in North America and Europe." *Br Med Bull*. 54 (3):647-58.

Barbour, A. G., and S. F. Hayes. 1986. "Biology of Borrelia species." *Microbiol Rev*. 50 (4):381-400.

Battisti, J. M., J. L. Bono, P. A. Rosa, M. E. Schrumpf, T. G. Schwan, and P. F. Policastro. 2008. "Outer surface protein A protects Lyme disease spirochetes from acquired host immunity in the tick vector." *Infect Immun*. 76 (11):5228-37.

Baum, S. G. 2008. "Zoonoses-with friends like this, who needs enemies?" *Trans Am Clin Climatol Assoc*. 119:39-51; discussion 51-52.

Bennet, L., and J. Berglund. 2002. "Reinfection with Lyme borreliosis: a retrospective follow-up study in southern Sweden." *Scand J Infect Dis*. 34 (3):183-86.

Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman. 1992. "Cultivation of *Borrelia burgdorferi* from erythema migrans lesions and perilesional skin." *J Clin Microbiol*. 30 (2):359-61.

Bergstrom, S., V. G. Bundoc, and A. G. Barbour. 1989. "Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochaete *Borrelia burgdorferi*." *Mol Microbiol*. 3 (4):479-86.

Bosler, E. M., B. G. Ormiston, J. L. Coleman, J. P. Hanrahan, and J. L. Benach. 1984. "Prevalence of the Lyme disease spirochete in populations of white-tailed deer and white-footed mice." *Yale J Biol Med*. 57 (4):651-59.

Bradley, J. F., R. C. Johnson, and J. L. Goodman. 1994. "The persistence of spirochetal nucleic acids in active Lyme arthritis." *Ann Intern Med*. 120 (6):487-89.

Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard. 1990. "Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins." *Infect Immun*. 58 (4):983-91.

Brisson, D., D. Drecktrah, C. H. Eggers, and D. S. Samuels. 2012. "Genetics of *Borrelia burgdorferi*." *Annu Rev Genet*. 46:515-36.

Brown, E. L., J. M. Rivas, S. E. Ullrich, C. R. Young, S. J. Norris, and M. L. Kripke. 1995. "Modulation of immunity to *Borrelia burgdorferi* by ultraviolet irradiation: differential effect on Th1 and Th2 immune responses." *Eur J Immunol*. 25 (11):3017-22.

Burgdorfer, W. 1984a. "Discovery of the Lyme disease spirochete and its relation to tick vectors." *Yale J Biol Med*. 57 (4):515-20.

Burgdorfer, W. 1984b. "The New Zealand white rabbit: an experimental host for infecting ticks with Lyme disease spirochetes." *Yale J Biol Med*. 57 (4):609-12.

Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis. 1982. "Lyme disease-a tick-borne spirochetosis?" *Science*. 216 (4552):1317-19.

Caimano, M. J., C. H. Eggers, C. A. Gonzalez, and J. D. Radolf. 2005. "Alternate sigma factor RpoS is required for the in vivo-specific repression of *Borrelia burgdorferi* plasmid lp54-borne ospA and lp6.6 genes." *J Bacteriol*. 187 (22):7845-52.

Caimano, M. J., R. Iyer, C. H. Eggers, C. Gonzalez, E. A. Morton, M. A. Gilbert, I. Schwartz, and J. D. Radolf. 2007. "Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle." *Mol Microbiol*. 65 (5):1193-217.

Canale-Parola, E. 1977. "Physiology and evolution of spirochetes." *Bacteriol Rev*. 41 (1):181-204.

Carey, A. B., W. L. Krinsky, and A. J. Main. 1980. "Ixodes dammini (Acari: Ixodidae) and associated ixodid ticks in South-central Connecticut, USA." *J Med Entomol*. 17 (1):89-99.

Casjens, S. 2000. "Borrelia genomes in the year 2000." *J Mol Microbiol Biotechnol*. 2 (4):401-10.

Chen, H., D. J. White, T. B. Caraco, and H. H. Stratton. 2005. "Epidemic and spatial dynamics of Lyme disease in New york State, 1990-2000." *J Med Entomol*. 42 (5):899-908.

de Silva, A. M., and E. Fikrig. 1997a. "Arthropod- and host-specific gene expression by *Borrelia burgdorferi*." *J Clin Invest*. 99 (3):377-79.

De Silva, A. M., and E. Fikrig. 1997b. "*Borrelia burgdorferi* genes selectively expressed in ticks and mammals." *Parasitol Today*. 13 (7):267-70.

de Silva, A. M., D. Fish, T. R. Burkot, Y. Zhang, and E. Fikrig. 1997. "OspA antibodies inhibit the acquisition of *Borrelia burgdorferi* by Ixodes ticks." *Infect Immun*. 65 (8):3146-50.

Dressler, F., J. A. Whalen, B. N. Reinhardt, and A. C. Steere. 1993. "Western blotting in the serodiagnosis of Lyme disease." *J Infect Dis*. 167 (2):392-400.

Drouin, E. E., L. J. Glickstein, and A. C. Steere. 2004. "Molecular characterization of the OspA(161-175) T cell epitope associated with treatment-resistant Lyme arthritis: differences among the three pathogenic species of *Borrelia burgdorferi* sensu lato." *J Autoimmun*. 23 (3):281-92.

Fingerle, V., U. Hauser, G. Liegl, B. Petko, V. Preac-Mursic, and B. Wilske. 1995. "Expression of outer surface proteins A and C of *Borrelia burgdorferi* in Ixodes ricinus." *J Clin Microbiol*. 33 (7):1867-69.

Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. Weidman, T. Utterback, L. Watthey, L. McDonald, P. Artiach, C. Bowman, S. Garland, C. Fuji, M. D. Cotton, K. Horst, K. Roberts, B. Hatch, H. O. Smith, and J. C. Venter. 1997. "Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*." *Nature*. 390 (6660):580-86.

Gajovic, O., Z. Todorovic, L. Nesic, and Z. Lazic. 2010. "[Lyme borreliosis--diagnostic difficulties in interpreting serological results]." *Med Pregl*. 63 (11-12):839-43.

Gerber, M. A., E. D. Shapiro, G. S. Burke, V. J. Parcells, and G. L. Bell. 1996. "Lyme disease in children in southeastern Connecticut. Pediatric Lyme Disease Study Group." *N Engl J Med*. 335 (17):1270-74.

Gomes-Solecki, M. J., J. J. Dunn, B. J. Luft, J. Castillo, D. E. Dykhuizen, X. Yang, J. D. Glass, and R. J. Dattwyler. 2000. "Recombinant chimeric Borrelia proteins for diagnosis of Lyme disease." *J Clin Microbiol*. 38 (7):2530-35.

He, M., T. Oman, H. Xu, J. Blevins, M. V. Norgard, and X. F. Yang. 2008. "Abrogation of *ospA* constitutively activates the Rrp2-RpoN-RpoS pathway (sigmaN-sigmaS cascade) in *Borrelia burgdorferi*." *Mol Microbiol*. 70 (6):1453-64.

Hinnebusch, J., and A. G. Barbour. 1992. "Linear- and circular-plasmid copy numbers in *Borrelia burgdorferi*." *J Bacteriol*. 174 (16):5251-57.

Ho, K., M. Melanson, and J. A. Desai. 2012. "Bell palsy in lyme disease-endemic regions of canada: a cautionary case of occult bilateral peripheral facial nerve palsy due to Lyme disease." *CJEM*. 14 (5):321-24.

Hofmann, H. 1996. "Lyme borreliosis--problems of serological diagnosis." *Infection*. 24 (6):470-72.

Hyde, J. A., D. K. Shaw, R. Smith Iii, J. P. Trzeciakowski, and J. T. Skare. 2009. "The BosR regulatory protein of *Borrelia burgdorferi* interfaces with the RpoS regulatory pathway and modulates both the oxidative stress response and pathogenic properties of the Lyme disease spirochete." *Mol Microbiol*. 74 (6):1344-55.

Kirkland, K. B., T. B. Klimko, R. A. Meriwether, M. Schriefer, M. Levin, J. Levine, W. R. Mac Kenzie, and D. T. Dennis. 1997. "Erythema migrans-like rash illness at a camp in North Carolina: a new tick-borne disease?" *Arch Intern Med*. 157 (22):2635-41.

Kornblatt, A. N., A. C. Steere, and D. G. Brownstein. 1984. "Experimental Lyme disease in rabbits: spirochetes found in erythema migrans and blood." *Infect Immun*. 46 (1):220-23.

Kornblatt, A. N., P. H. Urband, and A. C. Steere. 1985. "Arthritis caused by *Borrelia burgdorferi* in dogs." *J Am Vet Med Assoc*. 186 (9):960-64.

Krause, P. J., S. R. Telford, 3rd, A. Spielman, V. Sikand, R. Ryan, D. Christianson, G. Burke, P. Brassard, R. Pollack, J. Peck, and D. H. Persing. 1996. "Concurrent Lyme disease and babesiosis. Evidence for increased severity and duration of illness." *JAMA*. 275 (21):1657-60.

Lane, R. S., J. Piesman, and W. Burgdorfer. 1991. "Lyme borreliosis: relation of its causative agent to its vectors and hosts in North America and Europe." *Annu Rev Entomol*. 36:587-609.

Lebech, A. M. 2002. "Polymerase chain reaction in diagnosis of *Borrelia burgdorferi* infections and studies on taxonomic classification." *APMIS Suppl* (105):1-40.

Levine, J. F., M. L. Wilson, and A. Spielman. 1985. "Mice as reservoirs of the Lyme disease spirochete." *Am J Trop Med Hyg*. 34 (2):355-60.

Mitchell, P. D., K. D. Reed, M. F. Vandermause, and J. W. Melski. 1993. "Isolation of *Borrelia burgdorferi* from skin biopsy specimens of patients with erythema migrans." *Am J Clin Pathol*. 99 (1):104-07.

Mulay, V. B., M. J. Caimano, R. Iyer, S. Dunham-Ems, D. Liveris, M. M. Petzke, I. Schwartz, and J. D. Radolf. 2009. "*Borrelia burgdorferi* bba74 is expressed exclusively during tick feeding and is regulated by both arthropod- and mammalian host-specific signals." *J Bacteriol*. 191 (8):2783-94.

Nadelman, R. B., J. Nowakowski, D. Fish, R. C. Falco, K. Freeman, D. McKenna, P. Welch, R. Marcus, M. E. Aguero-Rosenfeld, D. T. Dennis, G. P. Wormser, and Group Tick Bite

Study. 2001. "Prophylaxis with single-dose doxycycline for the prevention of Lyme disease after an Ixodes scapularis tick bite." *N Engl J Med*. 345 (2):79-84.

Nadelman, R. B., J. Nowakowski, G. Forseter, N. S. Goldberg, S. Bittker, D. Cooper, M. Aguero-Rosenfeld, and G. P. Wormser. 1996. "The clinical spectrum of early Lyme borreliosis in patients with culture-confirmed erythema migrans." *Am J Med*. 100 (5):502-08.

Nadelman, R. B., and G. P. Wormser. 1998. "Lyme borreliosis." *Lancet*. 352 (9127):557-65.

Nocton, J. J., F. Dressler, B. J. Rutledge, P. N. Rys, D. H. Persing, and A. C. Steere. 1994. "Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis." *N Engl J Med*. 330 (4):229-34.

Ohnishi, J., J. Piesman, and A. M. de Silva. 2001. "Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks." *Proc Natl Acad Sci U S A*. 98 (2):670-75.

Oschmann, P., W. Dorndorf, C. Hornig, C. Schafer, H. J. Wellensiek, and K. W. Pflughaupt. 1998. "Stages and syndromes of neuroborreliosis." *J Neurol*. 245 (5):262-72.

Ouyang, Z., J. S. Blevins, and M. V. Norgard. 2008. "Transcriptional interplay among the regulators Rrp2, RpoN and RpoS in *Borrelia burgdorferi*." *Microbiology*. 154 (Pt 9):2641-58.

Ouyang, Z., R. K. Deka, and M. V. Norgard. 2011. "BosR (BB0647) controls the RpoN-RpoS regulatory pathway and virulence expression in *Borrelia burgdorferi* by a novel DNA-binding mechanism." *PLoS Pathog*. 7 (2):e1001272.

Ouyang, Z., M. Kumar, T. Kariu, S. Haq, M. Goldberg, U. Pal, and M. V. Norgard. 2009. "BosR (BB0647) governs virulence expression in *Borrelia burgdorferi*." *Mol Microbiol*. 74 (6):1331-43.

Pahl, A., U. Kuhlbrandt, K. Brune, M. Rollinghoff, and A. Gessner. 1999. "Quantitative detection of *Borrelia burgdorferi* by real-time PCR." *J Clin Microbiol*. 37 (6):1958-63.

Pal, U., X. Yang, M. Chen, L. K. Bockenstedt, J. F. Anderson, R. A. Flavell, M. V. Norgard, and E. Fikrig. 2004. "OspC facilitates *Borrelia burgdorferi* invasion of Ixodes scapularis salivary glands." *J Clin Invest*. 113 (2):220-30.

Paster, B. J., and F. E. Dewhirst. 2000. "Phylogenetic foundation of spirochetes." *J Mol Microbiol Biotechnol*. 2 (4):341-44.

Peavey, C. A., and R. S. Lane. 1995. "Transmission of *Borrelia burgdorferi* by Ixodes pacificus nymphs and reservoir competence of deer mice (Peromyscus maniculatus) infected by tick-bite." *J Parasitol*. 81 (2):175-78.

Philipp, M. T., and B. J. Johnson. 1994. "Animal models of Lyme disease: pathogenesis and immunoprophylaxis." *Trends Microbiol*. 2 (11):431-37.

Piesman, J., T. N. Mather, R. J. Sinsky, and A. Spielman. 1987. "Duration of tick attachment and *Borrelia burgdorferi* transmission." *J Clin Microbiol*. 25 (3):557-58.

Preac Mursic, V., E. Patsouris, B. Wilske, S. Reinhardt, B. Gross, and P. Mehraein. 1990. "Persistence of *Borrelia burgdorferi* and histopathological alterations in experimentally infected animals. A comparison with histopathological findings in human Lyme disease." *Infection*. 18 (6):332-41.

Radolf, J. D., M. J. Caimano, B. Stevenson, and L. T. Hu. 2012. "Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes." *Nat Rev Microbiol*. 10 (2):87-99.

Ribeiro, J. M., T. N. Mather, J. Piesman, and A. Spielman. 1987. "Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae)." *J Med Entomol*. 24 (2):201-05.

Rosa, P. A., K. Tilly, and P. E. Stewart. 2005. "The burgeoning molecular genetics of the Lyme disease spirochaete." *Nat Rev Microbiol*. 3 (2):129-43.

Samuels, D. S. 2011. "Gene regulation in *Borrelia burgdorferi*." *Annu Rev Microbiol*. 65:479-99.

Samuels, D. S., and J. D. Radolf. 2009. "Who is the BosR around here anyway?" *Mol Microbiol*. 74 (6):1295-99.

Schaible, U. E., S. Gay, C. Museteanu, M. D. Kramer, G. Zimmer, K. Eichmann, U. Museteanu, and M. M. Simon. 1990. "Lyme borreliosis in the severe combined immunodeficiency (scid) mouse manifests predominantly in the joints, heart, and liver." *Am J Pathol*. 137 (4):811-20.

Schaible, U. E., M. D. Kramer, C. W. Justus, C. Museteanu, and M. M. Simon. 1989. "Demonstration of antigen-specific T cells and histopathological alterations in mice experimentally inoculated with *Borrelia burgdorferi*." *Infect Immun*. 57 (1):41-47.

Schwan, T. G., and J. Piesman. 2000. "Temporal changes in outer surface proteins A and C of the lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice." *J Clin Microbiol*. 38 (1):382-88.

Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa. 1995. "Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding." *Proc Natl Acad Sci U S A*. 92 (7):2909-13.

Sonnesyn, S. W., J. C. Manivel, R. C. Johnson, and J. L. Goodman. 1993. "A guinea pig model for Lyme disease." *Infect Immun*. 61 (11):4777-84.

Spielman, A., C. M. Clifford, J. Piesman, and M. D. Corwin. 1979. "Human babesiosis on Nantucket Island, USA: description of the vector, Ixodes (Ixodes) dammini, n. sp. (Acarina: Ixodidae)." *J Med Entomol*. 15 (3):218-34.

Stanek, G., and M. Reiter. 2011. "The expanding Lyme Borrelia complex--clinical significance of genomic species?" *Clin Microbiol Infect*. 17 (4):487-93.

Steere, A. C. 1989. "Lyme disease." *N Engl J Med*. 321 (9):586-96.

Steere, A. C. 2001. "Lyme disease." *N Engl J Med*. 345 (2):115-25.

Steere, A. C. 2006. "Lyme borreliosis in 2005, 30 years after initial observations in Lyme Connecticut." *Wien Klin Wochenschr*. 118 (21-22):625-33.

Steere, A. C., N. H. Bartenhagen, J. E. Craft, G. J. Hutchinson, J. H. Newman, D. W. Rahn, L. H. Sigal, P. N. Spieler, K. S. Stenn, and S. E. Malawista. 1983. "The early clinical manifestations of Lyme disease." *Ann Intern Med*. 99 (1):76-82.

Steere, A. C., W. P. Batsford, M. Weinberg, J. Alexander, H. J. Berger, S. Wolfson, and S. E. Malawista. 1980. "Lyme carditis: cardiac abnormalities of Lyme disease." *Ann Intern Med*. 93 (1):8-16.

Steere, A. C., T. F. Broderick, and S. E. Malawista. 1978. "Erythema chronicum migrans and Lyme arthritis: epidemiologic evidence for a tick vector." *Am J Epidemiol*. 108 (4):312-21.

Steere, A. C., J. Coburn, and L. Glickstein. 2004. "The emergence of Lyme disease." *J Clin Invest*. 113 (8):1093-101.

Steere, A. C., R. L. Grodzicki, J. E. Craft, M. Shrestha, A. N. Kornblatt, and S. E. Malawista. 1984. "Recovery of Lyme disease spirochetes from patients." *Yale J Biol Med*. 57 (4):557-60.

Steere, A. C., R. E. Levin, P. J. Molloy, R. A. Kalish, J. H. Abraham, 3rd, N. Y. Liu, and C. H. Schmid. 1994. "Treatment of Lyme arthritis." *Arthritis Rheum*. 37 (6):878-88.

Steere, A. C., S. E. Malawista, D. R. Snydman, R. E. Shope, W. A. Andiman, M. R. Ross, and F. M. Steele. 1977. "Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities." *Arthritis Rheum*. 20 (1):7-17.

Steere, A. C., E. Taylor, G. L. McHugh, and E. L. Logigian. 1993. "The overdiagnosis of Lyme disease." *JAMA*. 269 (14):1812-16.

Stewart, P. E., R. Byram, D. Grimm, K. Tilly, and P. A. Rosa. 2005. "The plasmids of *Borrelia burgdorferi*: essential genetic elements of a pathogen." *Plasmid*. 53 (1):1-13.

Strother, K. O., E. Hodzic, S. W. Barthold, and A. M. de Silva. 2007. "Infection of mice with lyme disease spirochetes constitutively producing outer surface proteins a and B." *Infect Immun*. 75 (6):2786-94.

Tugwell, P., D. T. Dennis, A. Weinstein, G. Wells, B. Shea, G. Nichol, R. Hayward, R. Lightfoot, P. Baker, and A. C. Steere. 1997. "Laboratory evaluation in the diagnosis of Lyme disease." *Ann Intern Med*. 127 (12):1109-23.

Wallis, R. C., S. E. Brown, K. O. Kloter, and A. J. Main, Jr. 1978. "Erythema chronicum migrans and lyme arthritis: field study of ticks." *Am J Epidemiol*. 108 (4):322-27.

Wormser, G. P., R. Ramanathan, J. Nowakowski, D. McKenna, D. Holmgren, P. Visintainer, R. Dornbush, B. Singh, and R. B. Nadelman. 2003. "Duration of antibiotic therapy for early Lyme disease. A randomized, double-blind, placebo-controlled trial." *Ann Intern Med*. 138 (9):697-704.

Wormser, G. P., M. Schriefer, M. E. Aguero-Rosenfeld, A. Levin, A. C. Steere, R. B. Nadelman, J. Nowakowski, A. Marques, B. J. Johnson, and J. S. Dumler. 2013. "Single-tier testing with the C6 peptide ELISA kit compared with two-tier testing for Lyme disease." *Diagn Microbiol Infect Dis*. 75 (1):9-15.

Xu, H., M. J. Caimano, T. Lin, M. He, J. D. Radolf, S. J. Norris, F. Gherardini, A. J. Wolfe, and X. F. Yang. 2010. "Role of acetyl-phosphate in activation of the Rrp2-RpoN-RpoS pathway in *Borrelia burgdorferi*." *PLoS Pathog*. 6 (9):e1001104.

Xu, Q., K. McShan, and F. T. Liang. 2008. "Essential protective role attributed to the surface lipoproteins of *Borrelia burgdorferi* against innate defences." *Mol Microbiol*. 69 (1):15-29.

Xu, Q., Y. Shi, P. Dadhwal, and F. T. Liang. 2012. "RpoS regulates essential virulence factors remaining to be identified in *Borrelia burgdorferi*." *PLoS One*. 7 (12):e53212.

Yang, X. F., U. Pal, S. M. Alani, E. Fikrig and M. V. Norgard (2004). "Essential role for OspA/B in the life cycle of the Lyme disease spirochete." *J Exp Med 1*99(5): 641-648.

Yang, X. F., M. C. Lybecker, U. Pal, S. M. Alani, J. Blevins, A. T. Revel, D. S. Samuels, and M. V. Norgard. 2005. "Analysis of the ospC regulatory element controlled by the RpoN-RpoS regulatory pathway in *Borrelia burgdorferi*." *J Bacteriol*. 187 (14):4822-29.

Yoshinari, N. H., A. C. Steere, and W. Cossermelli. 1989. "[A review of Lyme disease]." *AMB Rev Assoc Med Bras*. 35 (1):34-38.

CHAPTER 2
DIRECT ROLE OF *BORRELIA BURGDORFERI* OXIDATIVE STRESS REGULATOR IN DOWNREGULATION OF OUTER SURFACE PROTEIN A [1]

Introduction

The enzootic infection cycle of *B. burgdorferi* has two phases: the tick phase and the mammalian phase. Each phase requires distinct physiological adaptations. Rapid adaptation in response to changing environmental signals by differential gene expression is the key survival strategy used by *B. burgdorferi* (Samuels 2011, Radolf et al. 2012). Most of the differentially expressed genes encode for the outer surface lipoproteins (Fraser et al. 1997). Thus, different outer surface proteins (osp) are expressed in the tick and mammalian phase (Barbour, Tessier, and Hayes 1984, Pollack, Telford, and Spielman 1993). A prototypical example of the phase differential expression of the surface lipoproteins can be seen in *B. burgdorferi* as it prepares to infect the mammalian host. This involves downregulation of the dominant tick phase osp, OspA, and upregulation of the mammalian phase osp, OspC (Schwan et al. 1995, Schwan and Piesman 2000).

The maintenance of coordinated outer surface lipoproteins requires intricate regulatory mechanisms. *B. burgdorferi* has a small genome, which encodes very few transcription regulators. Its only two known alternate sigma factors, RpoN (σ^N) and RpoS (σ^S), form a key regulatory cascade. In this regulatory pathway, *rpoS* depends on RpoN for transcription activation (Studholme and Buck 2000). Recent studies have added Rrp2 as the response regulator protein controlling activation of RpoN (Yang et al., 2003).

[1] A section (all materials pertaining to figures 2.4, 2.5, 2.6, & 2.7) of this chapter previously appeared as part of publication: (Wang et al. 2013); it is reprinted with permission of wiley & sons. See the permission letter in Appendix A & B

The most recent addition to the unique molecular regulators of *B. burgdorferi* is BB0647. It was initially known as borrelial Fur (Ferric uptake response) regulator protein, and is currently more well accepted as BosR (Borrelia oxidative stress response Regulator,) due to its shared homology with PerR protein (oxidative stress response repressor in *Bacillus subtillis*) (Hantke 1981, Bagg and Neilands 1987).

Boylan et al. showed BosR to be a transcriptional activator in 2003. They determined that BosR binds *in-vitro* to the upstream region of *dps*/*nap*A and activates it.[2] Furthermore, a 2009 study indicated that BosR may function as a universal repressor and activator in *B. burgdorferi* (Ouyang et al. 2009). The same study also showed that BosR is essential for causing infection in the mammalian host, but not for persistence in the *Ixodes* tick vector (Ouyang et al. 2009). While Ouyang et al. showed that loss of *bosR* also resulted in loss of expression of OspC and DbpA, a seminal study by J. A Hyde et al. showed that this loss was due to the absence of RpoS (Hyde et al. 2009, Ouyang et al. 2009). Thus, the role of BosR in RpoN-RpoS pathway was established as a transcriptional activator, with a more recent 2011 study showing that BosR binds directly to the upstream regulatory sequence of *rpoS* to effect its activation (Ouyang, Deka, and Norgard 2011). RpoS is required for OspC up- and OspA downregulation, respectively (Caimano et al. 2005, Yang et al. 2005); thus, BosR is considered a regulator protein that indirectly controls the crossregulation of OspC and OspA in *B. burgdorferi*.

However, the studies before identification of BosR indicated that an unidentified 23kDa repressor protein directly binds the *ospA* promoter, and therefore, may govern the OspA downregulation (Margolis and Samuels 1995). Additionally, some recent studies have predicted indirect RpoS mediated repression of *ospA* transcription via "a trans-acting DNA binding

[2] NapA protein is known to protect *B. burgdorferi* from reactive oxygen species (Boylan, Posey, and Gherardini 2003).

protein," (Caimano et al. 2005, Caimano et al. 2007). Furthermore, recent studies have shown that BosR is a 20 kDa DNA-binding protein required for the activation of the RpoS (Caimano et al. 2007), which has prompted investigation of BosR as a plausible repressor protein for OspA. The present study investigates the possible BosR mediated direct repression of OspA. Also, due to known similar expression pattern in enzootic cycle of *B. burgdorferi*, OspD is also investigated together with OspA repression in this study (Stewart et al. 2008).

The Outer surface proteins (Osps) A and B are encoded by a 2-gene operon (Fingerle et al. 2007). The expression of the *ospA* operon is driven by a (RpoD) σ^{70}-dependent promoter (Crother et al. 2004). *B. burgdorferi* cannot turn off this housekeeping σ factor in pursuit of the rapid turnoff of the *ospA* operon. To do so would likely be lethal to *B. burgdorferi*. A 1999 publication by Sohaskey et al. suggested involvement of the T-rich region immediately upstream of *ospA* promoter in regulation of *ospA* (Figure 2.1). A 2010 study in our lab identified two regulatory elements: *cisI* and *cisII*, flanking the *ospA* promoter (Figure 2.1). In this study, both these sequences were demonstrated to be required for *ospA* repression during the mammalian infection. Thus, these sequences were demonstrated to equip *B. burgdorferi* with a molecular mechanism for rapid adaptation within the distinct environments during its enzootic cycle (Barbour et al. 1986). The successful identification of two regulatory sequences, namely *cisI* and *cisII*, indicates the existence of a repressor(s), which should bind the two elements and shut off the *ospA* expression during the murine infection (Raetz and Whitfield 2002).

The current study first shows that BosR bound both *cisI* and *cisII*, and then demonstrates that increased BosR expression executed a complete switch off of OspA on the borrelial surface. The current study interestingly demonstrates that one of the two putative BosR-binding sites associated with the *ospA* operon is completely included within the previously identified *cisII*

regulatory sequence (Labandeira-Rey, Seshu, and Skare 2003), while the second is partially overlapped with the -10 region of the promoter (Grimm et al. 2004).

As an important regulator, BosR has a demonstrated function: it binds the *rpoS* promoter region and positively regulates the alternative σ factor, which, in turn, upregulates a number of osps, including OspC and DbpBA (Sears et al. 1991, Wang et al. 2013, Ouyang et al. 2009). Although in vitro cultured *B. burgdorferi* doesn't produce BosR during its early growth phase, once grown to late log phase, the pathogen dramatically upregulates the regulator. However, this dramatic BosR upregulation has been unable to associate with OspA downregulation, seriously challenging the notion that BosR, in addition to the identified function described above, may also function as a repressor of the *ospA* operon. The current study demonstrated that BosR binds both previously identified regulatory elements of the *ospA* operon. Furthermore, the study showed that increasing expression of BosR can completely turn the operon off when other major osps, such as OspC, DbpA and DbpB, were constitutively expressed. Thus, the current study adds a new important role of BosR as a repressor protein of *ospA* operon.

Material and methods

Growth conditions and harvest of *B. burgdorferi*

Borrelia burgdorferi was grown in 40 ml of Barbour-Stoenner-Kelly H (BSK) complete media in 50 ml Falcon tubes at 33°C (5% CO2) to about 10^8cell/ml. Cells were then harvested by centrifugation at 3711x g for 20 min at 4°C. The bacterial pellet was washed three times by suspended in 40 ml cold HN (10 mM HEPES, 50 mM NaCl, pH 8.0) and centrifuged. Supernatant were removed, pellet was further used to SDS-PAGE or 2-DE analysis or stored at -80˚C for future use.

Strains used for this study

The *B. burgdorferi* strains constructed previously from *B. burgdorferi* type strain B31 by Ouyang et al., 2009, and kindly provided by M.V. Norgard (University of Texas Southwestern Medical Center) were used (Ouyang et al. 2009). Mutant strains used included *bosR* lacking *B. burgdorferi* B31, and *bosR* complemented strain.

Construction of pIBM-*rpoS*$_{in}$, pME22-*bosR'* and pME22-*C'B'A'-bosR'*

As diagrammed in figure.3.2 A , to construct pIBM-*bosR*$_{in}$, a 554-bp DNA fragment covering the entire coding region of the *bosR* gene was amplified by using primers P1F and P1R (Table 3.1) and DNA template extracted from *B. burgdorferi* B31. The resultant PCR product was purified, digested with NcoI and XhoI, repurified and cloned into pIBM, which was created in a previous study (Xu et al. 2012), and pre-digested with the same enzymes.

To construct *pME22-bosR'*, a plasmid that conferred constitutive BosR expression, a 597-bp DNA fragment containing the entire coding sequence of the *bosR* gene was amplified using primers P2F and P2R (Table 2.1) and DNA template purified from *B. burgdorferi* B31. The resultant amplicon was purified, digested with BspHI and BamHI, repurified and cloned into pME22, which was created in an earlier study(Shi et al. 2008), and pre-digested with NcoI and BamHI. The construction process is summarized in figure. 2.2 B.

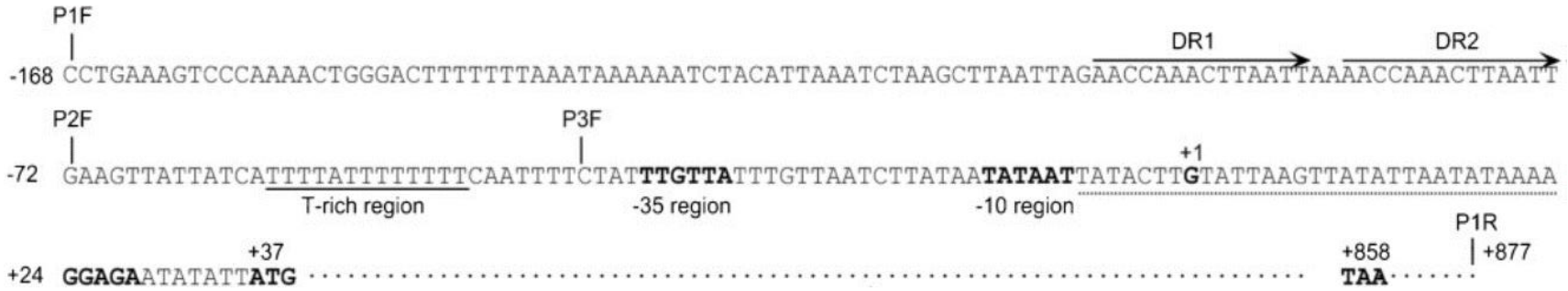

Figure 2.1 Potential regulatory sequences upstream of the coding region of the *ospA* operon, including a pair of direct repeats, DR1 and DR2 (labelled with long arrows), a T-rich region (underlined with a solid line), and a sequence between the”10 region and the start codon ATG (underlined with a dotted line). The ”35 and ”10 regions, putative ribosome-binding site (RBS), start codon ATG, and stop codon TAA (all in bold type) are indicated. The +1 marks thepreviously identified transcriptional initiation site (Jonsson et al., 1992). Picture from (Xu, McShan, and Liang 2010)

To construct pME22-*C'B'A'-bosR'*, a plasmid that was able to provide constitutive BosR, OspC, DbpA and DbpB expression simultaneously, two plasmids, pME22-*bosR'* and pME22-*C'B'A'* were used. The plasmid pME22-*C'B'A'* was constructed in an earlier study (Grimm et al. 2004). A 917-bp DNA fragment covering the promoterless *bosR* gene fused with the *flaB* promoter was amplified from pME22-*bosR* with use of primers P3F and P3R (Figure 2.3C; Table 2.1). A 10,059-bp DNA fragment harboring the promoterless *ospC*, *dbpB* and *dbpA* fused with the same *flaB* promoter was amplified from pME22-*C'B'A'* using primers P4F and P4R (Figure 2.3C; Table 2.1). The resultant PCR products were pooled, purified, digested with NheI and PstI, and ligated to complete construction of pME22-*C'B'A'-bosR'*.

Table 2.1: Primers used in this study

Primer	Sequences (5' to 3') [a]
P1F	AAATTCATGCCATGGACGACAACATAATAGACG
P1R	TTTCCGCTCGAGTCATAAAGTGATTTCCTTGTTCTC
P2F	AAATCATGAACGACAACATAATAGACGTACATTCC
P2R	AAAGGATCCACCAGTATTAAGAGTAATAAGAATATAAG
P3F	AAAGCTAGCAGGAAACAGCTATGACCATGATTAC
P3R	TGCCAAGCTTGCATGCCTGCAG
P4F	GACCTGCAGGCATGCAAGCTTGG
P4R	AAAGCTAGCGTCTTGATTATCGGGCGAAGAG
P5F	TAATTCCATATGAACGACAACATAATAGACG
P5R	TTTCCGCTCGAGTAAAGTGATTTCCTTGTTCTC

[a]The underlined sequences are restriction enzyme sites: a BamHI sit (P2R), a BspHI site (P2F), a NdeI site (P5F), a NcoI site (P1F), NheI sites (P3F and P4R), PstI sites (P3R and P4F), and XhoI sites (P1R and P5R).

Generation of transformants

The *rpoS* mutant, *ΔrpoS*, which was generated in our previous study (Grimm et al. 2004, Xu et al. 2012), was grown to late logarithmic (log) phase in Barbour-Stoenner-Kelly H (BSK-H) complete medium (Sigma). Spirochetes were harvested from approximately 40 ml of culture and transformed with pIBM-*bosR*$_{in}$, pME22-*bosR'* or pME22-*C'B'A'-bosR'* as described previously (Xu et al. 2005). Transformants were identified by PCR using a primer pair specific for either streptomycin or kanamycin cassette and their plasmid content was analyzed as described previously (Xu et al. 2005).

Growth rate estimation

The spirochete culture was grown at 33°C to late log phase (approximately 10^8 cells/ml) in BSK-H complete medium and diluted to 10^5 cells/ml with the medium. A total of fifteen 1.3-ml aliquots were prepared and IPTG was then added to final concentrations at 0, 0.02, 0.05, 0.10 and 0.20 mM. Each inducer concentration was in triplicate. All aliquots were incubated at 33°C and cell numbers were counted daily for 10 days. Either the parental clone 13A or the transformant *ΔrpoS/ΔrpoS* was used as a control.

Host adaption of spirochaetes in DMCs

Mammalian host adapted spirochetes were prepared using dialysis membrane chambers (DMCs) implanted intraperitonealy in Sprague Dawley as described by Akins previously (Akins et al. 1998). The rats used were were 6-8 weeks old and obtained from Division of Laboratory Animal Medicine at Louisiana State University, Baton Rouge. All animal procedures described were approved by the Institutional Animal Care and Use Committee at Louisiana State University.

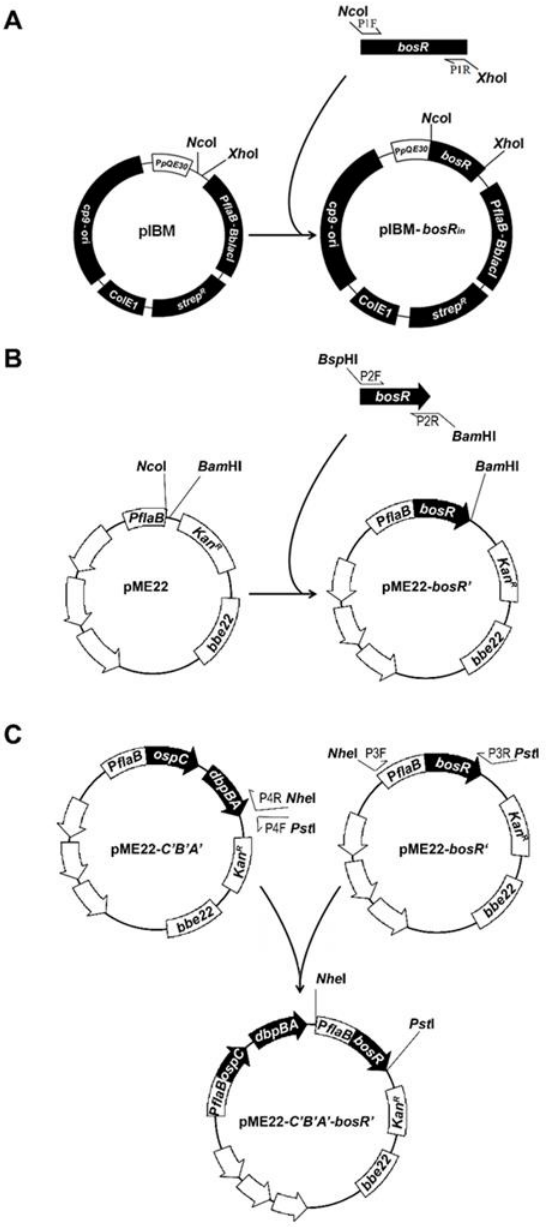

Figure 2.2. Construction of pIBM-*bosRin*, pME22-*bosR'* and pME22-*C'B'A'-bosR'*. (A) To construct pIBM-bosRin, a 554-bp DNA fragment covering the entire coding region of the *bosR* gene was amplified. The resultant PCR product was cloned into pIBM. (B) To construct pME22-*bosR'*, a 597-bp DNA fragment containing the entire coding sequence of the *bosR* gene was amplified and cloned into pME22. (C) To construct pME22-*C'B'A-bosR'*, a 917-bp DNA fragment covering the promoterless *bosR* gene fused with the *flaB* promoter was amplified from pME22-*bosR'*. A 10,059-bp DNA fragment harboring the promoterless *ospC, dbpB* and *dbpA* fused with the *flaB* promoter was amplified from pME22-*C'B'A'*. The resultant PCR products were ligated to complete construction of pME22-*C'B'A'-bosR'*.

The Spectra/Por® 6 Standard Grade Regenerated Cellulose dialysis membrane with molecular weight cut-off of 8 kDa (Spectrum Laboratories, Rancho Dominguez, CA) was used for making the dialysis membrane chambers. The dialysis membrane was treated with 5 mM EDTA to remove any heavy metal impurities and was subsequently autoclaved. The dialysis membrane chambers were filled with 5 ml of 10^3 spirochaetes per ml of spirochete containing BSK-H complete media. The spirochetes were first cultured to late log phase and then diluted to 10^3/ ml. Using standard aseptic techniques, sterile DMC was implanted into the peritoneum of rat, after 4 weeks of adaptation in DMCs, approximately 5×10^7 of the *bosR* mutant and 2×10^7 of the complemented *bosR* mutant were recovered and subjected to immunoblot and q RT-PCR analyses as described below.

Immunoblot and Coomassie analysis

Spirochetes were harvested from culture (both DMC & BSK-H) and pelleted by centrifugation at 5,000×g at 4ºC. The spirochetes were then suspended in Laemmli buffer (Laemmli 1970) at spirochetes to 2×10^5 spirochaete cells/µl. The sample was incubated at 100°C for 10 min. SDS-PAGE was run using 5 µl of each sample. The SDS run samples were transferred to nitrocellulose membrane and were incubated with a mixture of FlaB mAb and mouse anti-BosR sera or OspC mAb alone as described in our previous study (Xu et al. 2006) or pooled sera (antibodies for FlaB, BosR, OspC, OspA, or OspD) from mice infected with *B. burgdorferi* B31 strain via tick infestation. Immunoblot reaction was performed using goat α-mouse IgG-horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories) at a 1:10000 dilution and developed using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) and captured on X-ray films. For protein analysis, proteins separated on SDS-PAGE gels were directly stained with Coomassie Brilliant Blue G-250 (Amresco, Inc., Solon, and OH).

Quantitative RT-PCR and PCR

Total RNA was extracted from harvested spirochaetes using TRIZOL reagent (Life Technologies) and treated with RNase-free DNase I (Roche) for complete digestion of possible DNA contamination. In order to achieve highest purity RNA preparation was purified using RNeasy mini spin column (Qiagen) following the manufacturers' instructions.

For q RT-PCR analysis, RNA was first reverse transcribed into cDNA using Superscript™ III reverse transcriptase (Life Technologies) and random hexamers. A mock reaction without reverse transcription was also performed to ensure that preparation is DNA free. A standard curve was generated by using a 10-fold dilutions of genomic DNA from known concentrations *B. burgdorferi* and a linear standard curve was achieved. These standards were assayed in triplicates to generate a linear calculation curve, which was used to convert the threshold cycle (Ct) values into gene copy numbers of the test samples. Quantitative PCR was performed in 96-well plates using SYBR® green PCR master mix (Applied Biosystems) and primers targeting specific sequences (Table 2.1) using ABI 9500 thermal cycler and negative control in duplicates were also added to the plate (Grimm et al. 2004), (Liang et al. 2004).

Expression and purification of recombinant BosR

The entire *bosR*-coding region was amplified from genomic DNA of *B. burgdorferi* B31 using primers P5F and P5R (Table 2.1). Resultant PCR product was digested, purified and cloned into pET-23a vector (EMD Chemicals Inc., Darmstadt, Germany), generating a construct that contained the *bosR*-coding region fused with a C-terminal His_6 tag. One Shot® BL21(DE3)pLysS Chemically Competent *E. coli* cells (Life Technologies, Grand Island, NY) were transformed with the construct and induced with 1.0 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, MO).

Recombinant BosR was affinity-purified using HiTrap Chelating HP following the manufacturer's instruction (GE Healthcare Bio- Sciences, Pittsburgh, PA). Protein concentration were measured using Quick Start™ Bradford Dye Reagent following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

Table 2.2: Oligos used for quantitative PCR assay

Name	**Sequence (5' – 3')**
flab + 441 Forward	AGCTGAAGAGCTTGGAATGC
flab + 543 Reverse	TTGGTTTGCTCCAACATGAA
bosR + 41 Forward	AAGTCGGCATTACAAACGAT
bosR +153 Reverse	TTTTGGGTTTGATGCTATGTAT
ospA +166 Forward	GCAACAGTAGACAAGCTTGAGC
ospA +295 Reverse	GTGTGGTTTGACCTAGATCGTCA
ospC +12 Forward	TACATTAAGTGCAATATTAATGACT
ospC +125 Reverse	AGATTAGGCCCTTTAACAGA
rpoS +108 Forward	AGGCAATGCAAAAGCAAAAA
rpoS +232 Reverse	ATCCCAAGTTGCCTTCTTGA

Electrophoretic mobility shift assay

To date, BosR has been shown to recognize sequences upstream of at least three other genes, *rpoS*, *bosR* itself, and the gene encoding neutrophil activating protein A (NapA) (Liang et al. 2002, Katona et al. 2004, Ouyang, Deka, and Norgard 2011). Primers (Table 2.2) spanning 330 bps upstream and 20 bps downstream of the start of the *ospA* gene and 231 bps upstream and 68 bps downstream of the start of the *ospD* gene were designed (Table 2.2) and single band PCR

products were used as probes for EMSA. Primers targeting upstream region of *B. burgdorferi* genes *ospA, ospD, rpoS, bicA, bosR,* and *ospC* were designed and targeted regions were amplified from *B. burgdorferi* type strain B31 using iProof DNA polymerase (Bio-Rad). Primers labeled with biotin at 5′-end were used to generate biotinylated PCR fragments, which were then purified on a 1.5% SeaPlaque (Lonza) agarose gel using the QIAquick gel extraction kit (Qiagen) and DNA concentration was determined by absorbance at 260 nm and used as a probe in EMSA. Each strand of the probes *CisI, CisII,* and IRs (Figure 2.3) was synthesized, respectively, by Integrated DNA Technologies, Inc. (Coralville, Iowa). One strand of each complementary pair was incorporated with 5' Digoxigenin during synthesis. Complementary strands were allowed to anneal to form a double-stranded probe.

Both the His6-tagged and the tag-free BosR were affinity-purified from *E. coli* as a soluble recombinant protein and found to be active, His6-tagged BosR was used for EMSA assays.

For the density dependent competiton assay (Figure 2.6), the probes (1 nM) were incubated at room temperature for 10 min with 5-, 10-, 20-, 40-, 80-, 160-, 320- or 640-fold molar excess of BosR dimer in 20 µl reactions containing 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM DTT, 5% glycerol, 50 µg /ml salmon sperm DNA, and 100 µg/ ml of BSA. Annealing pairs of complementary oligos were used as double-stranded competitor oligos (Table 2.2) in equi-molar ratio. For competitor EMSA, BosR (320 nM of dimer) was incubated with 2-, 4-, 8-, or 16-fold molar excess of a competitor prior to incubation with a probe (1 nM) and the binding reactions were separated by electrophoresis on 6% polyacrylamide gels prepared in 0.5× TBE (Bio-Rad; 45 mM Tris, pH 8.3, 45 mM boric acid, 1 mM EDTA) and then transferred to nylon membranes. The biotin signal was detected using Light Shift chemiluminescent EMSA kit (Thermo Scientific) following the manufacturer's instructions.

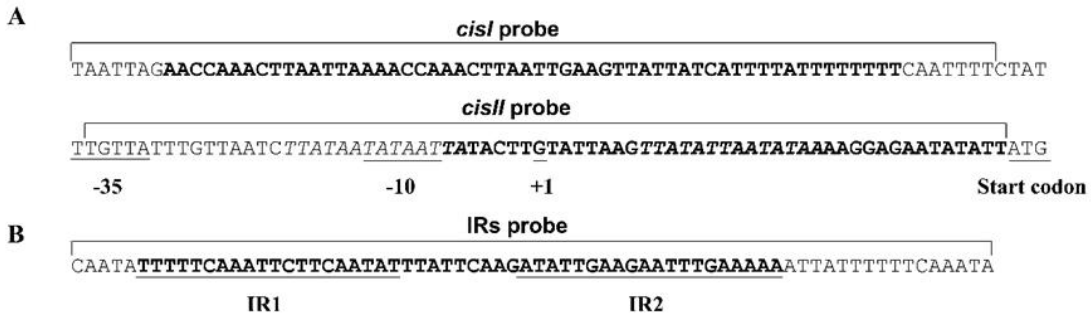

Figure 2.3. Diagram of *cisI*, *cisII* and putative BosR-binding sites of the *ospA* operon and three probe sequences used in the study. (A) The location of *cisI*, *cisII* and putative BosR-binding sites and the sequences of *cisI* and *cisII* probes. The regulatory sequences upstream of the coding region of the *ospA* operon include *cisI* and *cisII* (both in bold) and two putative BosR-binding sites (italic). One putative site is overlapped with the -10 region and the other is contained within *cisII*. The -35 and -10 regions of the promoter, transcriptional initiation site, and start codon ATG are underlined. The sequences of *cisI* and *cisII* probes are marked with brackets. (B) The IRs were identified as the *ospC* operator in a previous study (Xu, McShan, and Liang 2007), and used as a control probe. The sequence of the probe is marked with a bracket

The binding assay (Figure 2.8) reaction volume was set in a 20 µl, in which 1.0 nM DNA probe was allowed to bind 400 nM of recombinant BosR at room temperature for 10 min. The binding buffer contained 50µg/ml salmon sperm DNA, 100µg/ml BSA, 1.0 mM DTT, 50 mM KCl, 10 mM Tris (pH, 7.5) and 5% glycerol. In competition assay, 10 nM DNA competitor was first added to a reaction volume of 20 µl and allowed to interact with 400 nM of recombinant BosR at room temperature for 10 min before 1.0 nM DNA probe was supplemented and incubated for an additional 10 min. Resultant mixtures were separated by electrophoresis on 10% polyacrylamide gels prepared with 0.5× TBE buffer. Separated DNA was transferred to a nylon membrane and probed using DIG High Prime DNA Labeling and Detection Starter Kit I as per the manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

Table 2.3 Primers used to generate probes and competitive oligos for Electrophoretic mobility shift assay (EMSA)

Name	**5'-Label**	**Sequence (5' – 3')**
53% G+C For	Biotin	CTATTCTGGACTACCTGCTGA
53% G+C Rev		CAATGTCTTTGCGTTTCGCTT
ospD -231 For	Biotin	AGCATCATTAAACATCCTTTCAATA CTCA
ospD +68 Rev	Biotin	ATCATGAACACAAGATATTGAGAGCAAT
Rnd Rev		CCCAGTCATCTTCATTGACTCATACCC
Fur For		GGGAAATGATAATAATTATCATTTGGG
Fur Rev		CCCAAATGATAATTATTATCATTTCCC
Per For		GGGAATTTATAATTATTATAAATTGGG
Per Rev		CCCAATTTATAATAATTATAAATTCCC
DR For		GGGTAAATTAAATTAAATTAAATGGG
DR Rev		CCCATTTAATTTAATTTAATTTACCC
RndAT Rev		CCCTTTATTTTAAATATATTATACCCTA
Per/7-0-7 For		GGGAATTTATAATATTATAAATTGGG
Per/7-1-7 Rev		CCCAATTTATAATATTATAAATTCCC
Per/7-2-7 For		GGGAATTTATAATTTATTATAAATTGGG
Per/7-2-7 Rev		CCCAATTTATAATAAATTATAAATTCCC
Rnd For		GGGTATGAGTCAATGAAGATGACTGGG
RndAT For		GGGTATAATATATTTAAAATAAAGGGTA
ospA -330 For	Biotin	ATCAAGACAAACATTGCTGCTTTAA
ospA +20 Rev	Biotin	CCCAATAAATATTTTTTCATAATATATTCTCCTT

Table 2.3 Continued

Name	**5'-Label**	**Sequence (5' – 3')**
rpoS -277 For	Biotin	CTTGTGTTCTCTTACTGATTTTAAATATATGTTT
rpoS +60 Rev	Biotin	TGCTAAACGGAGGCCAAGTA
bosR -183 For	Biotin	TGTCGTTCATATGATTATACCTTTTTTGT
bosR +10 Rev	Biotin	CTGAATTCAAAAATAAAAAATTTAATTTTTTATACT
bicA -170 For	Biotin	TCTTCTTTTGTATCTATTTTATGCATT GT
bicA +159 Rev	Biotin	AGTTTTTTTGTGAATAACAAAGAAATTGGT
Rnd Rev	FAM	CCCAGTCATCTTCATTGACTCATACCC
RndAT Rev	FAM	CCCTTTATTTTAAATATATTATACCC
ospA -60 For	FAM	TAATCTTATAATATAATTATACTT
ospA -37 Rev		AAGTATAATTATATTATAAGATTA
ospD -85 For	FAM	ATAATTGATATTAAAATATAATTGAT
ospD -60 Rev		ATCAATTATATTTTAATATCAATTAT
ospA -330 For	FAM	ATCAAGACAAACATTGCTGCTTTAA
ospA +20 Rev	VIC	TTCCCAATAAATATTTTTTCATAATATATTCTCCTT

Results

Repression of OspA and OspD in mammals requires BosR

To determine the OspA repression in BosR lacking *B. burgdorferi*, the *bosR* mutant and the isogenic complemented *bosR* mutant were cultivated in the DMCs implanted within the rat peritoneal cavity. The *bosR* mutant did not express OspC and several other lipoproteins that are expressed in the mammalian host, but the levels of these proteins were restored in the

complemented *bosR* mutant (Figure 2.4). Also OspA and OspD protein levels were different for the two variants ,while the *bosR* complement repressed OspA and OspD expression when adapted in DMC just like its parental B31 background (Akins et al. 1998, Brooks et al. 2003) the *bosR* mutant failed to do so (Figure. 2.4).

Quantification of the immunoblot bands showed that the relative OspA and OspD protein levels were 3.8-fold and 3.5-fold higher, respectively, in the *bosR* mutant than in the complemented *bosR* mutant (after normalization against the FlaB protein level) (Figure 2.5A).

Quantitative reverse transcription-polymerase chain reaction (q RT-PCR) was performed to determine whether these differences at the protein levels could be observed at mRNA. After normalization against the *flaB* mRNA level, the relative *ospA* and *ospD* mRNA levels were 2.7-fold and 5.5-fold higher, respectively, in the *bosR* mutant than in the complemented *bosR* mutant (Figure 2.5B).

The data above clearly shows that absence of BosR causes significant increase in the *ospA* and *ospD* mRNA levels, which may be responsible for increase in the OspA and OspD protein levels. The experimental results shown here suggest that OspA and OspD fail to be repressed in absence of BosR. As absence of BosR causes absence of RpoS, these results are consistent with previous studies (Hyde et al. 2009, Ouyang et al. 2009, Caimano et al. 2005).

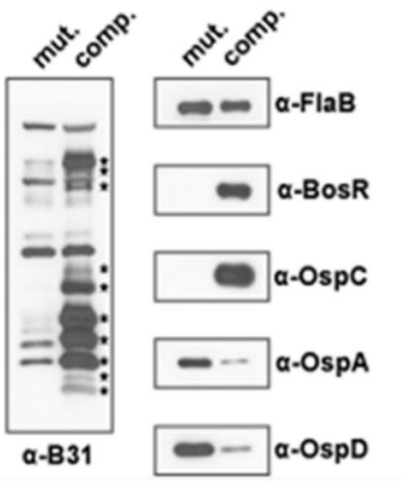

Figure 2.4. OspA and OspD repression in DMC requires BosR. The bosR mutant (mut.) and the complemented bosR mutant (comp.) were cultivated for 4 weeks in DMCs implanted in rat peritoneal cavities and immunoblot analyses were performed using pooled sera from mice infected with *B. burgdorferi* type strain B31 through tick infestation (α-B31) as well as antibodies specific to FlaB, BosR, OspC, OspA, and OspD.

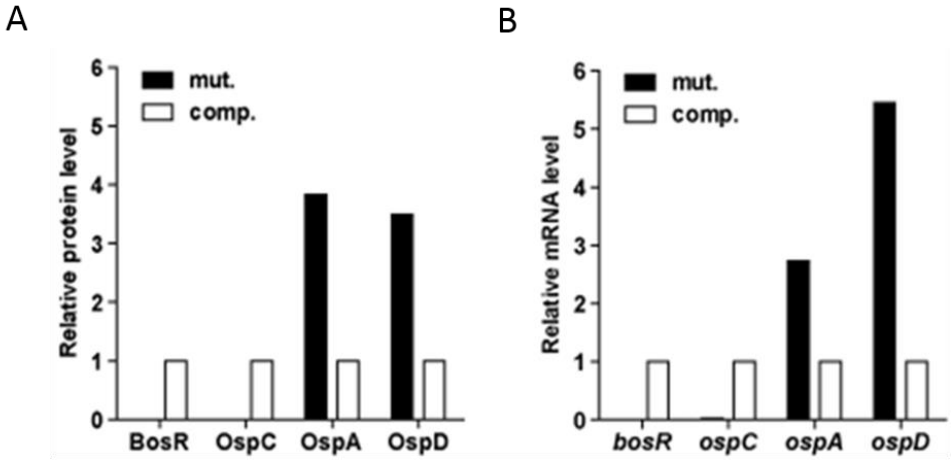

Figure 2.5: BosR dependent down regulation of OspA and OspD DMC: The bosR mutant (mut.) and the complemented *bosR* mutant (comp.) were cultivated for 4 weeks in DMCs implanted in rat peritoneal cavities and then subjected to protein and RNA analyses. (A) Relative protein levels of BosR, OspC, OspA, and OspD (after normalization against the FlaB level) in the *bosR* mutant as compared with those in the complemented bosR mutant. Data above is based on quantification of the immunoblot results shown in figure 2.4. (B) The mRNA levels of *bosR*, *ospC*, *ospA*, and *ospD* (after normalization against the *flaB* mRNA level sample) in the *bosR* mutant as relative to their respective levels in the complemented *bosR* mutant. Data were based on triplicate qRT-PCR analysis of the RNA samples obtained from the same DMC-adapted bacterial samples that were subjected to immunoblot analyses in Figure 2.4.

The above results indicate indirect role of BosR in downregulation of OspA and OspD. However, BosR homology to family of transcriptional regulators (FuR) known to function as repressors (Cullen, Haake, and Adler 2004, Fung et al. 1994) and more common role of sigma factors like RpoS in transcription activation suggest an alternative explanation for the increased *ospA* and *ospD* mRNA levels, which is, BosR may be repressing the *ospA* and *ospD* transcription direclty by interacting with the promoter regions .

BosR binds directly to sequences upstream of the *ospA* operon and the *ospD* gene

BosR-binding to P_{rpoS}, P_{bosR}, and P_{bicA} was compared to P_{ospAB} and P_{ospD} binding as to date, BosR has been shown to recognize sequences upstream of at least three other genes, *rpoS*, *bosR* itself, and the gene `encoding neutrophil activating protein A (NapA) now called BicA, Borrelia iron- and copper-binding protein A (Liang et al. 2002, Katona et al. 2004, Ouyang, Deka, and Norgard 2011). Sequences upstream of the ospC gene (as it is RpoS regulated) and two unrelated genes from Ehrlichia chaffeensis (ech193 and ech818) were included as negative controls in this experiment. Weaker but nonetheless dose-dependent BosR binding to P_{ospC}, P_{ech193}, and P_{ech818} was detected. As BosR did not bind to the synthetic probe that was 53% G+C probe (P_{G+C}) while it did bind to all other probes in this study (which were AT rich) the minimal BosR binding to P_{ospC}, $P_{ech193,}$ and P_{ech818} could be attributed to propensity of BosR to bind AT- rich sequences (Figure 2.6A). Thus conclusively to the EMSA results it can be stated that despite having a propensity for AT-rich sequences, BosR binds to sequences upstream of the *ospA* operon and the *ospD* gene, much more strongly than to the upstream of the *ospC* gene.

The EMSA for dose dependent BosR binding to probes based upon the upstream sequences of *ospA* operon and the *ospD* gene (PospAB and PospD) demonstrated that BosR readily binds to these regulatory regions (Figure 2.6 A, B & C).

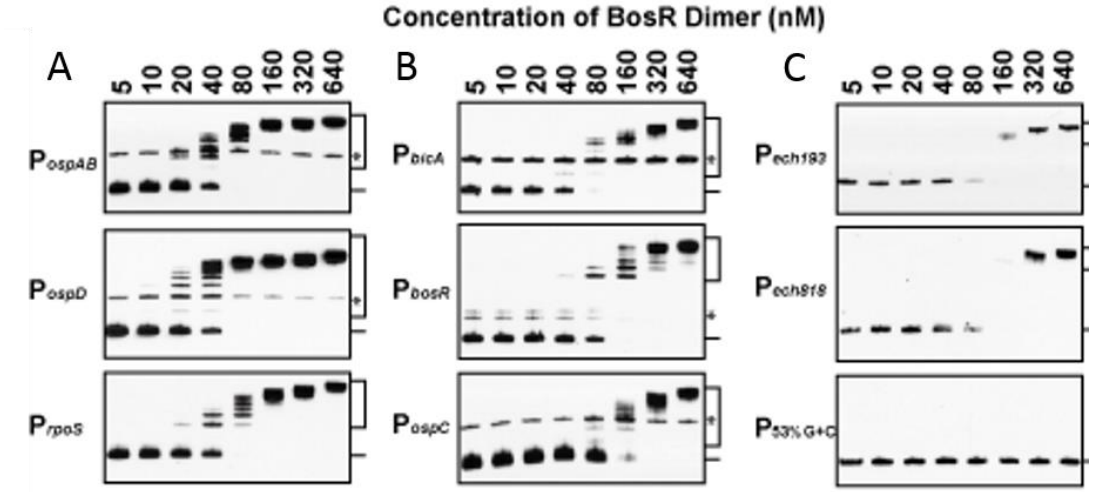

Figure 2.6. BosR directly binds to sequences upstream of the *ospA* and the *ospD* genes. BosR binding to P_{ospAB}, P_{ospD}, P_{rpoS}, P_{bicA}, P_{bosR}, P_{ospC}, P_{ech193}, P_{ech818}, and $P_{53\%G+C}$ was analysed by EMSA. Probes (1 nM) were first incubated with BosR (5–640 nM of dimer) and then separated on 6% polyacrylamide gels in 0.5× TBE.

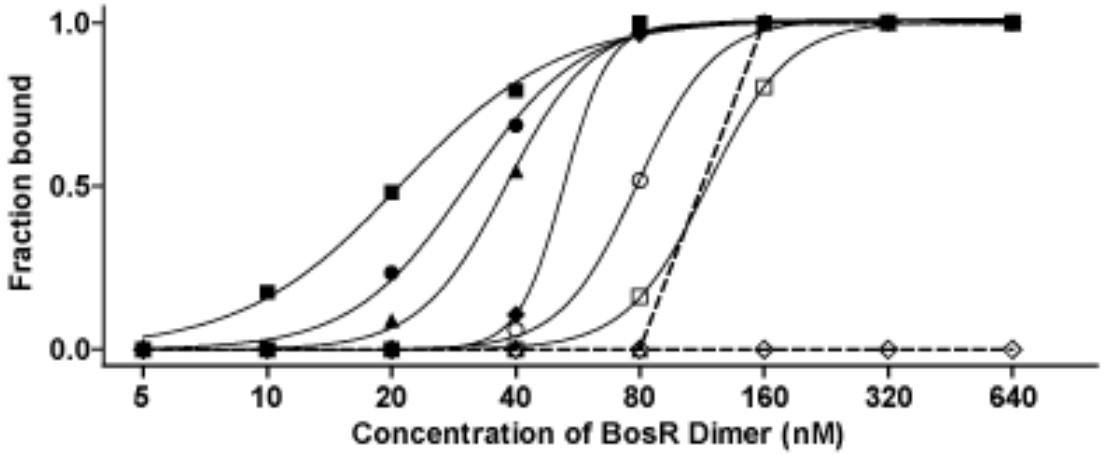

Figure 2.7. Relative binding of BosR to P_{ospA} and P_{ospD}: BosR binding curves for P_{ospAB} (closed circle), P_{ospD} (closed square), P_{rpoS} (closed triangle), P_{bicA} (closed diamond), P_{bosR} (open circle), P_{ospC} (open square), P_{ech193} or P_{ech818} (open triangle), and $P_{53\%G+C}$ (open diamond) were generated based on quantification of the EMSA data shown in Figure 2.6. Fraction of probe bound by BosR in each reaction was calculated by dividing the intensity of all shifted bands (indicated with a bracket) with the total intensity of both the shifted bands and the unbound band (indicated with a line). In some probes, there were minor isomers (indicated with asterisks) that migrated slower and were often not recognized by BosR. These isomers were excluded in the quantitative analysis. For clarity, curves for the *B. burgdorferi* probes were indicated with solid lines, and curves for the non- *B. burgdorferi* probes were indicated with dashed lines.

The *ospA* regulatory sequences, *cisI* and *cisII*, bind BosR, albeit *cisII* shows much stronger binding

Our previous study identified two regulatory sequences, one located upstream of the *ospA* promoter, *cisI*, and the second between the promoter and the translational start codon, *cisII*, regulating the downregulation of *ospA* expression as *B. burgdorferi* prepares to infect the mammalian host (Xu, McShan, and Liang 2010). Also the above results (Figure 2.6) have indicated the existence of two putative BosR-binding sites associated with the *ospA* locus. One of the sites is completely included within *cisII* and the second covers the entire -10 region of the *ospA* promoter and the first two base pairs of *cisII* (Figure 2.1 & 2.3). As the second site of the 14-bp putative BosR-binding sequence is overlapped with the -10 region, it is impossible to entirely remove it without inactivation of the *ospA* promoter. For this particular reason, only 2 bps of this putative binding site were identified as a part of *cisII* in the previous study (Liang et al. 2002).

The regulatory sequence *cisI* contains no putative BosR-binding site but showed a critical role in repression of *ospA* transcription during murine infection, albeit it is less effective than *cisII* in this regard (Xu, McShan, and Liang 2010). To examine if the previously identified regulatory elements were able to bind BosR, three 70-bp DNA probes, namely probes *cisI*, *cisII* and IRs, respectively, were synthesized as diagrammed in figure 2.3 Probe *cisI* covered the entire *cisI* sequence and additional 14 bps down- and upstream sequences. Probe *cisII* included the entire *cisII* sequence and extended upstream to include the -35 region of the *ospA* promoter. Probe IRs contained the *ospC* IRs and extended few bps up- and down-stream to make up a total of 70 bps. The IRs sequence was previously identified as an operator of the *ospC* gene and was not expected to bind BosR, and thus was chosen as a negative control (Xu, McShan, and Liang 2007). To minimize nonspecific DNA interaction with BosR, the binding buffer was

supplemented with 50μg/ml salmon sperm DNA. As shown in figure2.8 the presence of sperm DNA completely eliminated the interaction of IRs probe with BosR. In contrast, the mobility of both *cisI* and *cisII* probes was completely restrained by BosR, a result that clearly indicates that both *cisI* and *cisII* are able to effectively bind BosR.

Excessive BosR expression causes cell death in *B. burgdorferi*

To artificially regulate BosR expression, pIBM-$bosR_{in}$ was constructed as illustrated in figure2.2. Within the construct, BosR expression was under control of an inducible promoter. The BosR production cannot occur due to the construct in the absence of the inducer IPTG, although the native *bosR* gene may produce BosR as normal.

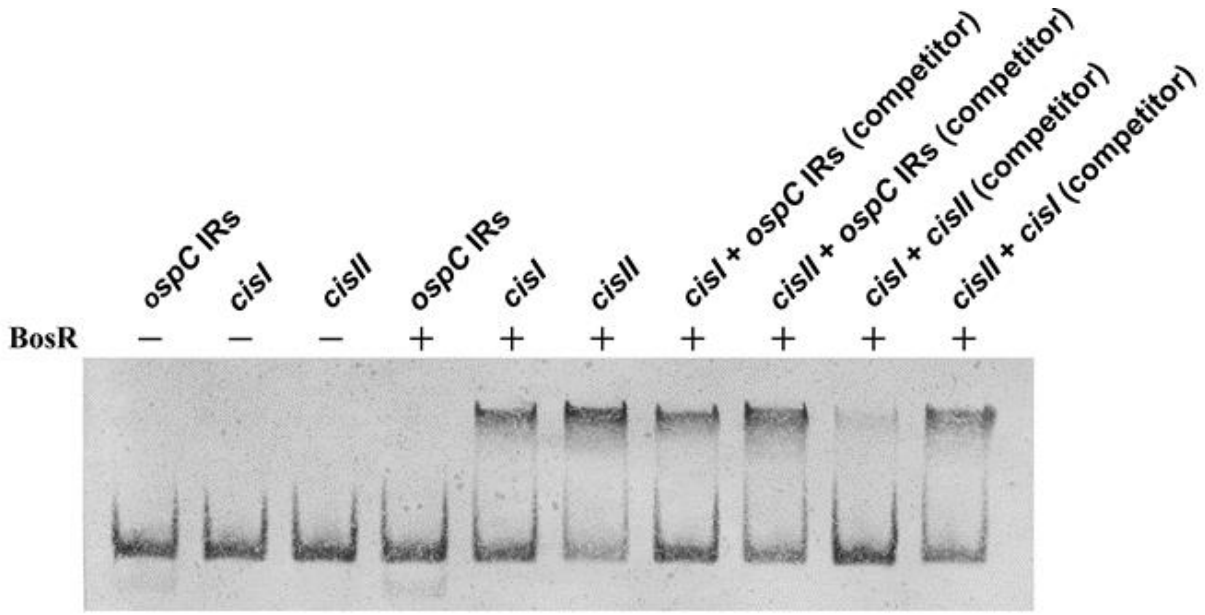

Figure 2.8. Both the regulatory sequences of the *ospA* operon, *cisI* and *cisII*, bind BosR and *cisII* shows stronger binding: The binding buffer contained 50μg/ml salmon sperm DNA. The ratio of a DNA probe and a competitor was set at 1:10 (1.0/10 nM). Mobility shift analysis was performed with 10% polyacrylamide gels.

The plasmid pIBM-*bosR*$_{in}$ was easily introduced into *ΔrpoS*, which was used because any RpoS involvement in OspA downregulation was readily ruled out in this way. The plasmid pIBM-*bosR*$_{in}$ was also electroporated into 13A, the parental clone of *ΔrpoS*, as a control. In a single transformation experiment with *ΔrpoS*, five transformants were obtained. Plasmid analyses led to the identification of one clone, namely, *ΔrpoS/bosR*$_{in}$, which lost cp9, lp5, lp21, lp25 and lp56 as *ΔrpoS*, in addition to lp28-1. Transformation of 13A led to selection of 7 transformants, one of which, namely 13A/*bosR*$_{in}$, lost cp9, lp5, lp21, lp25, lp56 and lp28-1 and was chosen for further studies. There had been no evidence that any of the lost plasmids affects gene regulation although both lp25 and lp28-1 are critical for murine infection (Xu, McShan, and Liang 2007).

A recent study reported that increasing RpoS expression causes cell death (Chen at el 2013). To investigate whether high BosR expression is toxic, both *ΔrpoS/bosR*$_{in}$ and 13A/*bosR*$_{in}$ were grown to early log phase (10^7 cells/ml) in BSK-H medium and diluted to 10^5 cells/ml before IPTG was added to a final concentrations ranging from 0 to 0.2 mM. When IPTG reached as low as 0.05 mM, *ΔrpoS/bosR*$_{in}$ growth was affected negatively (Figure 2.9A). When the concentration increased to 0.1 mM, growth was essentially arrested within a couple of days after induction and all spirochetes eventually died within a week. Although at lower concentrations IPTG did not affect early growth, its presence reduced the stationary cell density.

13A/*bosR*$_{in}$ was little less sensitive than *ΔrpoS/bosR*$_{in}$ to induction with IPTG. At 0.1 mM, the inducer showed little effect on growth (Figure 2.9B). Even at 0.2 mM, spirochetes continued to grow for a couple of days and then started to die and induced spirochetes became uncountable within a week.

Inducing BosR expression leads to dramatic downregulation of OspA

As shown above, excessive BosR expression caused a lethal consequence to *B. burgdorferi*. At 0.2 mM, IPTG significantly inhibited both *ΔrpoS/bosR*$_{in}$ and *13A/bosR*$_{in}$ shortly after the induction and eventually killed them within a week. This concentration was chosen to investigate how induction of BosR influenced OspA expression.

The *ΔrpoS/bosR*$_{in}$ and *13A/bosR*$_{in}$ spirochetes were grown to 10^7 cells/ml and then IPTG was supplemented to 0.2 mM. The IPTG induction was allowed for 3 days before spirochetes were harvested and analyzed. During this time, the spirochete density reached 10^8 cells/ml, reflecting a 3-fold increase. As shown in figure 2.10, as BosR was induced, OspA expression was dramatically reduced in ΔrpoS/*bosR*$_{in}$. A less prominent effect of induced BosR expression on OspA downregulation was observed in *13A/bosR*$_{in}$.

It should be noted that OspA may be pretty stable in live cells and thus reduction in detected protein amount might primarily depend on the dilution effect of cell division. The total bacterial number increased approximately 3 times after induction. Therefore, reduction in OspA amount might not be more than 3-fold even if the transcriptional process could be fully stopped immediately upon induction. Moreover, the abundant *ospA* mRNA accumulated before induction would continue to be translated into protein until they were degraded.

Total RNA was also prepared and analyzed. As shown in figure 2.10 C, induced BosR expression led to more than 86% reduction in *ospA* transcription in *ΔrpoS/bosR*$_{in}$ and 65% in *13A/bosR*$_{in}$. Unlike protein, mRNA is less stable and its level would better reflect the magnitude in *ospA* gene downregulation resulting from increased BosR expression.

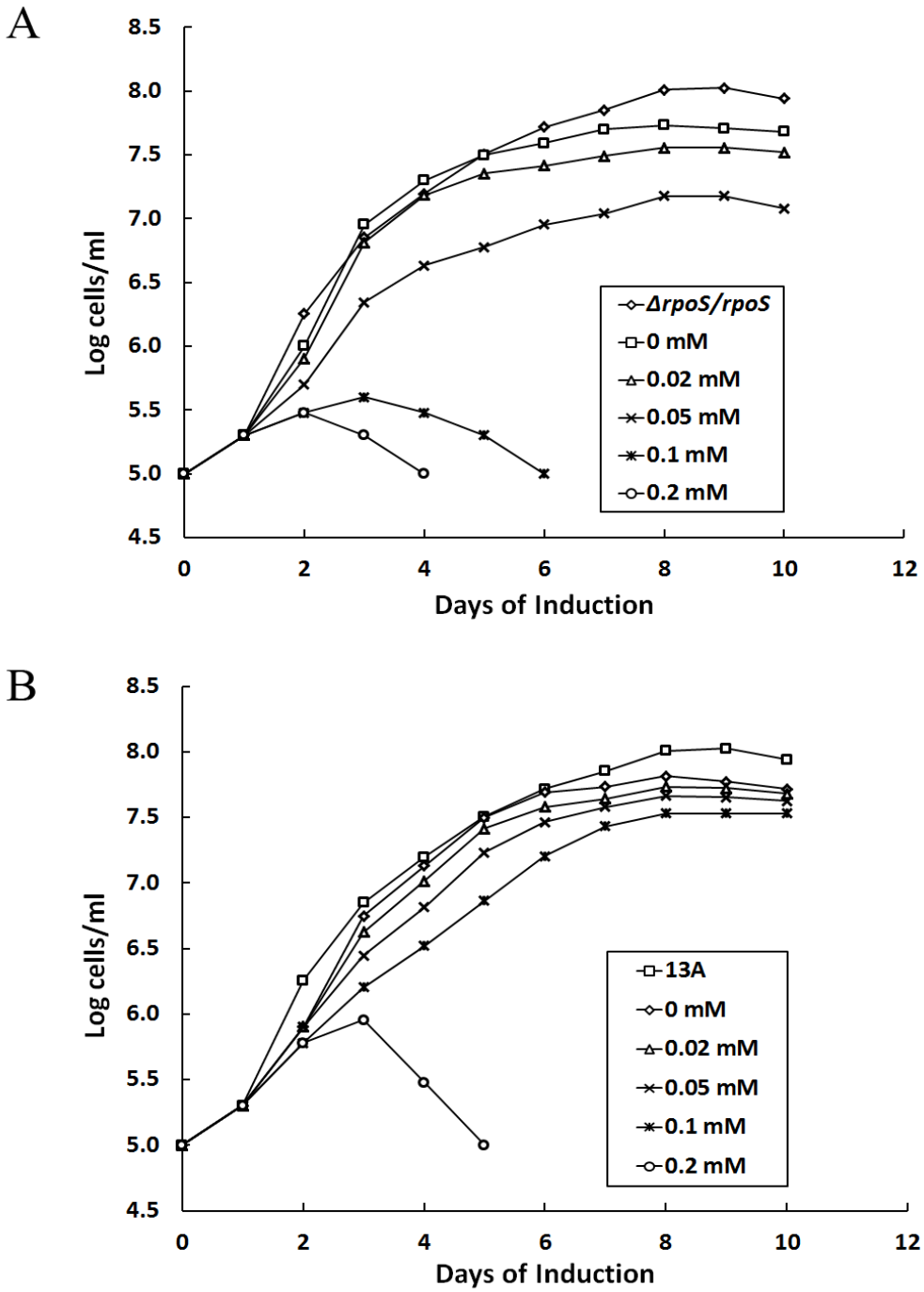

Figure 2.9. Excessive BosR expression causes cell death: (A) A total of 15 1.3-ml aliquots of *ΔrpoS*/*bosR*$_{in}$ spirochetes at a density of 10^5 cells/ml were prepared and supplemented with IPTG to final concentrations at 0, 0.02, 0.05, 0.10, and 0.20 mM. Each sample was incubated at 33ºC and assayed in triplicate. The cell numbers were counted daily for 10 days. Mean counts presented here were calculated from the triplicates of each treatment. The *ΔrpoS/rpoS* spirochetes were used as a control. (B) The same experimental design was used to examine the 13A/*bosR'* spirochetes when the parental clone 13A was used as a control.

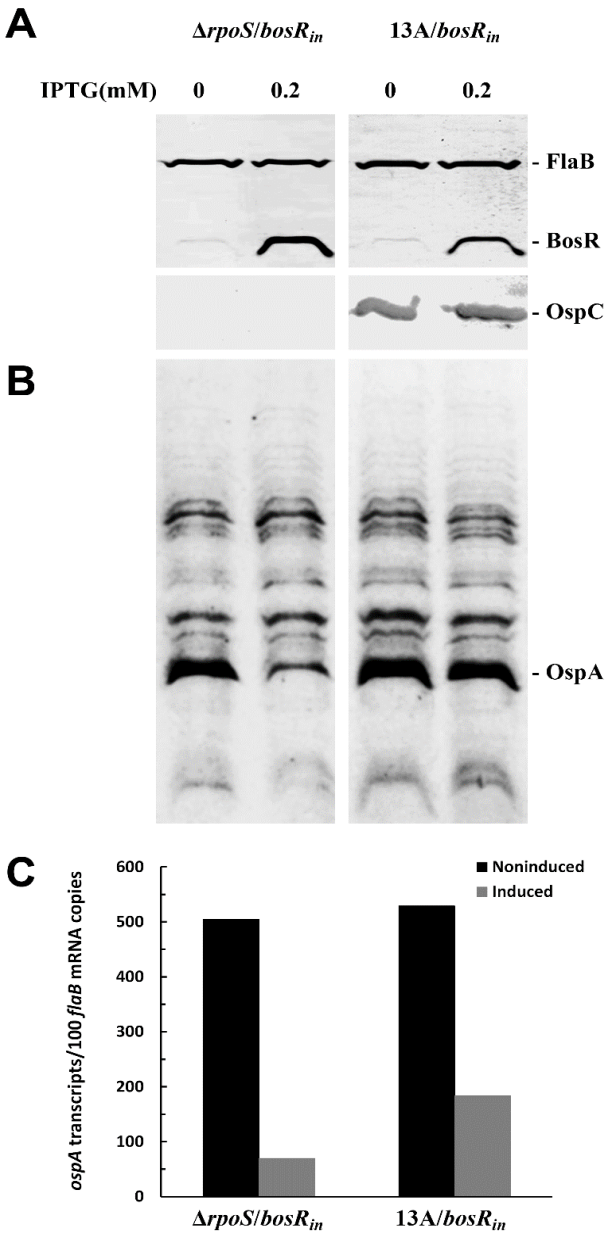

Figure 2.10. Inducing BosR expression leads to dramatic downregulation of OspA: (A&B). Both *ΔrpoS/bosR*$_{in}$ and *13A/bosR*$_{in}$ spirochetes were grown to a density of 10^7 cells/ml and then supplemented with IPTG to a final concentrations at of 0 or 0.2 mM. Induction was allowed for 3 days. Treated bacteria were harvested and analyzed either by immunoblotting probed with a mixture of FlaB mAb and mouse BosR antibodies (A, upper panel) or with OspC mAb alone (A, lower panel), or Coomassie staining (B). (C) Total RNA was extracted from induced and noninduced spirochetes 24 hours after initial treatment, converted to cDNA and analyzed by qRT-PCR.

Although induction led to a dramatic increase in BosR production, OspC expression didn't significantly increase (Figure 2.10), probably because RpoS was also positively regulated by other regulators, such as RpoN, small RNAs and Badr (Miller, Karna, and Seshu 2013, Lybecker and Samuels 2007, Hubner et al. 2001, Samuels 2011). An unbalanced increase in BosR expression might not overwhelm RpoS expression if expression of RpoN was not dramatically mobilized.

Increasing BosR expression completely shutoffs OspA production when constitutive expression of other Osps simultaneously occurs

Increasing BosR expression leads to dramatic downregulation of OspA but is never able to shut off expression, even when BosR expression was induced to a toxic level. To completely downregulate OspA with increased BosR, we designed an experiment based on our hypothesis that the Osp layer homeostasis of *B. burgdorferi* overwrites the normal regulatory programs. In other words, as OspA/B are so dominantly expressed osps in cultured spirochetes, *B. burgdorferi* cannot completely downregulate them without dramatically increasing expression of other osps. To continue to address the hypothesis, two additional constructs, pBBE22-*bosR'* and pBBE22-*C'B'A'-bosR'*, were created as shown in figure 2.2 B & C. Within the former, the fused *bosR* gene was designed to express under the control of *flaB* promoter, while in the latter, in addition to a fused *bosR* gene, three osp genes, *ospC, dbpA* and *dbpB*, were all engineered to transcribe under control of a fused *flaB* promoter. After the two constructs were introduced into *ΔrpoS*, five and eight transformants were obtained, respectively. Plasmid analysis led to identification of two clones, namely, *ΔrpoS/bosR'* and *ΔrpoS/C'B'A'-bosR'*, which lost cp9, lp5, lp21, lp25 and lp56 as *ΔrpoS*, in addition to lp-28-1. As shown in figure 2.11B, a high level of BosR expression driven by the fused *flaB* promoter greatly downregulated OspA but was unable to completely shutoff its expression, consistent with the results obtained above with the inducible

promoter shown in figure 2.10 B. Whereas the simultaneous expression of OspC, DbpA and DbpB, along with the constitutive BosR production led to successful shutoff of OspA production (Figure 2.11B).

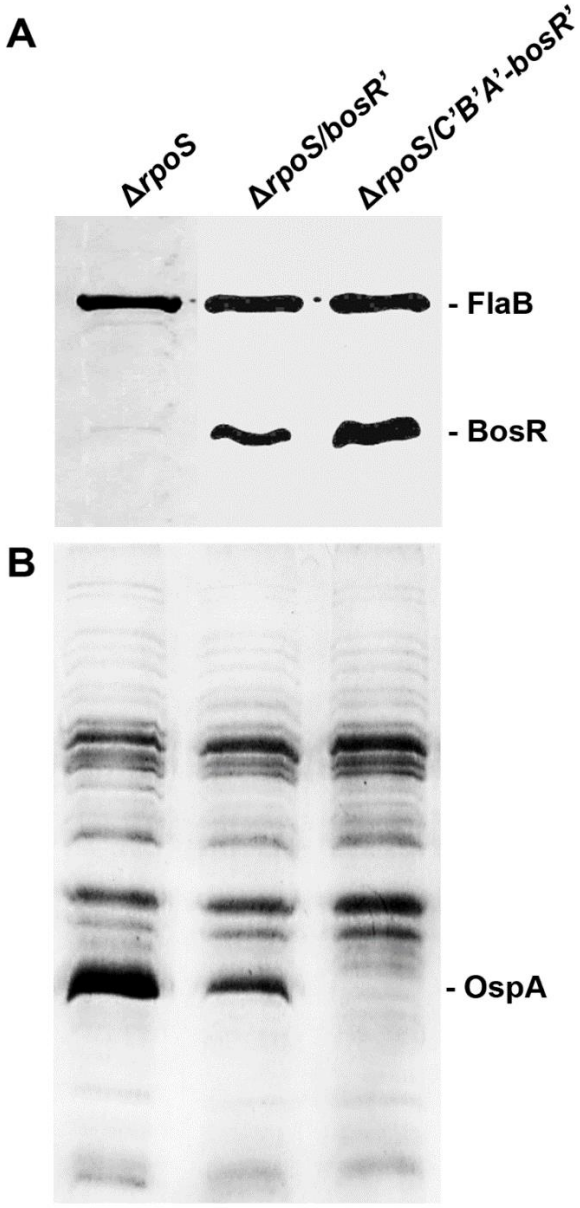

Figure 2.11. Increasing BosR expression shuts off OspA production only when other osps, OspC, DbpA and DbpB, are constitutively expressed: The *ΔrpoS*, *ΔrpoS/bosR'* and *ΔrpoS/C'B'A'-bosR'* were grown to early log phase (10^8 cells/ml), harvested and analyzed either by immunoblotting & probed with FlaB and BosR antibodies (A), or by Coomassie staining (B).

Discussion

The *in-vitro* BSK-H cultivated *B.burgdorferi* shows simultaneous expression of OspA, OspC, and OspD. Downregulation of OspA requires mammalian host specific signals, even in nature only a part of the spirochetes colonizing tick vector midgut downregulate OspA in response to blood meal and migrate to the salivary glands and infect the mammalian host. Thus in order to understand the mechanism of OspA downregulation it is very important to provide mammalian host environment to *B. burgdorferi* strains involved in this study. In order to see difference in expression of the outer surface lipoprotein layer between *bosR* mutant and *bosR* complement strain, both were incubated in DMCs implanted in the rat peritoneum (a technique providing mammalian physiological signals). Consistent with previous reports, OspC expression was absent in host adapted *bosR* mutant while it was expressed in *bosR* complement.This further confirms the requirement of BosR for OspC expression (Hyde et al. 2009, Ouyang et al. 2009). Further, just like the wild type *B. burgdorferi* B31 adapted in DMC, OspA and OspD were downregulated in *bosR* complement (Akins et al. 1998, Brooks et al. 2003). Based on these expression differences for OspC, OspA, and OspD it was confirmed that both *bosR* mutant and *bosR* complement spirochetes were optimally mammalian host adapted for further analysis. Based on the electrophoretic mobility shift assay results, this study demonstrated that BosR directly binds to sequences upstream of the *ospA* and the *ospD*. This upstream region of *ospA* operon have been shown in a previous 2010 study by our lab to be involved in OspA regulation (Xu, McShan, and Liang 2010). The present study indicates that BosR is capable of producing repression of OspA by direct binding to its promoter region and hence acts as a repressor for it. Until now OspA downregulation has been considered to be regulated by RpoS, as the study by Caimano et al. had demonstrated that loss of *rpoS* in *B. burgdorferi* also resulted in lack of OspA

repression within the mammalian host (Caimano et al. 2007). The requirement of RpoS for downregulation of OspA in addition to newly found direct OspA repressor function of BosR suggests that, BosR alone is not sufficient for OspA repression. In an unfed tick OspA is the most abundantly expressed osp on the borrelia surface. A previous study in our lab showed that expression of osps plays an important protective role in maintenance of viability of spirochetes. As shown in various studies, the outer surface lipoproteins may be expressed in accordance to a certain level of homeostasis of the outer surface. *B. burgdorferi* mutants have shown compensatory upregulation of osps supporting the surface homeostasis theory for example, a 2008 study by He et al. demonstrated that the deletion of *ospA* operon in *B. burgdorferi* lead to constitutive activation of Rrp2-RpoN-RpoS pathway and thus constitutive expression of OspC (He et al. 2008). However, in a similar study, parental B31 as well *ospA* mutant did not show OspC expression, showing increased level of several other outer surface proteins (Battesti, Majdalani, and Gottesman 2011). This study also indicated that OspC was not a unique compensatory osp. Another study also reported example of compensatory osp upregulation in *B. burgdorferi* (297 strain) *ospC* mutant, in which several other lipoproteins were found to be upregulated under the RpoS regulon like DbpA, DbpB, and BBK32 (Pal et al. 2004).

In each of the above mentioned studies, different strains of *B. burgdorferi* appeared to compensate the loss of one or the other lipoprotein with increased expression of other lipoproteins. Therefore, it is possible that deficiency of RpoS-mediated outer surface lipoproteins cause immense pressure on spirochete to maintain expression of tick phase surface lipoproteins (OspA and OspD). Nonetheless, BosR most probably acts as a repressor for *ospA*.

The lipoprotein nature and dominant expression of OspA makes it highly immunogenic and thus elicits a strong mammalian immune response (Barthold et al. 1995, Weis, Ma, and Erdile 1994).

Though, the mammalian OspA antibodies may not effectively eliminate *B. burgdorferi* from host tissues but can still prove fatal for spirochetes when acquired by the infected tick vector along with blood meal, this may eventually lead to disruption of borrelial enzootic cycle (de Silva et al. 1996, de Silva et al. 1999). Thus, in order to maintain itself in the natural cycle *B. burgdorferi* must rapidly downregulate OspA and evade immune detection in the mammalian host. The present study elucidates the mechanism of OspA downregulation and demonstrates that BosR binds the regulatory elements namely *cisI* and *cisII* on the *ospA* operon and shuts down the OspA expression on borrelial surface. Hence we can state that BosR functions as a repressor and the two regulatory elements, *cisI* and *cisII*, serve as operators of the *ospA* operon.

A previous study in our lab identified *cisI* and *cisII* as the two regulatory sequences flanking *ospA* operon that are required to produce maximum downregulation during murine infection (Xu, McShan, and Liang 2010). This study also demonstrated that downregulation mediated by *cisII* was four fold more effective than by *cisI* (Xu, McShan, and Liang 2010). Hence the findings of present study are in congruence with the previous results. It was observed that both *cisI* and *cisII* elements bind BosR and indeed *cisII* binding was much stronger than that mediated by *cisI*. This double-operator system, comprised of *cisI* and *cisII* sequences, use BosR as a common repressor and enables the complete shutoff of *ospA* operon, and thus provides *B. burgdorferi* with a protective mechanism to hide from host immune system and maintain its enzootic infection cycle.

BosR is required for expression of RpoS, which in turn activates *ospC*, *dbpBA* and many other osp genes (Hyde et al. 2009, Ouyang et al. 2009, Wang et al. 2013). The regulator is not expressed in early growth phases but is dramatically upregulated in late log phase. There has been no significant OspA downregulation observed in cultured spirochetes although BosR

expression is considered reaching the highest level in late log phase. Even in the vector tick's midgut, no more than 50% of the spirochete population downregulate OspA during any period of blood meal although most of the remaining population express OspC abundantly, an indication that BosR is actively expressed (Purser and Norris 2000). It is not an easy task to downregulate OspA although *B. burgdorferi* readily shuts off OspA either during mammalian infection or while being grown in host-adapted conditions (Barthold et al. 1995, Akins et al. 1998). These previous observations foretold what we experienced in the present study. To induce OspA downregulation, we increased BosR expression to a level that nearly kills *B. burgdorferi*. Under this *in-vitro* condition OspA expression is significantly downregulated but is not completely shut down.

The survival of *B. burgdorferi* within its two very distinct host environments demands deployment of complex survival strategies. All the adaptive changes taken up within its enzootic cycle are a consequence of dramatic differential gene expression of *B. burgdorferi*. Under the normal conditions, complete OspA downregulation occurs only during mammalian infection, in which OspC and other many RpoS-dependent osps are dramatically upregulated. The mammalian host apparently provides an extreme environment as these specific alternation signals might be unachievable under any *in-vitro* conditions. While either excessive RpoS or BosR expression is lethal to *B. burgdorferi in-vitro,* these high levels of expression may be essential for the pathogen to achieve downregulation of the *ospA* operon and to greatly upregulate RpoS-dependent virulence factors, and ultimately allow better survival of *B. burgdorferi* in the mammalian host. To achieve OspA downregulation in *B. burgdorferi* grown *in-vitro,* a high level of BosR expression in combination with simultaneous expression of OspC and other osps may be required.

As a key regulator, BosR, like RpoS, must be strictly regulated. The present study showed that BosR causes cell death when expressed at a very high level, just like RpoS (Chen et al. 2013). The cell death caused by excessive RpoS expression may be simply attributed to σ factor competition but there is no simple explanation for BosR-related death. While the biological significance of induced cell death remains to be addressed, at least one possibility should be considered, that is to control cell populations less diverse in the same environment. For instance, the death strategy would select out subpopulations with a phenotype that overexpresses RpoS- or BosR-dependent genes during the stage in the tick vector. However, cell death resulting from increased RpoS or BosR expression is observed *in-vitro*. Given that *B. burgdorferi* cycles between the extremely different environments encountered in the tick vector and a mammal, *in-vitro* growth conditions may only constitute an abnormal environment. It is possible that a high level of BosR and RpoS expression may provide an essential strategy for the pathogen to survive. Especially during infection of the mammalian host, *B. burgdorferi* may have to upregulate produce high levels of BosR to shutoff OspA expression and upregulate RpoS-dependent virulence genes in order to adapt to the hostile environment inside host tissues.

An RpoS-deficient background was chosen initially to rule out any involvement of RpoS in OspA downregulation, as a previous study suggested the alternative σ factor may be involved in regulation (Eggers, Caimano, and Radolf 2004). BosR positively affects expression of many osps, including OspC and DbpA/B through activation of RpoS expression (Hyde et al. 2009, Liang, Nelson, and Fikrig 2002). We speculated that induced BosR expression might cause downregulation of OspA more effectively in 13A/*bosR*$_{in}$ than *ΔrpoS/bosR*$_{in,}$ as BosR also increases RpoS expression, which increases OspC and DbpAB expression and increased production of these osps would compensate for the loss of OspA. However, the present study

showed that induced BosR causes OspA downregulation in *ΔrpoS/bosR*$_{in}$ than in the 13A/*bosR*$_{in}$ mutants, suggesting that gene regulation in *B. burgdorferi* is more complicated than believed previously. Our immunoblotting experiment showed that BosR expression is significantly stronger in the RpoS-deficient background than in the 13A/*bosR*$_{in}$, suggesting that the presence of RpoS might exert a feedback pressure on BosR expression.

BosR acts as a critical regulator as it directly binds the *rpoS* promoter region and activates *rpoS* transcription along with other regulators, RpoN and Rrp2. The current study demonstrated that BosR shuts off OspA expression by directly binding to the two regulatory sequences of the *ospA* operon. The question that how BosR can function as an activator as well as repressor remains to be investigated and thus may form a basis for future studies in the field. A previous study in our lab which identified *cisI* and *cisII* showed that these two elements could enhance *ospA* transcription in spirochetes cultured under *in-vitro* conditions. Although this enhancement was very minor, it supports the activator function of BosR. Thus there is another aspect to the regulatory function of BosR which remains to be uncovered when it is expressed at relatively low levels.

In continuation to the previous publication from our lab which identified *cisI* and *cisII* as the genetic elements required for downregulation of *ospA* within the murine hosts (Xu, McShan, and Liang 2010); the present study elaborately demonstrates the repressor function of BosR for the *ospA* operon. This study demonstrates direct binding of BosR to both *cisI* and *cisII* regulatory elements and resulting into a complete shut down of OspA expression. This data contribute further to the series of studies by other groups showing that BosR directly binds to *rpoS* promoter region and causes upregulation of several RpoS regulated osps on the borrelial surface(Sears et al. 1991, Ouyang, Deka, and Norgard 2011). In conclusion BosR acts as an

important gene coordinator, which on one hand causes upregulation of RpoS-dependent OspC, DbpA, and DbpB and on the other hand leads to a direct repression of OspA expression.

References

Akins, D. R., K. W. Bourell, M. J. Caimano, M. V. Norgard, and J. D. Radolf. 1998. "A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state." *J Clin Invest* . 101 (10):2240-50.

Bagg, A., and J. B. Neilands. 1987. "Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in Escherichia coli." *Biochemistry* . 26 (17):5471-77.

Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumpf, and S. L. Tessier. 1986. "A Borrelia-specific monoclonal antibody binds to a flagellar epitope." *Infect Immun* . 52 (2):549-54.

Barbour, A. G., S. L. Tessier, and S. F. Hayes. 1984. "Variation in a major surface protein of Lyme disease spirochetes." *Infect Immun* . 45 (1):94-100.

Barthold, S. W., E. Fikrig, L. K. Bockenstedt, and D. H. Persing. 1995. "Circumvention of outer surface protein A immunity by host-adapted *Borrelia burgdorferi*." *Infect Immun* . 63 (6):2255-61.

Battesti, A., N. Majdalani, and S. Gottesman. 2011. "The RpoS-mediated general stress response in Escherichia coli." *Annu Rev Microbiol* . 65:189-213.

Boylan, J. A., J. E. Posey, and F. C. Gherardini. 2003. "Borrelia oxidative stress response regulator, BosR: a distinctive Zn-dependent transcriptional activator." *Proc Natl Acad Sci U S A* . 100 (20):11684-89.

Brooks, C. S., P. S. Hefty, S. E. Jolliff, and D. R. Akins. 2003. "Global analysis of *Borrelia burgdorferi* genes regulated by mammalian host-specific signals." *Infect Immun* . 71 (6):3371-83.

Caimano, M. J., C. H. Eggers, C. A. Gonzalez, and J. D. Radolf. 2005. "Alternate sigma factor RpoS is required for the in vivo-specific repression of *Borrelia burgdorferi* plasmid lp54-borne ospA and lp6.6 genes." *J Bacteriol* . 187 (22):7845-52.

Caimano, M. J., R. Iyer, C. H. Eggers, C. Gonzalez, E. A. Morton, M. A. Gilbert, I. Schwartz, and J. D. Radolf. 2007. "Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle." *Mol Microbiol* . 65 (5):1193-217.

Chen, L., Q. Xu, J. Tu, Y. Ge, J. Liu, and F. T. Liang. 2013. "Increasing RpoS expression causes cell death in *Borrelia burgdorferi*." *PLoS One* . 8 (12):e83276.

Crother, T. R., C. I. Champion, J. P. Whitelegge, R. Aguilera, X. Y. Wu, D. R. Blanco, J. N. Miller, and M. A. Lovett. 2004. "Temporal analysis of the antigenic composition of *Borrelia burgdorferi* during infection in rabbit skin." *Infect Immun* . 72 (9):5063-72.

Cullen, P. A., D. A. Haake, and B. Adler. 2004. "Outer membrane proteins of pathogenic spirochetes." *FEMS Microbiol Rev* . 28 (3):291-318.

de Silva, A. M., S. R. Telford, 3rd, L. R. Brunet, S. W. Barthold, and E. Fikrig. 1996. "*Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine." *J Exp Med* . 183 (1):271-75.

de Silva, A. M., N. S. Zeidner, Y. Zhang, M. C. Dolan, J. Piesman, and E. Fikrig. 1999. "Influence of outer surface protein A antibody on *Borrelia burgdorferi* within feeding ticks." *Infect Immun* . 67 (1):30-35.

Eggers, C. H., M. J. Caimano, and J. D. Radolf. 2004. "Analysis of promoter elements involved in the transcriptional initiation of RpoS-dependent *Borrelia burgdorferi* genes." *J Bacteriol* . 186 (21):7390-402.

Fingerle, V., G. Goettner, L. Gern, B. Wilske, and U. Schulte-Spechtel. 2007. "Complementation of a Borrelia afzelii OspC mutant highlights the crucial role of OspC for dissemination of Borrelia afzelii in Ixodes ricinus." *Int J Med Microbiol* . 297 (2):97-107.

Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. Weidman, T. Utterback, L. Watthey, L. McDonald, P. Artiach, C. Bowman, S. Garland, C. Fuji, M. D. Cotton, K. Horst, K. Roberts, B. Hatch, H. O. Smith, and J. C. Venter. 1997. "Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*." *Nature* . 390 (6660):580-86.

Fung, B. P., G. L. McHugh, J. M. Leong, and A. C. Steere. 1994. "Humoral immune response to outer surface protein C of *Borrelia burgdorferi* in Lyme disease: role of the immunoglobulin M response in the serodiagnosis of early infection." *Infect Immun* . 62 (8):3213-21.

Grimm, D., K. Tilly, R. Byram, P. E. Stewart, J. G. Krum, D. M. Bueschel, T. G. Schwan, P. F. Policastro, A. F. Elias, and P. A. Rosa. 2004. "Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals." *Proc Natl Acad Sci U S A* . 101 (9):3142-7.

Hantke, K. 1981. "Regulation of ferric iron transport in Escherichia coli K12: isolation of a constitutive mutant." *Mol Gen Genet* . 182 (2):288-92.

He, M., T. Oman, H. Xu, J. Blevins, M. V. Norgard, and X. F. Yang. 2008. "Abrogation of *ospA* constitutively activates the Rrp2-RpoN-RpoS pathway (sigmaN-sigmaS cascade) in *Borrelia burgdorferi*." *Mol Microbiol* . 70 (6):1453-64.

Hubner, A., X. Yang, D. M. Nolen, T. G. Popova, F. C. Cabello, and M. V. Norgard. 2001. "Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway." *Proc Natl Acad Sci U S A* . 98 (22):12724-29.

Hyde, J. A., D. K. Shaw, R. Smith Iii, J. P. Trzeciakowski, and J. T. Skare. 2009. "The BosR regulatory protein of *Borrelia burgdorferi* interfaces with the RpoS regulatory pathway and modulates both the oxidative stress response and pathogenic properties of the Lyme disease spirochete." *Mol Microbiol* . 74 (6):1344-55.

Katona, L. I., R. Tokarz, C. J. Kuhlow, J. Benach, and J. L. Benach. 2004. "The fur homologue in *Borrelia burgdorferi*." *J Bacteriol* . 186 (19):6443-56.

Labandeira-Rey, M., J. Seshu, and J. T. Skare. 2003. "The absence of linear plasmid 25 or 28-1 of *Borrelia burgdorferi* dramatically alters the kinetics of experimental infection via distinct mechanisms." *Infect Immun* . 71 (8):4608-13.

Laemmli, U. K. 1970. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature* . 227 (5259):680-85.

Liang, F. T., M. J. Caimano, J. D. Radolf, and E. Fikrig. 2004. "*Borrelia burgdorferi* outer surface protein (osp) B expression independent of ospA." *Microb Pathog* . 37 (1):35-40.

Liang, F. T., M. B. Jacobs, L. C. Bowers, and M. T. Philipp. 2002. "An immune evasion mechanism for spirochetal persistence in Lyme borreliosis." *J Exp Med* . 195 (4):415-22.

Liang, F. T., F. K. Nelson, and E. Fikrig. 2002. "Molecular adaptation of *Borrelia burgdorferi* in the murine host." *J Exp Med* . 196 (2):275-80.

Lybecker, M. C., and D. S. Samuels. 2007. "Temperature-induced regulation of RpoS by a small RNA in *Borrelia burgdorferi*." *Mol Microbiol* . 64 (4):1075-89.

Margolis, N., and D. S. Samuels. 1995. "Proteins binding to the promoter region of the operon encoding the major outer surface proteins OspA and OspB of *Borrelia burgdorferi*." *Mol Biol Rep* . 21 (3):159-64.

Miller, C. L., S. L. Karna, and J. Seshu. 2013. "Borrelia host adaptation Regulator (BadR) regulates rpoS to modulate host adaptation and virulence factors in *Borrelia burgdorferi*." *Mol Microbiol* . 88 (1):105-24.

Ouyang, Z., R. K. Deka, and M. V. Norgard. 2011. "BosR (BB0647) controls the RpoN-RpoS regulatory pathway and virulence expression in *Borrelia burgdorferi* by a novel DNA-binding mechanism." *PLoS Pathog* . 7 (2):e1001272.

Ouyang, Z., M. Kumar, T. Kariu, S. Haq, M. Goldberg, U. Pal, and M. V. Norgard. 2009. "BosR (BB0647) governs virulence expression in *Borrelia burgdorferi*." *Mol Microbiol* . 74 (6):1331-43.

Pal, U., X. Li, T. Wang, R. R. Montgomery, N. Ramamoorthi, A. M. Desilva, F. Bao, X. Yang, M. Pypaert, D. Pradhan, F. S. Kantor, S. Telford, J. F. Anderson, and E. Fikrig. 2004. "TROSPA, an Ixodes scapularis receptor for *Borrelia burgdorferi*." *Cell* . 119 (4):457-68.

Pollack, R. J., S. R. Telford, 3rd, and A. Spielman. 1993. "Standardization of medium for culturing Lyme disease spirochetes." *J Clin Microbiol* . 31 (5):1251-55.

Purser, J. E., and S. J. Norris. 2000. "Correlation between plasmid content and infectivity in *Borrelia burgdorferi*." *Proc Natl Acad Sci U S A* . 97 (25):13865-70.

Radolf, J. D., M. J. Caimano, B. Stevenson, and L. T. Hu. 2012. "Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes." *Nat Rev Microbiol* . 10 (2):87-99.

Raetz, C. R., and C. Whitfield. 2002. "Lipopolysaccharide endotoxins." *Annu Rev Biochem* . 71:635-700.

Samuels, D. S. 2011. "Gene regulation in *Borrelia burgdorferi*." *Annu Rev Microbiol* . 65:479-99. doi: 10.1146/annurev.micro.112408.134040.

Schwan, T. G., and J. Piesman. 2000. "Temporal changes in outer surface proteins A and C of the lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice." *J Clin Microbiol* . 38 (1):382-88.

Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa. 1995. "Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding." *Proc Natl Acad Sci U S A* . 92 (7):2909-13.

Sears, J. E., E. Fikrig, T. Y. Nakagawa, K. Deponte, N. Marcantonio, F. S. Kantor, and R. A. Flavell. 1991. "Molecular mapping of Osp-A mediated immunity against *Borrelia burgdorferi*, the agent of Lyme disease." *J Immunol* . 147 (6):1995-2000.

Shi, Y., Q. Xu, K. McShan, and F. T. Liang. 2008. "Both decorin-binding proteins A and B are critical for the overall virulence of *Borrelia burgdorferi*." *Infect Immun* . 76 (3):1239-46.

Stewart, P. E., A. Bestor, J. N. Cullen, and P. A. Rosa. 2008. "A tightly regulated surface protein of *Borrelia burgdorferi* is not essential to the mouse-tick infectious cycle." *Infect Immun* . 76 (5):1970-78.

Studholme, D. J., and M. Buck. 2000. "The alternative sigma factor sigma(28) of the extreme thermophile Aquifex aeolicus restores motility to an Escherichia coli fliA mutant." *FEMS Microbiol Lett* . 191 (1):103-7.

Wang, P., P. Dadhwal, Z. Cheng, M. R. Zianni, Y. Rikihisa, F. T. Liang, and X. Li. 2013. "*Borrelia burgdorferi* oxidative stress regulator BosR directly represses lipoproteins primarily expressed in the tick during mammalian infection." *Mol Microbiol* . 89 (6):1140-53.

Weis, J. J., Y. Ma, and L. F. Erdile. 1994. "Biological activities of native and recombinant *Borrelia burgdorferi* outer surface protein A: dependence on lipid modification." *Infect Immun* . 62 (10):4632-36.

Xu, Q., K. McShan, and F. T. Liang. 2007. "Identification of an ospC operator critical for immune evasion of *Borrelia burgdorferi*." *Mol Microbiol* . 64 (1):220-31.

Xu, Q., K. McShan, and F. T. Liang. 2010. "Two regulatory elements required for enhancing ospA expression in *Borrelia burgdorferi* grown in vitro but repressing its expression during mammalian infection." *Microbiology* . 156 (Pt 7):2194-204.

Xu, Q., S. V. Seemanapalli, L. Lomax, K. McShan, X. Li, E. Fikrig, and F. T. Liang. 2005. "Association of linear plasmid 28-1 with an arthritic phenotype of *Borrelia burgdorferi*." *Infect Immun* . 73 (11):7208-15.

Xu, Q., S. V. Seemanapalli, K. McShan, and F. T. Liang. 2006. "Constitutive expression of outer surface protein C diminishes the ability of *Borrelia burgdorferi* to evade specific humoral immunity." *Infect Immun* . 74 (9):5177-84.

Xu, Q., Y. Shi, P. Dadhwal, and F. T. Liang. 2012. "RpoS regulates essential virulence factors remaining to be identified in *Borrelia burgdorferi*." *PLoS One* . 7 (12):e53212.

Yang, X. F., M. C. Lybecker, U. Pal, S. M. Alani, J. Blevins, A. T. Revel, D. S. Samuels, and M. V. Norgard. 2005. "Analysis of the ospC regulatory element controlled by the RpoN-RpoS regulatory pathway in *Borrelia burgdorferi*." *J Bacteriol* . 187 (14):4822-29.

CHAPTER 3
OUTER SURFACE LIPOPROTEIN LAYER HOMEOSTASIS OVERRIDES NORMAL REGULATORY PROGRAMS IN *BORRELIA BURGDORFERI*

Introduction

Unlike other Gram-negative bacteria, which make a thick LPS (Lipopolysaccharide) coat or Gram-positive microorganisms, which cover and protect themselves with a strong cell wall, *B. burgdorferi* expresses lipoproteins and anchors them to the outer membranous surface through lipidation (Cullen, Haake, and Adler 2004, Fung et al. 1994, Xu et al. 2005). Accumulating evidence indicates that *B. burgdorferi* maintains its overall surface lipoprotein expression during the entire enzootic life cycle i.e. travelling between the tick vector and a mammal, and during the course of mammalian infection. The Lyme disease pathogen abundantly expresses outer surface protein A (OspA) in the unfed tick (Takayama, Rothenberg, and Barbour 1987, Liang et al. 2002, He et al. 2008, Xu et al. 2012). A fresh blood meal induces downregulation of OspA and the upregulation of OspC and other osps, a process that prepares *B. burgdorferi* for infection in mammals (Schwan et al. 1995, Ohnishi, Piesman, and de Silva 2001, Liang, Nelson, and Fikrig 2002, Purser et al. 2003). Abundant OspC expression ultimately induces a robust early humoral response that imposes tremendous pressure on the pathogen (Schwan and Piesman 2000, Sears et al. 1991). To evade the specific humoral response and cause persistent infection, *B. burgdorferi* down-regulates OspC, and dramatically upregulates other surface lipoproteins, including VlsE and BBF01 (Liu et al. 2009, Grimm et al. 2004, Mbow, Gilmore, and Titus 1999, Pal et al. 2004).

The outer surface lipoprotein expression is vital for *B. burgdorferi* as the failure to maintain appropriate surface lipoprotein expression can be of a lethal consequence (Xu, McShan, and

Liang 2008). *B. burgdorferi* highly expresses OspA in culture and shuts off the genes in response to overwhelming *ospA* repressing mammalian signals after introduction into the host. The inability to timely upregulate OspC to compensate for the loss of OspA and other osps results in quick elimination of the *ospC* mutant (Ohnishi, Piesman, and de Silva 2001). This occurs when OspC-deficient spirochetes are inoculated into mice. Modification of the mutant with constitutive expression of any of the four lipoproteins, OspA, DbpA, VlsE or OspE, successfully rescues the *ospC* mutant with infectivity (Purser and Norris 2000).

The importance of surface lipoprotein layer homeostasis was also observed in vitro, although interpreted in a different way, Yang and colleagues reported that inactivation of the *ospA* operon leads to constitutive activation of RpoS expression (de Silva et al. 1996) . This occurs most likely as the alternative σ factor, RpoS, controls many outer surface lipoproteins, including OspC, DbpA/B, and BBK32 (Caimano et al. 2004, Caimano et al. 2005, Caimano et al. 2007). Thus, the deletion of the operon that encodes the two most abundantly expressed Osps in vitro (*ospA*) could be fatal to *B.burgdorferi*, unless other Osp(s) is dramatically upregulated to compensate for the loss of OspA. As a result, only *ospA* mutants that happen to dramatically upregulate other osps can survive and be selected for further propagation.

Although accumulating evidence supports the hypothesis that the outer surface lipoprotein layer homeostasis is an overwhelming force to override the normal regulatory programs, no experiments have been ever designed to specifically address this. In the present study, a series of experiments were designed to directly examine this hypothesis, in addition to showing osp homeostasis exists in *B. burgdorferi*.

Material & methods

Strains and constructs that were generated previously and used in the current study

B. burgdorferi B31 (strain 13A) was used as wild type strain. The *ospA* mutant (Δ*ospA*) and *ospA* complement (*ΔospA/ospA*) were generated in a previous study (Xu, McShan, and Liang 2008). The shuttle vector pBBE22 was a gift from S. Norris (Liang et al. 2004).

2-D electrophoresis

This analysis was performed using the Bio-Rad 2-D system (Bio-Rad, Hercules, and CA) following the manufacturer's instructions. Briefly, *B. burgdorferi* was grown to late log phase (approximately 10^8 cell/ml) in 1.0 ml BSK-H complete medium at 33ºC and harvested by centrifugation 5,000 ×g for 3 min. Resultant pellets were washed twice with 2-D washing buffer by centrifugation and resuspended in ~150 µl preheated (95°C) SDS sample solubilization buffer. After sonication, the sample was heated at 95°C for 5 min, cooled to 20°C, diluted with ~500 µl of 2-D sample solution, kept at room temperature for 20 min, and centrifuged at 14,000 ×g for 30 min at 20ºC. The protein concentration of the supernatant was determined and was adjusted at ~5 µg/µl with 2-D sample solution.

First-dimension separation is performed by isoelectric focusing (IEF) electrophoresis. The pre-made IPG strip from Bio-Rad were used. IPG strip was rehydrated in rehydration/equilibration trays followed by IEF with gel-side up.

Second-dimension separation is SDS-page. Equilibrate the IPG strips twice, each time for 10 min, in two different equilibration buffers. In sealing IPG Strips onto SDS-PAGE Gels, the equilibrated IPG strips were placed on the top of polyacrylamide gels and SDS-PAGE was performed.

Total Protein Staining was performed with Coomassie or SYPRO Ruby Protein Gel Stain.

Mass spectrometry

Selected proteins from 2-D gel were analyzed by gel trypsin digestion and Matrix-assisted laser desorption/ionization (MALDI TOF/TOF). MALDI was done in Nevada Proteomics Center, in Reno, Nevada. The Spots or bands were excised with robot and digested using a previously described protocol (Rosenfeld et al. 1992, Finehout and Lee 2003). Selected proteins from this step were also analyzed by gel trypsin digestion and MALDI TOF/TOF. Samples were washed twice with 25mM ammonium bicarbonate (ABC) and 100% acetonitrile, reduced and alkylated using 10 mM dithiothreitol and 100 mM iodoacetamide and incubated with 75 ng sequencing grade modified porcine trypsin (Promega) in 25 mM ABC overnight at 37^{o} C. Digested samples were spotted onto a MALDI target with ZipTipμ-C18 (Millipore Corp., MA) and eluted with 70% acetonitrile, 0.2% formic acid and overlaid with 0.5 μL of 5 mg/ml MALDI matrix (α-Cyano-4 hydroxycinnamic acid 10 mM ammonium phosphate). All mass spectrometric data were collected using an ABI 4700 Proteomics Analyzer MALDI TOF/TOF mass spectrometer (Applied Biosystems, CA), using their 4000 Series Explorer software v. 3.6. The peptide masses were acquired in reflectron positive mode (1-keV accelerating voltage) from a mass range of 650 – 4000 Daltons, 1250 laser shots were averaged for each mass spectrum. Each sample was internally calibrated on trypsin's autolysis peaks 842.51 and 2211.10 to within 20ppm. Any sample failing to internally calibrate was analyzed under default plate calibration conditions of 150ppm. Raw spectrum filtering/peak detection settings were S/N threshold of 3, and cluster area S/N optimization enabled at S/N threshold 10, baseline subtraction enabled at peak width 50. The twenty most intense ions from the MS analysis, which were not on the exclusion list, were subjected to MS/MS. The MS/MS exclusion list included known trypsin masses: 842.51, 870.54,

1045.56, 1126.56, 1420.72, 1531.84, 1940.94, 2003.07, 2211.10, 2225.12, 2239.14, 2283.18, 2299.18, 2678.38, 2807.31, 2914.51, 3094.62, 3337.76, and 3353.75. For MS/MS analysis the mass range was 70 to precursor ion with a precursor window resolution of -1 to +4 Da with an average 2500 laser shots for each spectrum, CID ON, metastable suppressor ON. Raw spectrum filtering/peak detection settings were S/N threshold of 5, and cluster area S/N optimization enabled at S/N threshold 6, baseline subtraction enabled at peak width 50. The data was then stored in an Oracle database (Oracle database schema v. 3.19.0 Data version 3.90.0).

MALDI data analysis

The data was extracted from the Oracle database and a peak list was created by GPS Explorer software v 3.6 (Applied Biosystems) from the raw data generated from the ABI 4700. Analyses were performed as combination of MS + MS/MS. MS peak filtering included mass range 650-4000 Da, minimum S/N filter 10. A peak density filter of 50 peaks per 200 Da with a maximum number of peaks set to 65. MSMS peak filtering included mass range of 60 Da to 20 Da below each precursor mass. Minimum S/N filters 10, peak density filter of 50 peaks per 200 Da, cluster area filter used with maximum number of peaks 65. The filtered data were searched by Mascot v 2.2.04 (Matrix Science) using NCBI nr database (NCBI 2009), containing 8,080,522 sequences. Searches were performed without restriction to protein species, Mr, or pI and with variable oxidation of methionine residues and carbamidomethylation of cysteines. Maximum missed cleavage was set to 1 and limited to Trypsin cleavage sites. Precursor mass tolerance and fragment mass tolerance were set to 20 ppm and ± 0.2 Da, respectively. These files were then analyzed using Proteome Software's Scaffold software.

RNA preparation

Spirochetes grown to late log phase (approximately 10^8 cell/ml) in 40 ml of BSK-H medium at 33°C were harvested by centrifugation. Total RNA was extracted using RNA Isolation Kit following the manufacturer's instruction & (Qiagen). Genomic DNA was removed through DNase I digestion. The rRNAs were depleted using Microb Express Kit (Ambion). The RNA concentration was determined by using ND-1000 (Nano Drop Technologies).

Real time RT-PCR

The real time RT-PCR was performed using SYBR green PCR Kit and following its instructions. After extracting RNA from borrelia culture, cDNA from total RNA was synthesized using, 2 µg RNA and oligo-dT for reverse transcription reaction. The DNA-free RNA preparation was first annealed with oligo-dT (*In-vitro*gen) at 42˚C for 2 min, in presence of reverse transcription (RT) buffer (*In-vitro*gen). Deoxynucleoside triphosphates and SuperScript II Rnase H- reverse transcriptase was added, RT was conducted at 42˚C for 1 h, and then reaction was terminated by heat inactivation of enzyme at 70˚C for 15 min according to manufacturer's instructions. The cDNA preparation was diluted with nuclease free water and aliquoted into small tubes and stored at -80˚C until further use.

Using the real time PCR primers (Table 3.1) and iTaq™ SYBR® Green Supermix with ROX (Bio-Rad, Hercules, CA), real time PCR was performed on Applied Biosystems GeneAmp PCR System 7900. The primers were designed to amplify sequences ranging from100-200 bps. The absolute and relative quantitation of target RNA was calculated by comparing result data of samples with standard curve of target and reference gene. The borrelia *flaB* gene was used as the reference gene. All cDNA amplification reactions were carried out in 20µl volume in a 96-well

PCR plate, each sample was run in triplicate. Amplification was achieved with following parameters: 95˚C for 10 min, and 40 cycles of 95˚C for 15 s and 60˚C for 1 min. The mean cDNA copy numbers of each sample, transcripts of each cDNA pool were automatically calculated from triplicate wells using SDS 1.4 software. Gene expression levels were presented as mRNA copy number per 10^3 *flaB* mRNA transcripts.

Protein extraction and SDS polyacrylamide gel electrophoresis (SDS-PAGE)

For one dimensional SDS-PAGE, cell pellets were suspended in HN buffer containing protease inhibitor cocktail (Roche), sonicated with a Branson Digital Sonifier for 4 cycles each of 15 sec burst followed by 1 min rest. After centrifugation at 10,000xg, supernatants were analyzed by electrophoresis according to Laemli method using 12% TGX gel (BioRad) in Tris-glycine buffer system for 2h at 80 volts. Gels were then stained with Bio-Safe Coomassie G-250 stain for visualization of protein bands.

Immunoblot Analysis

The protein bands in SDS-PAGE gel were electrotransferred onto a nitrocellulose membrane (Thermo Scientific, Rockford, IL). Different borrelial proteins (Figure 3.4 &3.5) were probed with either a mixture or individual Monoclonal Antibodies (MAbs). The α-FlaB, were used as positive control and used at 1:100 dilution (Barbour et al. 1986). The α-OspC, α-DbpA, α-RpoS, and α-BosR MAbs were used in 1:10000 dilution. The α-OspC were developed by Mbow et al. in 1999 (Mbow, Gilmore, and Titus 1999). The α-RpoS was kindly provided by F. Yang (Indiana University School of Medicine) and the α-BosR MAbs were provided by Xin Li (Department of Veterinary Biosciences, The Ohio State University).

The secondary antibody used was the commercially available goat anti-mouse IgG conjugated with horseradish peroxidase (hrp) at 1:5000 final dilution (ThermoScientific, Waltham, MA). The commerically available Coloroimetric blotting substrate (4CN) was used to develop nitrocellulose membrane (Kirkegaard and Perry Laboratories, Gaithesburg, MD).

Table 3.1. Primers used in the study: All the primers designed for qPCR covered 100-200 bps of the respective genes.

Primer	Sequences (5' to 3')	Purpose
flaBqf	CATATCTTGGAGACAGACGTAGACTTATACACGG	qPCR
flaBqr	CTGCATTCCAAGCTCTTCAGCTGTTC	qPCR
$ospDf_1$	AGGCAAATAAAGTTGTAGAAGCGGT	qPCR
$ospDr_2$	TTCTGCCATTTGAGCTAAATCATGC	qPCR
bbj41f	AACCGAAGAATAGCTGGTCAA	qPCR
bbj41r	TTGTAGCAACTTTTCGGCTTCT	qPCR
$ospDf_2$	GAA GGC GCA AAT TCA AAT TAC GAA TC	lp38 identification
$ospDr_2$	CAG CAG AAT CAG AAT AGT CAG ATT C	lp38 identification

Selection of *B. burgdorferi* with lost lp38 (ospA/lp38$^-$)

The *ospA* knockout mutant was cultivated to late logphase in 10 ml BSK-H media at 33ºC, pelleted by centrifugation and resuspended after washing with media. The pellet was resuspended in 50 ml of fresh media and aliquoted for obtaining single borrelial colonies. All the resultant clones were screened by PCR for presence of lp38 using primers as shown in table 3.1. The PCR conditions included: 94 °C for 1 min; 94 °C for 64 seconds, 54 °C for 30 seconds, 72 °C for 1 min, 35 cycles; 72 °C for 10 min. PCR products were separated on an ethidium

bromide-agarose gel. The selected clones were first surveyed for the presence of lp28-1 because this plasmid is essential for persistent infection of immunocompetent hosts (Fingerle et al. 2007, Crother et al. 2004, Barbour et al. 1986). Only clones containing lp28-1 were further analyzed for Osp expression study.

In-vitro characterization of selected clones

The selected *ospA* mutants lacking lp38 were grown in Barbour-Stoenner-Kelly H (BSK-H) complete medium (Sigma Chemical Co., St. Louis, MO) to late-log phase at 33ºC, and harvested by centrifugation. Spirochete lysates were subjected to immunoblot analysis probed with FlaB (Raetz and Whitfield 2002), OspA (Xu, McShan, and Liang 2007) or OspC mAb (Xu, McShan, and Liang 2007). Immunoblotting was performed as described (Sears et al. 1991).

Microscope image

The wild type *B. burgdorferi* 13A, *ospA* mutant (*ΔospA),* both strains with and without lp38 (*ΔospA*/lp38^{+} & *ΔospA*/lp38^{-}) and the ospA complement (*ΔospA/ospA*) spirochetes were grown to early log phase (10^{7} cells/ml) in BSK-H medium at 33°C and harvested by centrifugation from 1.0 ml culture. The harvested spirochetes were resuspended in 20µl PBS and then charged onto fresh glow-discharged holey carbon grids for 1 min. A filter paper was used to blott the Grids were and then rapidly frozen in liquid ethane, using a homemade gravity-driven plunger apparatus. Polara G2 electron microscope, with field emission gun and a 16 megapixel camera, operated at 300 kV was used for imaging the frozen-hydrated samples at -170°C (FEI Co., Hillsboro, OR).

Statistical analysis

The qPCR data was analyzed using a one-way analysis of variance (ANOVA), followed by a two-tailed Student t test to compare both conditions and calculate P values of ≤ 0.05 were considered to be significant. Fisher's exact test was used to analyze tissue colonization data. GraphPad Prism 5.0 was used for calculations (GraphPad Software Inc., La Jolla, CA)

Results

Deletion of the *ospA* locus leads to dramatic up-regulation of OspD and BBJ41 and complementation reduces their expression to an undetectable level

The protein analysis of an *ospA* mutant showed two protein bands that were substantially up-regulated (Figure 3.1). Complementation of the mutant with the *ospA* gene alone repressed the two polypeptides to an undetectable level. As OspA are such highly expressed Osps, not regulators, when *B. burgdorferi* is grown in vitro, these observations led us to hypothesize in previous study that the outer surface lipoprotein layer is required for basic survival of the spirochete and its homeostasis is an overwhelming force to gene regulation (Xu, McShan, and Liang 2008). Based on the previous study we hypothesised that the two highly upregulated protein bands as shown in figure 3.1 may be that of some other outer surface lipoproteins that might have been up-regulated in *ospA* mutant to compensate for the loss of OspAB and maintainence of the outer surface lipoprotein layer homeostasis.

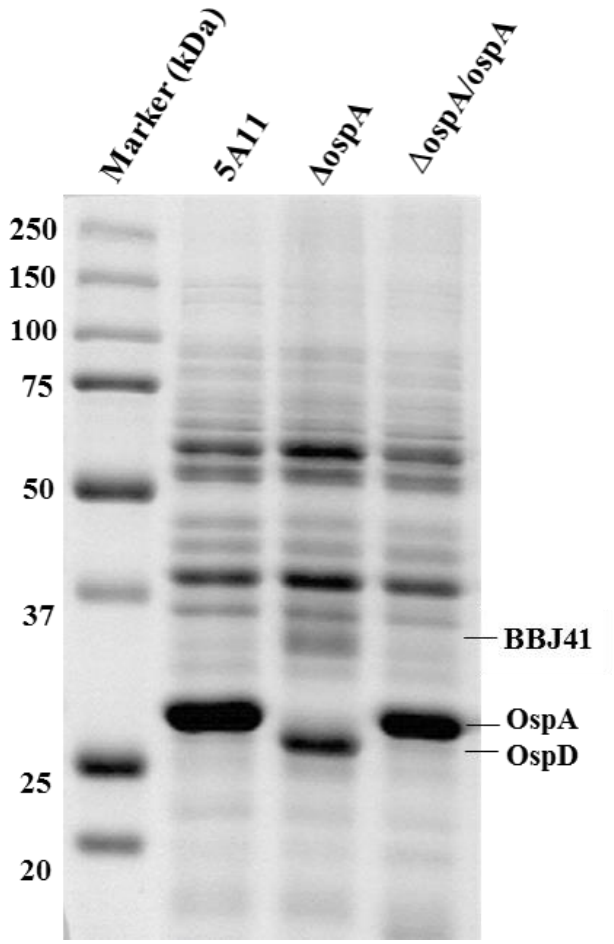

Figure3.1. Disruption of the *ospA* locus leads to dramatic upregulation of OspD and BBJ41, and complementation with *ospA* causes their downregulation to an undetectable level. The two greatly upregualated protein bands were initially unidentified. Proteins were further purified by using 2-D gel electrophoresis, and identified as outer surface lipoproteins OspD and BBJ41by MS-Spect analysis.

Identification of two highly upregulated proteins as OspD & BBJ41 in *ospA* knockout (Δ*ospA*):

The two highly upregulated polypeptides were separated using 1-D (Figure 3.1) and 2-D gel electrophoresis (Figure3.2). Purified protein Spots or bands were excised with robot and analyzed by MS-Spect. The MS-Spect analysis identified the two highly upregulated proteins to be 28kDa OspD (spot 1'/1, 2'/2, and 3'/3) and 35kDa BBJ41 (spot 5'/5) both expressed as outer

surface lipoproteins in *B. burgdorferi.* Additionally, OspD and BBJ41 were also identified on 1-D gel directly using same method (Figure 3.1).

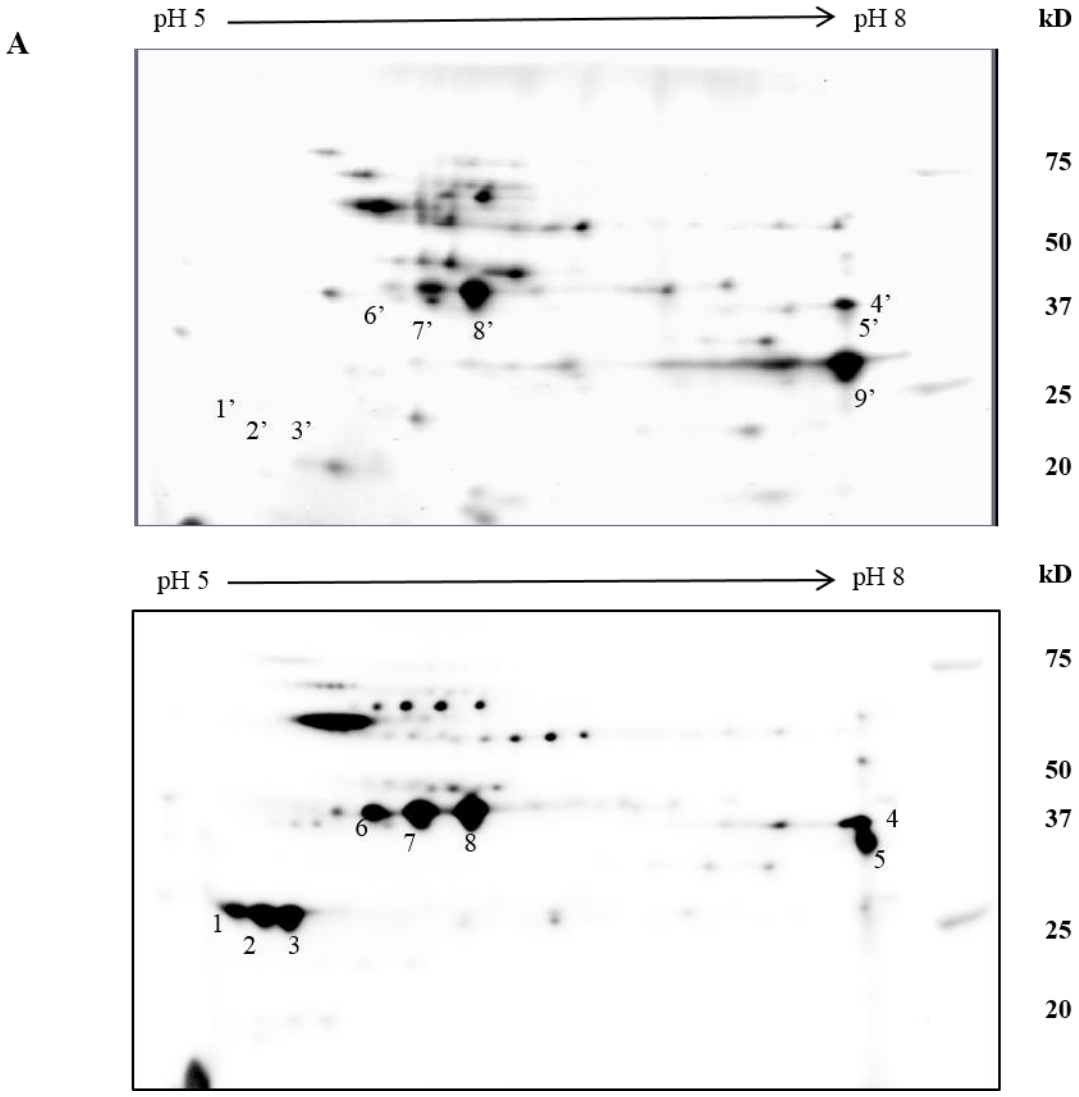

Figure 3.2. The 2-D gel electrophoresis for wild type *B. burgdorferi* 13A & OspA knock out.(A) 2-D gel image for wild type strain 13 A of *Borrelia burgdorferi.*(B)2-D gel image for *ospA* mutant. MS-Spec analysis revealed Spots 1'/1, 2'/2, and 3'/3 as OspD; Spot 4'/4 as GAPDH. Spot 5'/5 as BBJ41; Spot 6'/6, 7'/7, 8'/8 as FlaB & Spot 9' as OspA.

Over expression of identified protein at mRNA level

The dramatic regulation of OspD and BBJ41 was further confirmed at the mRNA level. RNA was extracted from the parental strain 13A, Δ*ospA* and Δ*ospA/ospA* and analyzed by qRT-PCR. As shown in figure 3.3, the expression level changed 16 and 21 times for *ospD* and *bbj41*, respectively. These results clearly show that expression of OspD and BBJ41 is highly increased at the mRNA level in absence of *ospA*, thus indicating that absence of OspA is compensated by upregulation of OspD and BBJ41.

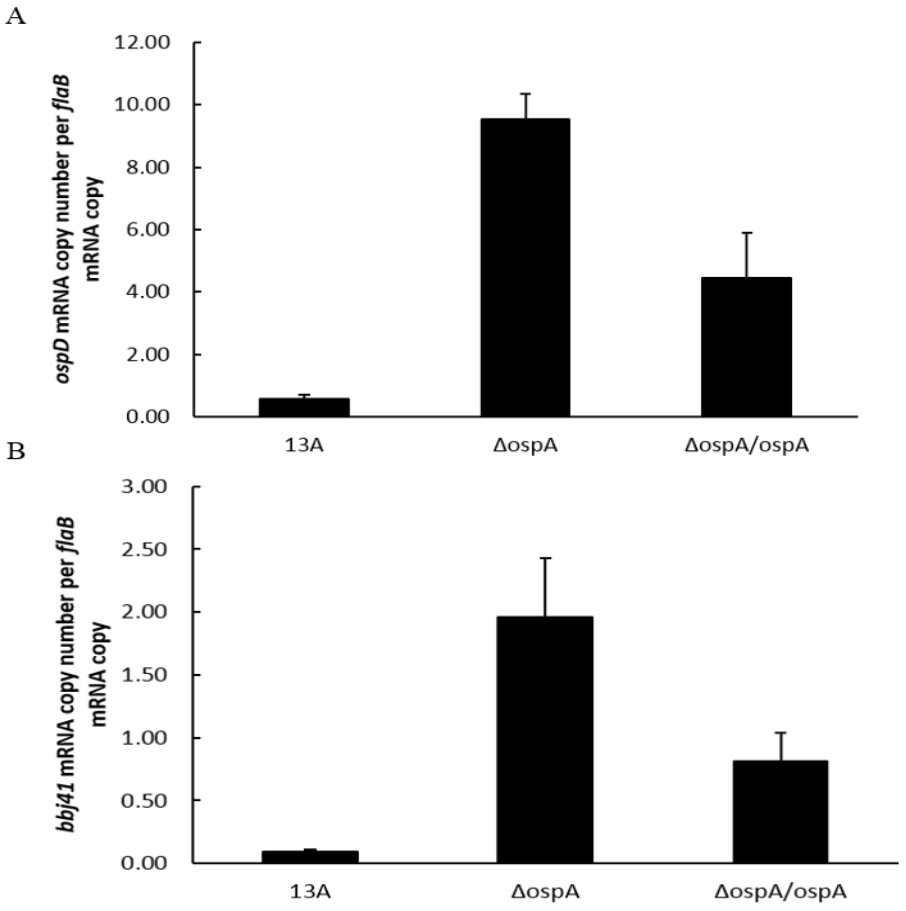

Figure 3.3. Absence of *ospA* causes increased expression of Outer surface lipoproteins, OspD & BBJ41 at mRNA level: (A) qRTPCR results for ospD show 16 fold rise in mRNA copy and (B) qRTPCR results for bbj41 show 21 fold rise in mRNA copy in comparison to the wild type 13A strain of *B. burgdorferi*.

Deprivation of lp38, the plasmid that carries the *ospD* and *bbj41* genes, leads to constitutive expression of RpoS and its dependent Osps

In order to continue to address the hypothesis that homeostasis of the Osp layer overrides the normal regulatory program, we initially attempted to inactivate both *ospD* and *bbj41* genes. As the plasmid lp38, which carries both the genes, is not essential for infection and may be easily lost, we designed experiments to isolate clones that lose the plasmid. A total of 56 clones were selected; only one of them, namely *ΔospA*/lp38^{-}, had lost lp38. The 13A, *ΔospA*, and *ΔospA* (lp38^{-}) spirochetes were grown to early log phase, harvested, analyzed by SDS-PAGE, and stained with Coomassie staining (Figure3.4A) and probed by immunoblotting with α-FlaB & α-OspC (Figure 3.4 B & C). The protein analysis below clearly show that OspC is induced in *ospA* mutant lacking lp38, these results indicate that OspC is upregulated in response to an absence of OspA, OspD & BBJ41. As OspC expression is induced by RpoS, we further analysed *ospA* mutant without lp38 for presence of other RpoS induced Osps. Hence immunoblotting with α-DbpA was done (Figure3.4D). Taken together these results indicate that absence of major Osps caused induction of RpoS and hence the RpoS regulated Osps, OspC & DbpA, thus maintaining the surface homeostasis and overriding usual regulatory mechanisms and ensuring the survival of *B. burgdorferi*.

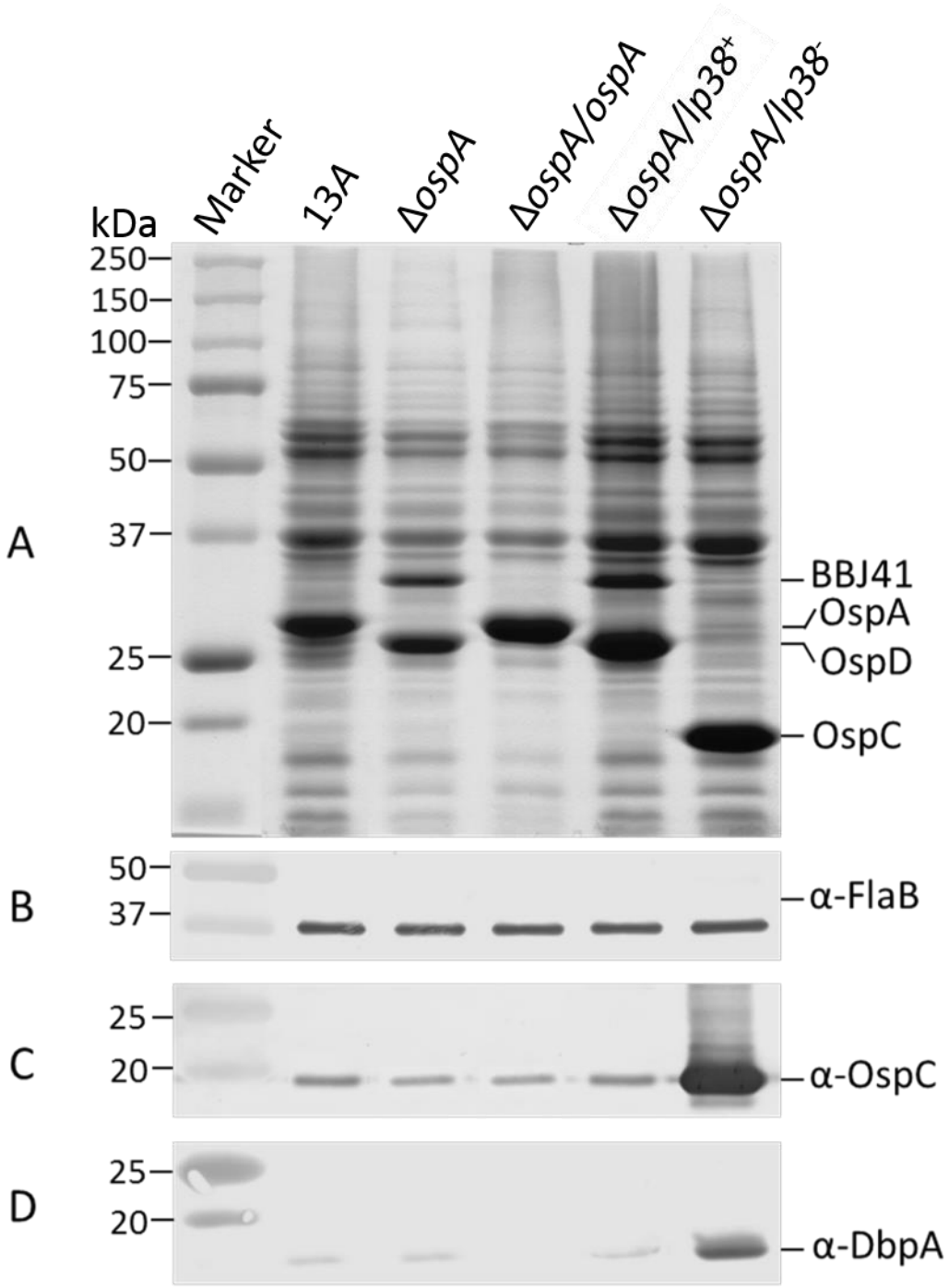

Figure 3.4. Depletion of lp38, the plasmid that carries both *ospD* and *bbj41* genes, leads to constitutive RpoS activation as well as dramatic upregulation of RpoS-dependent osps. The Δ*ospA* culture was subcloned through a serial dilution. A total of 56 clones were selected; only one of them, namely Δ*ospA* (lp38-), lost lp38. The 13A, Δ*ospA*, and Δ*ospA* (lp38$^-$) spirochetes were grown to early log phase, harvested, analyzed by SDS-PAGE, and stained with Coomassie staining (A) and immunoblotting probed with (B) *flaB*, (C) RpoS, and (D) DbpA antibodies.

Complementation of *ΔospA* (lp38^{-}) with *ospA* represses constitutive expression of RpoS and its dependent osps

RpoS is continuosly expressed throughout the mammalian phase of Lyme disease, it regulates many important mammalian phase Osps, including OspC, DbpA/B and BBK32. The experimental results above show that RpoS is induced in vitro in the absence of major tick phase Osps like OspA & OspD. To ensure that RpoS was induced in a compensatory response to the absence of major Osps like OspA, we complemented *ospA* mutant lacking lp38 with *ospA* plasmid. Complementation of ospA mutant resulted in reduced expression of RpoS upregulator BosR, hence RpoS and OspC (Figure 3.5). These results clearly indicate that RpoS was induced in *ospA* mutant lacking lp38 as the osp layer homeostatic response in *B.burgdorferi*.

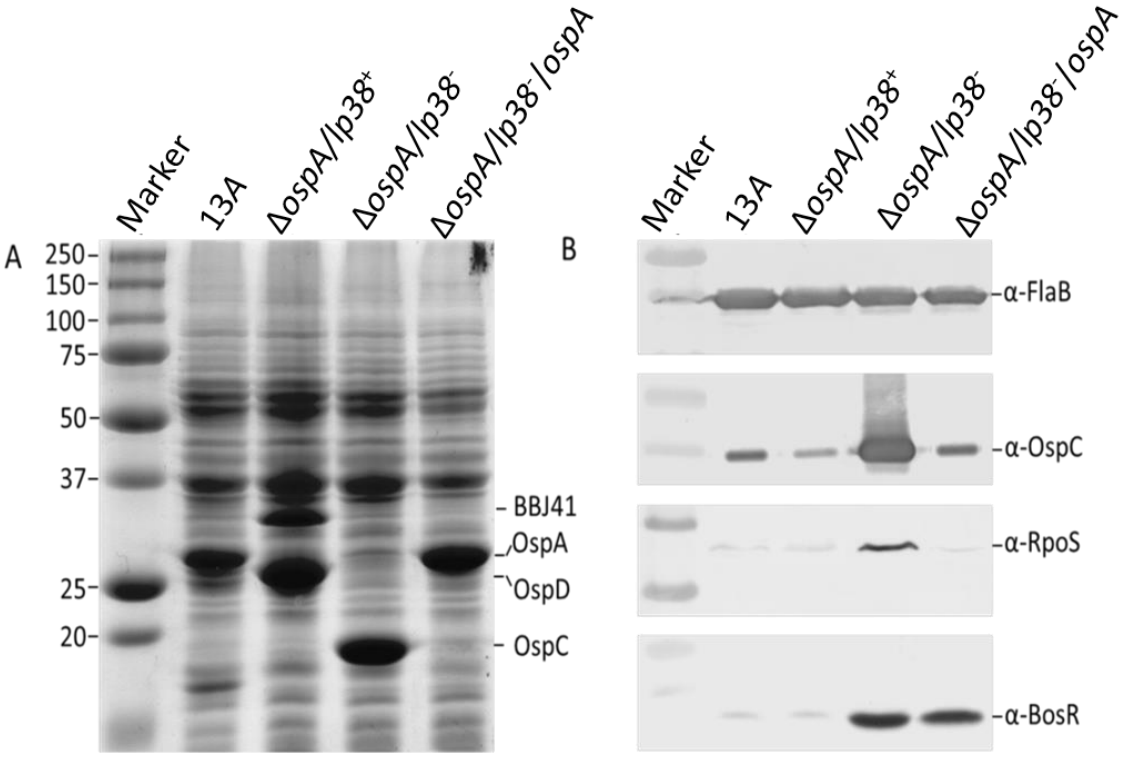

Figure 3.5. Complementation of *ospA* mutant represses BosR which represses RpoS and hence the RpoS induced OspC. (A) Coomassie stained SDS-PAGE showing clear reversal of compensatory OspC expression on complementation of *ospA* mutant with pBBE22-*ospA*. (B) Immunoblot probing with α-FlaB, α-OspC, α-RpoS, and α–BosR antibodies indicate significant reduction in BosR, RpoS, and OspC expression upon complementation of *ospA* mutant with *ospA*.

Homeostasis of the Osp layer

The advanced technology of cryo-electron microscopy allowed the observation of the density of the outer surface lipoprotein layer directly under cro-te. (Figure 3.6). The microscopic images clearly show that the density of osp layer did not change under distinct conditions with regard to the different dominant osps. All the *B. burgdorferi* strains including wild type 13A, *ospA* mutant lacking lp38 (consequently lacking OspD, BBJ41 & OspA) and *ospA* mutant retaining lp38 show similar density of osp layer (Figure 3.6). The same results are supported by the above experimental results which showed that the *ospA* mutant lacking lp38 induces RpoS and hence RpoS regulated osps like OspC & DbpA. This enables *ospA* mutant to retain contant osp layer density. Similarly in the *ospA* mutant with lp38, the overexpression of OspD and BBJ41 compensates for the loss of OspA and the density of the osp layer remains unchanged even in the absence of dominant *ospA* locus.

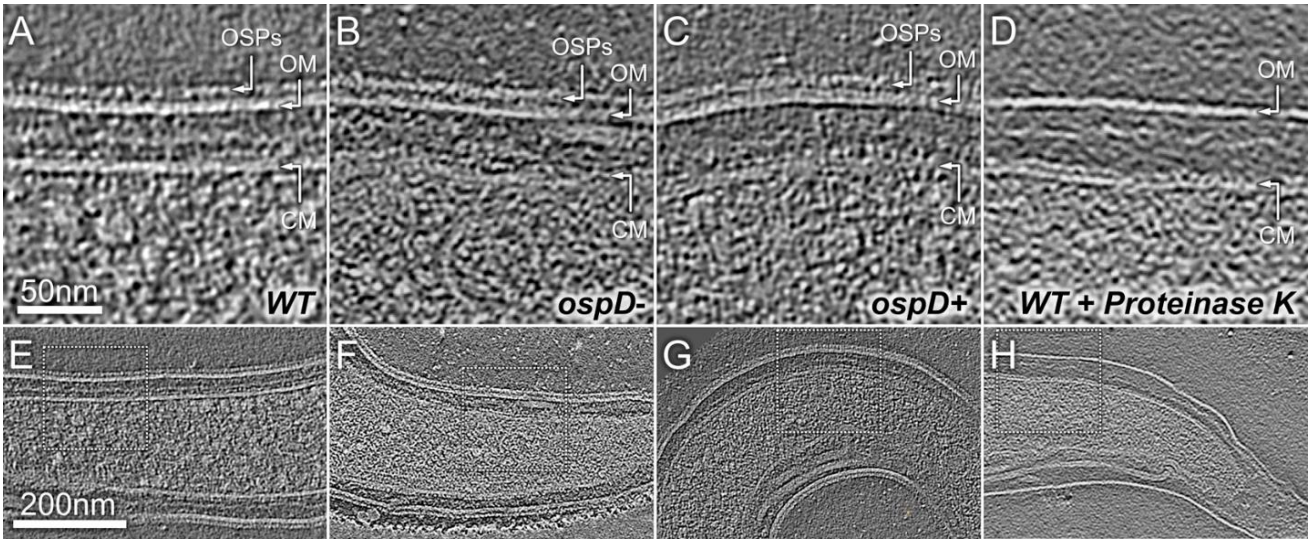

Figure 3.6. The overall density of the osp layer remains unchanged irrespective of the expression levels of each individual osp. The 13A, Δ*ospA* and *ΔospA/ospA* spirochetes were grown to early log phase and imaged using the Polara G2 electron microscope. Images A & E show osp layer for wild type *B. burgdorferi* 13A, Images B & F show osp layer for *ΔospA* lacking *ospD* containing lp38, Images C and G show osp layer for Δ*ospA/ospA,* and Images D and H shows proteinase treated wild type *B. burgdorferi* completely lacking the osp layer.

All these results taken together indicate that the osp layer in *B. burgdorferi* exists in a state of homeostasis. The regulation of this homeostasis takes priority over the normal osp expression and loss of one osp is compensated by expression of another osp.

Discussion

One feature of the Lyme disease spirochete, *Borrelia burgdorferi*, is to abundantly express outer surface lipoproteins (osps) and to form a lipoprotein layer to coat itself (Beck et al. 1985, Radolf et al. 1994). It has been known for years that the spirochete constantly modifies the component of the antigen layer to adapt to diverse environments and evade the immune system (Schwan 2003, Gutierrez Fernandez et al. 1997, Barbour 1991, Koomey 1997). A previous study has shown that maintenance of surface lipoprotein coat is central to survival of spirochete (Xu, McShan, and Liang 2008). However, the knowledge of interplay amongst several Outer surface lipoproteins is still incomplete and is a matter of investigation in borrelia research.

OspA is the most dominantly expressed osp when *B. burgdorferi* resides in tick and is grown *in-vitro*. During the generation of the *ospA* mutants in a previous stuy, it was noticed that, inactivation of *ospA* gene was more difficult than any other osp genes. Furthermore, it was observed that the *ospA* mutants grow unhealthily in vitro (Barbour et al. 1986). One explanation to these observations during the generation of *ospA* mutant can be that absence of OspA would cause a dramatic reduction in the density of the osp layer and hence *ospA* mutants that are not able to upregulate other osps in compensation of OspA fail to thrive well. Hence, deletion of *ospA* is lethal if the spirochetes do not increase the expression of other osps to compensate for the loss (Barbour et al. 1986).

The current study reconfirmed that the integrity of the lipoprotein layer is crucial for the basic survival of *B. burgdorferi* and that the requirement for maintaining this integrity is above the normal regulatory programs. The deletion of the *ospA* locus, which encodes the two most abundantly expressed osps when *B. burgdorferi* is grown in vitro or inhabits the tick vector (Howe, Mayer, and Barbour 1985, Beck et al. 1985), led to dramatic increase in expression of two other osps, namely OspD and BBJ41. Hence it can be stated that osps compensate for each other in order to maintain constant surface lipoprotein coat.

This was further corroborated as the complementation with the *ospA* gene alone completely repressed the two highly upregulated osps to an undetectable level and depletion of lp38, the plasmid that carries both *ospD* and *bbj41*, resulted in a constitutive expression of RpoS and its dependent osps like OspC. In addition to the above observations, the complementation of the lp38-deficient *ospA* mutant with *ospA* led to a significant repression of the constitutively expressed RpoS and its dependent osps.

Finally, the hypothesis regarding constant homeostatic maintenance of surface lipoprotein coat was confirmed by cryo-electron microscopy, which showed that the density of the osp layer remains constant regardless of expression levels of individual osps. Taken together, the study demonstrated that outer surface lipoprotein layer homeostasis overwhelms regulatory programs in *B. burgdorferi*. This theory should help in interpretation of many experimental results, such as inactivation of a major osp would lead to great upregulation of other ones. The understanding of interplay amongst different osps in biology of *B. burgdorferi*, may lead to new approaches for prevention and treatment of enigmatic Lyme disease.

References

Barbour, A. G. 1991. "Molecular biology of antigenic variation in Lyme borreliosis and relapsing fever: a comparative analysis." *Scand J Infect Dis Suppl*. 77:88-93.

Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumpf, and S. L. Tessier. 1986. "A Borrelia-specific monoclonal antibody binds to a flagellar epitope." *Infect Immun*. 52 (2):549-54.

Beck, G., G. S. Habicht, J. L. Benach, and J. L. Coleman. 1985. "Chemical and biologic characterization of a lipopolysaccharide extracted from the Lyme disease spirochete (*Borrelia burgdorferi*)." *J Infect Dis*. 152 (1):108-17.

Caimano, M. J., C. H. Eggers, C. A. Gonzalez, and J. D. Radolf. 2005. "Alternate sigma factor RpoS is required for the in vivo-specific repression of *Borrelia burgdorferi* plasmid lp54-borne ospA and lp6.6 genes." *J Bacteriol*. 187 (22):7845-52.

Caimano, M. J., C. H. Eggers, K. R. Hazlett, and J. D. Radolf. 2004. "RpoS is not central to the general stress response in *Borrelia burgdorferi* but does control expression of one or more essential virulence determinants." *Infect Immun*. 72 (11):6433-45.

Caimano, M. J., R. Iyer, C. H. Eggers, C. Gonzalez, E. A. Morton, M. A. Gilbert, I. Schwartz, and J. D. Radolf. 2007. "Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle." *Mol Microbiol*. 65 (5):1193-217.

Crother, T. R., C. I. Champion, J. P. Whitelegge, R. Aguilera, X. Y. Wu, D. R. Blanco, J. N. Miller, and M. A. Lovett. 2004. "Temporal analysis of the antigenic composition of *Borrelia burgdorferi* during infection in rabbit skin." *Infect Immun*. 72 (9):5063-72.

Cullen, P. A., D. A. Haake, and B. Adler. 2004. "Outer membrane proteins of pathogenic spirochetes." *FEMS Microbiol Rev*. 28 (3):291-318.

de Silva, A. M., S. R. Telford, 3rd, L. R. Brunet, S. W. Barthold, and E. Fikrig. 1996. "*Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine." *J Exp Med*. 183 (1):271-75.

Finehout, E. J., and K. H. Lee. 2003. "Comparison of automated in-gel digest methods for femtomole level samples." *Electrophoresis*. 24 (19-20):3508-16.

Fingerle, V., G. Goettner, L. Gern, B. Wilske, and U. Schulte-Spechtel. 2007. "Complementation of a Borrelia afzelii OspC mutant highlights the crucial role of OspC for dissemination of Borrelia afzelii in Ixodes ricinus." *Int J Med Microbiol*. 297 (2):97-107.

Fung, B. P., G. L. McHugh, J. M. Leong, and A. C. Steere. 1994. "Humoral immune response to outer surface protein C of *Borrelia burgdorferi* in Lyme disease: role of the immunoglobulin M response in the serodiagnosis of early infection." *Infect Immun*. 62 (8):3213-21.

Grimm, D., K. Tilly, R. Byram, P. E. Stewart, J. G. Krum, D. M. Bueschel, T. G. Schwan, P. F. Policastro, A. F. Elias, and P. A. Rosa. 2004. "Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals." *Proc Natl Acad Sci U S A*. 101 (9):3142-47.

Gutierrez Fernandez, J., M. Rodriguez Fernandez, F. Nunez Murillo, and M. C. Maroto Vela. 1997. "Antigenic and genetic structure of *Borrelia burgdorferi*." *Microbios*. 91 (368-369):165-74.

He, M., T. Oman, H. Xu, J. Blevins, M. V. Norgard, and X. F. Yang. 2008. "Abrogation of *ospA* constitutively activates the Rrp2-RpoN-RpoS pathway (sigmaN-sigmaS cascade) in *Borrelia burgdorferi*." *Mol Microbiol*. 70 (6):1453-64.

Howe, T. R., L. W. Mayer, and A. G. Barbour. 1985. "A single recombinant plasmid expressing two major outer surface proteins of the Lyme disease spirochete." *Science*. 227 (4687):645-46.

Koomey, M. 1997. "Bacterial pathogenesis: a variation on variation in Lyme disease." *Curr Biol*. 7 (9):R538-40.

Liang, F. T., M. B. Jacobs, L. C. Bowers, and M. T. Philipp. 2002. "An immune evasion mechanism for spirochetal persistence in Lyme borreliosis." *J Exp Med*. 195 (4):415-22.

Liang, F. T., F. K. Nelson, and E. Fikrig. 2002. "Molecular adaptation of *Borrelia burgdorferi* in the murine host." *J Exp Med*. 196 (2):275-80.

Liang, F. T., J. Yan, M. L. Mbow, S. L. Sviat, R. D. Gilmore, M. Mamula, and E. Fikrig. 2004. "*Borrelia burgdorferi* changes its surface antigenic expression in response to host immune responses." *Infect Immun*. 72 (10):5759-67.

Liu, J., T. Lin, D. J. Botkin, E. McCrum, H. Winkler, and S. J. Norris. 2009. "Intact flagellar motor of *Borrelia burgdorferi* revealed by cryo-electron tomography: evidence for stator ring curvature and rotor/C-ring assembly flexion." *J Bacteriol*. 191 (16):5026-36.

Mbow, M. L., R. D. Gilmore, Jr., and R. G. Titus. 1999. "An OspC-specific monoclonal antibody passively protects mice from tick-transmitted infection by *Borrelia burgdorferi* B31." *Infect Immun*. 67 (10):5470-72.

Ohnishi, J., J. Piesman, and A. M. de Silva. 2001. "Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks." *Proc Natl Acad Sci U S A*. 98 (2):670-75.

Pal, U., X. Yang, M. Chen, L. K. Bockenstedt, J. F. Anderson, R. A. Flavell, M. V. Norgard, and E. Fikrig. 2004. "OspC facilitates *Borrelia burgdorferi* invasion of Ixodes scapularis salivary glands." *J Clin Invest*. 113 (2):220-30.

Purser, J. E., M. B. Lawrenz, M. J. Caimano, J. K. Howell, J. D. Radolf, and S. J. Norris. 2003. "A plasmid-encoded nicotinamidase (PncA) is essential for infectivity of *Borrelia burgdorferi* in a mammalian host." *Mol Microbiol*. 48 (3):753-64.

Purser, J. E., and S. J. Norris. 2000. "Correlation between plasmid content and infectivity in *Borrelia burgdorferi*." *Proc Natl Acad Sci U S A*. 97 (25):13865-70.

Radolf, J. D., K. W. Bourell, D. R. Akins, J. S. Brusca, and M. V. Norgard. 1994. "Analysis of *Borrelia burgdorferi* membrane architecture by freeze-fracture electron microscopy." *J Bacteriol*. 176 (1):21-31.

Raetz, C. R., and C. Whitfield. 2002. "Lipopolysaccharide endotoxins." *Annu Rev Biochem*. 71:635-700.

Rosenfeld, J., J. Capdevielle, J. C. Guillemot, and P. Ferrara. 1992. "In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis." *Anal Biochem*. 203 (1):173-79.

Schwan, T. G. 2003. "Temporal regulation of outer surface proteins of the Lyme-disease spirochaete *Borrelia burgdorferi*." *Biochem Soc Trans*. 31 (Pt 1):108-12.

Schwan, T. G., and J. Piesman. 2000. "Temporal changes in outer surface proteins A and C of the lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice." *J Clin Microbiol*. 38 (1):382-88.

Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa. 1995. "Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding." *Proc Natl Acad Sci U S A*. 92 (7):2909-13.

Sears, J. E., E. Fikrig, T. Y. Nakagawa, K. Deponte, N. Marcantonio, F. S. Kantor, and R. A. Flavell. 1991. "Molecular mapping of Osp-A mediated immunity against *Borrelia burgdorferi*, the agent of Lyme disease." *J Immunol*. 147 (6):1995-2000.

Takayama, K., R. J. Rothenberg, and A. G. Barbour. 1987. "Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*." *Infect Immun*. 55 (9):2311-13.

Xu, Q., K. McShan, and F. T. Liang. 2007. "Identification of an ospC operator critical for immune evasion of *Borrelia burgdorferi*." *Mol Microbiol*. 64 (1):220-31.

Xu, Q., K. McShan, and F. T. Liang. 2008. "Essential protective role attributed to the surface lipoproteins of *Borrelia burgdorferi* against innate defences." *Mol Microbiol*. 69 (1):15-29.

Xu, Q., S. V. Seemanapalli, L. Lomax, K. McShan, X. Li, E. Fikrig, and F. T. Liang. 2005. "Association of linear plasmid 28-1 with an arthritic phenotype of *Borrelia burgdorferi*." *Infect Immun*. 73 (11):7208-15.

Xu, Q., Y. Shi, P. Dadhwal, and F. T. Liang. 2012. "RpoS regulates essential virulence factors remaining to be identified in *Borrelia burgdorferi*." *PLoS One*. 7 (12):e53212.

CHAPTER 4
GENERAL CONCLUSIONS AND DISCUSSION

Lyme disease is currently the most commonly reported vector borne disease in the United States (Bacon et al. 2008). The causative agent of Lyme disease *B. burgdorferi* sensu stricto, is mainly responsible for the disease in North America. In its North American cycle, *B. burgdorferi* mostly gets transmitted amongst small mammals (Levine, Wilson, and Spielman 1985). However, the Lyme disease transmission to humans is accidental and they act as the dead-end host. The bite of infected *Ixodes scapularis* on the east coast (and mid central) and *Ixodes pacificus* on the west coast (Anderson 1989, Anderson and Magnarelli 1980, Barbour 1998) acts as the main mode of transmission of Lyme disease.

The need of rapid adaptation to the discrete invertebrate and mammalian environments elicit impressive genetic manipulation by *B. burgdorferi*. The small genome of *B. burgdorferi* is coordinated by an intricate molecular mechanism still incompletely known and understanding the molecular mechanism of borrelial adaptation may lead to identification of suitable targets for development of effective Lyme disease prevention strategies.

In order to adapt and survive in the disparate environments *B. burgdorferi* has to differentially regulate its whole transcriptome, this global adjustment is accomplished through the use of RNA polymerase sigma factors. The genome of *B. burgdorferi* encodes only three sigma factors, the constitutive sigma factor RpoD (σ^{70}), and two alternate sigma factors, RpoN (σ^{54}) and RpoS (σ^{S}) (Fraser et al. 1997). The alternate sigma factors form a well-coordinated cascade where *rpoS* depends on RpoN for its transcription, which in turn relies on Rrp2 a phosphorylation activated protein (Yang, Alani, and Norgard 2003, Smith et al. 2007).

BosR is a recent addition to the key regulatory molecules known for the regulation of RpoN-RpoS pathway. Other than *bosR* itself, *rpoS* and *napA*/*bicA* are the only two other known genes that are directly activated by BosR (Ouyang et al. 2009, Wang et al. 2012). The previous studies have shown that the downregulation of OspA is mediated indirectly through RpoS via a DNA binding repressor molecule (Caimano et al. 2005). The present study shows that BosR can directly bind to the upstream regulatory sequences of *ospA* and *ospD* genes. It also proves that BosR causes direct repression of OspA by binding to *cisI* and *cisII* regulatory elements (Xu, McShan, and Liang 2010). Thus, this study provide sufficient evidence to support the hypothesis, that OspA downregulation is not essentially RpoS dependent and BosR acts as a direct repressor for OspA.

Furthermore, the present study also indicates that BosR mediates complete shut off of OspA only when the constitutive expression of other surface lipoproteins (OspC, DbpA and DbpB) is provided. This can be explained by the putative homeostasis existing on the outer surface membrane of *B. burgdorferi*. The surface lipoproteins play a protective role and hence downregulation of a major surface lipoprotein like OspA may not be complete till borrelia has other Osps to compensate (Yang et al. 2004, Xu, McShan, and Liang 2008). Even in the natural infectious cycle OspA downregulation is not 100%. As *B. burgdorferi* prepares to infect the mammalian host, downregulation of OspA occurs only in about 50% of the total spirochete population colonizing the tick midgut before migration to the tick salivary glands (Ohnishi, Piesman, and de Silva 2001). Hence OspA shutoff is difficult for *B. burgdorferi* even in nature.

Most of the differentially regulated genes in borrelia that participate in the adaptive response to an external environmental change, encode for one or the other outer surface lipoprotein. Being an interface between external and internal environment, outer surface lipoproteins play a central

role in survival adaptations of *B. burgdorferi* (Xu, McShan, and Liang 2008). Furthermore, there have been several studies which have pointed the homeostatic balance in osp expression on the outer membrane of *B. burgdorferi*. One such study showed that the deletion of the *ospA* operon caused constitutive activation of the Rrp2-RpoN-RpoS pathway and hence osps controlled by it like OspC, DbpA and DbpB (He et al. 2008). In another such study, the *ospA* mutant appeared to have increased expression of several outer surface lipoproteins, but none of these were RpoS regulated (Battisti et al. 2008). Similarly, an *ospC* mutant showed upregulation of several other lipoproteins regulated by the RpoS regulon (Pal et al. 2004). The present study proves the existence of osp layer homeostasis and presents two new osps, OspD and BBJ41, wich compensate for the absence of dominat OspA and maintain outer surface homeostasis.

Many putative lipoproteins encoded by *B. burgdorferi* have been implicated in corroborating tick vector colonization and disease transmission, however, precise mechanisms and function of most of these remains largely unknown. The present study adds to knowledge of osp regulation as it shows how BosR acts a repressor for OspA.

In the natural infectious cycle the downregulation of OspA on surface is compensated by upregulation of OspC as *B. burgdorferi* prepares to infect the host. This crossregulation of OspA-OspC has been demonstrated as a key factor for causing infection in mammalian host. Further in its infection phase, borrelia downregulates even OspC and replaces it by VlsE outer surface protein. Thus, broadly, Osps of borrelia protect it from mammalian immune system, enable its successful persistence in tick vector and ensures continuation of the natural infection cycle (Xu, McShan, and Liang 2008).

Exact regulatory mechanisms involved in controlling the osp expression are complex and still largely unknown. However, OspA is one such significant osp which forms a dominant

expression on the surface while *B. burgdorferi* lies dormant in an unfed tick midgut, it is rapidly downregulated in response to blood meal from an uninfected mammalian host. The rapid downregulation of OspA is important to protect borrelia from mammalian immune system as the antibodies against OspA once produced can eliminate *B.burgdorferi* from it enzootic cycle. Therefore, OspA has been an attractive target for development of vaccine against Lyme disease and, also a vaccine using an immunogenic recombinant *B. burgdorferi* outer surface protein (OspA) was licensed by the US Food and Drug Administration (FDA). Unfortunately, vaccine was withdrawn citing multiple concerns. Understanding the differential regulation of *ospA* may lead us for identification of additional target for disruption of the homeostasis of outer surface proteins required for the survival of borrelia in different host environment. The present study deals specifically with questions pertaining to downregulation of OspA and what happens on borrelial surface in its absence.

While the incidence of Lyme disease continues to increase along with its expanding endemic area, the ambiguous clinical manifestations still pose a challenge to early diagnosis of Lyme disease. Though antibiotic therapy is currently available for treating Lyme disease in early stages, it is still not successful in treating antibiotic refractory Lyme arthritis and post lyme disease syndrome (chronic lyme disease). Hence the current tools for prevention, diagnose, and treatment of Lyme disease are limited.

The main goal for the studies described in this dissertation is to delve further into the molecular mechanisms of differential gene regulation and function of outer surface lipoprotein in the infectious life cycle of *B. burgdorferi*. One part of this study adds to such observations where the absence of OspA in an *ospA* deficient *B. burgdorferi* is compensated by increased OspD and BBJ41 expression. Both these tick phase surface lipoproteins are not expressed in mammalian

host and need to be investigated for their potential as vaccine targets. The electron microscopy results clearly show maintenance of constant lipoprotein density on the outer membrane layer in *B. burgdorferi* irrespective of the osp gene mutation. This study for the first time clarifies well speculated homeostatic osp maintenance on the borrelial surface, which is critical for development of intervention strategies for the Lyme disease. Our study has re-emphasized the need for further investigations into the alternative mechanisms of downregulation of OspA, independent to RpoS.

Understanding the gene regulation and outer surface lipoprotein layer functioning can potentially provide important targets for interrupting *B. burgdorferi* infectious cycle and development of vaccine against Lyme disese.

References

Anderson, J. F. 1989. "Epizootiology of Borrelia in Ixodes tick vectors and reservoir hosts." *Rev Infect Dis*. 11 Suppl 6:S1451-9.

Anderson, J. F., and L. A. Magnarelli. 1980. "Vertebrate host relationships and distribution of ixodid ticks (Acari: Ixodidae) in Connecticut, USA." *J Med Entomol*. 17 (4):314-23.

Bacon, R. M., K. J. Kugeler, P. S. Mead, Control Centers for Disease, and Prevention. 2008. "Surveillance for Lyme disease United States, 1992-2006." *MMWR Surveill Summ*. 57 (10):1-9.

Barbour, A. G. 1998. "Fall and rise of Lyme disease and other Ixodes tick-borne infections in North America and Europe." *Br Med Bull*. 54 (3):647-58.

Battisti, J. M., J. L. Bono, P. A. Rosa, M. E. Schrumpf, T. G. Schwan, and P. F. Policastro. 2008. "Outer surface protein A protects Lyme disease spirochetes from acquired host immunity in the tick vector." *Infect Immun*. 76 (11):5228-37.

Caimano, M. J., C. H. Eggers, C. A. Gonzalez, and J. D. Radolf. 2005. "Alternate sigma factor RpoS is required for the in vivo-specific repression of *Borrelia burgdorferi* plasmid lp54-borne ospA and lp6.6 genes." *J Bacteriol*. 187 (22):7845-52.

Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg,

M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. Weidman, T. Utterback, L. Watthey, L. McDonald, P. Artiach, C. Bowman, S. Garland, C. Fuji, M. D. Cotton, K. Horst, K. Roberts, B. Hatch, H. O. Smith, and J. C. Venter. 1997. "Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*." *Nature*. 390 (6660):580-86.

He, M., T. Oman, H. Xu, J. Blevins, M. V. Norgard, and X. F. Yang. 2008. "Abrogation of *ospA* constitutively activates the Rrp2-RpoN-RpoS pathway (sigmaN-sigmaS cascade) in *Borrelia burgdorferi*." *Mol Microbiol*. 70 (6):1453-64.

Levine, J. F., M. L. Wilson, and A. Spielman. 1985. "Mice as reservoirs of the Lyme disease spirochete." *Am J Trop Med Hyg*. 34 (2):355-60.

Ohnishi, J., J. Piesman, and A. M. de Silva. 2001. "Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks." *Proc Natl Acad Sci U S A*. 98 (2):670-75.

Ouyang, Z., M. Kumar, T. Kariu, S. Haq, M. Goldberg, U. Pal, and M. V. Norgard. 2009. "BosR (BB0647) governs virulence expression in *Borrelia burgdorferi*." *Mol Microbiol*. 74 (6):1331-43.

Pal, U., X. Yang, M. Chen, L. K. Bockenstedt, J. F. Anderson, R. A. Flavell, M. V. Norgard, and E. Fikrig. 2004. "OspC facilitates *Borrelia burgdorferi* invasion of Ixodes scapularis salivary glands." *J Clin Invest*. 113 (2):220-30.

Smith, A. H., J. S. Blevins, G. N. Bachlani, X. F. Yang, and M. V. Norgard. 2007. "Evidence that RpoS (sigmaS) in *Borrelia burgdorferi* is controlled directly by RpoN (sigma54/sigmaN)." *J Bacteriol*. 189 (5):2139-44.

Wang, P., A. Lutton, J. Olesik, H. Vali, and X. Li. 2012. "A novel iron- and copper-binding protein in the Lyme disease spirochaete." *Mol Microbiol*. 86 (6):1441-51.

Xu, Q., K. McShan, and F. T. Liang. 2008. "Essential protective role attributed to the surface lipoproteins of *Borrelia burgdorferi* against innate defences." *Mol Microbiol*. 69 (1):15-29.

Xu, Q., K. McShan, and F. T. Liang. 2010. "Two regulatory elements required for enhancing ospA expression in *Borrelia burgdorferi* grown in vitro but repressing its expression during mammalian infection." *Microbiology*. 156 (Pt 7):2194-204.

Yang, X. F., S. M. Alani, and M. V. Norgard. 2003. "The response regulator Rrp2 is essential for the expression of major membrane lipoproteins in *Borrelia burgdorferi*." *Proc Natl Acad Sci U S A*. 100 (19):11001-06.

Yang, X. F., U. Pal, S. M. Alani, E. Fikrig, and M. V. Norgard. 2004. "Essential role for OspA/B in the life cycle of the Lyme disease spirochete." *J Exp Med*. 199 (5):641-48.

APPENDIX A
PERMISSION FOR USE OF THE PUBLISHED TEXT

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3400451391736
License date	Jun 01, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	Molecular Microbiology
Licensed content title	Borrelia burgdorferi oxidative stress regulator BosR directly represses lipoproteins primarily expressed in the tick during mammalian infection
Licensed copyright line	© 2013 John Wiley & Sons Ltd
Licensed content author	Peng Wang,Poonam Dadhwal,Zhihui Cheng,Michael R. Zianni,Yasuko Rikihisa,Fang Ting Liang,Xin Li
Licensed content date	Aug 14, 2013
Start page	1140
End page	1153
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Text extract
Number of Pages	2
Will you be translating?	No
Title of your thesis / dissertation	Outer surface lipoprotein layer homeostasis and gene regulation in Borrelia burgdorferi
Expected completion date	Aug 2014

APPENDIX B
PERMISSION FOR USE OF THE PUBLISHED FIGURES

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3400451172073
License date	Jun 01, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	Molecular Microbiology
Licensed content title	Borrelia burgdorferi oxidative stress regulator BosR directly represses lipoproteins primarily expressed in the tick during mammalian infection
Licensed copyright line	© 2013 John Wiley & Sons Ltd
Licensed content author	Peng Wang,Poonam Dadhwal,Zhihui Cheng,Michael R. Zianni,Yasuko Rikihisa,Fang Ting Liang,Xin Li
Licensed content date	Aug 14, 2013
Start page	1140
End page	1153
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	4
Original Wiley figure/table number(s)	Figure1, Figure2, Figure3, and Figure4
Will you be translating?	No
Title of your thesis / dissertation	Outer surface lipoprotein layer homeostasis and gene regulation in Borrelia burgdorferi
Expected completion date	Aug 2014

VITA

Poonam Dadhwal was born in India, in a small village in Punjab. She completed her basic high school education in Chandigarh, India and qualified entrance examination for Veterinary Sciences in 2002. She was awarded a Bachelor of Veterinary Sciences and Animal Husbandry (equivalent to a DVM in the United States) by Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) in Ludhiana, India in July of 2008. During her veterinary studies, she was awarded a university scholarship, awarded only to the top 10% of students, one the most prestigious fellowships for veterinary students in the country. She was accepted for PhD program in the Department of Pathobiological Sciences at Louisiana State University with a full LSU Flagship Fellowship in 2008. She joined Dr. Fang-Ting Liang's laboratory in the Department of Pathobiological Sciences, at the School of Veterinary Medicine.

Under the guidance of Dr. Fang-Ting Liang, she studied the role of the outer surface protein C (OspC) in self-regulation and in facilitating the dissemination of *Borrelia burgdorferi* during murine infection. In December 2014, Dadhwal will be finishing her dissertation research and comple the requirements for the degree of Doctor of Philosophy in Veterinary Medical Sciences.