THE ROLE OF THE *BORRELIA* OXIDATIVE STRESS REGULATOR PROTEIN IN VIRULENCE GENE EXPRESSION OF THE LYME DISEASE SPIROCHETE

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For my mother, who left us too suddenly and too soon…

ACKNOWLEDGEMENTS

To my amazing family, thank you for keeping me grounded and for putting up with my insanity from the moment I learned to talk. We have had a rough few years but we found smiles and laughter no matter how hard it got and now the rainbow is peeking out behind the last dark cloud!

Eric, my soulmate, I am so glad I found you… The hard right turn we took at high speed has brought us to the greatest adventure of our lives!

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I sincerely thank my committee, Dr. Stanley Spinola, Dr. Margaret Bauer and Dr. Raymond Johnson, for helping to steer my research in the right direction.

I would also like to convey my appreciation to the ladies in our department offices, especially Cindy Booth, Janis Stringer and Cathy Collins, for making sure that everything runs smoothly for the rest of us.

"I can be changed by what happens to me, but I refuse to be reduced by it."

— *Maya Angelou*

ABSTRACT

Joleyn Yean Chern Khoo

THE ROLE OF THE *BORRELIA* OXIDATIVE STRESS REGULATOR PROTEIN IN VIRULENCE GENE EXPRESSION OF THE LYME DISEASE SPIROCHETE

The Lyme disease agent, *Borrelia burgdorferi*, has a complex system that allows it to thrive in the harsh and distinct environments of its tick vector and mammalian host. Although it has been known for some time that the *Borrelia* oxidative stress regulator protein (BosR) plays a necessary role in mammalian infectivity and functions as a transcriptional regulator of alternative sigma factor RpoS, very little is known about its mechanism of action, other than the suggestion that BosR activates *rpoS* transcription by binding to certain upstream regions of the gene. In our studies, we performed protein degradation assays and luciferase reporter assays for further understanding of BosR function. Our preliminary findings suggest that BosR is post-transcriptionally regulated by an unknown protease and may not need to bind to any *rpoS* upstream regions in order to activate transcription. We also describe the construction of luciferase reporter systems that will shed light on BosR's mechanism of action. We postulate the provocative possibility that unlike its homologs Fur and PerR in other bacterial systems, BosR may not utilize a DNA-binding mechanism in order to fulfill its role as a transcriptional regulator to modulate virulence gene expression.

X. Frank Yang, Ph.D, Chair

TABLE OF CONTENTS

INTRODUCTION

Borrelia burgdorferi, the deer tick and the accidental host

Lyme disease is the most common arthropod-borne disease in the United States of America. Statistics published by the Centers for Disease Control and Prevention (CDC) show that the number of confirmed cases have been on a general upward trend since 1992, when 9,908 cases were reported, to 2011, when 24,364 cases were reported. Most of the cases tend to be concentrated in the Northeast and North Central regions although it must be noted that the cases are reported by county of residence, not county of infection (Bacon *et al.*, 2008; CDC, 2012). Lyme disease was first characterized in 1977 when cases of juvenile rheumatoid arthritis inflicting both adults as well as children in three communities were reported (Steere *et al.*, 1977) and subsequently found to correlate with the presence of the deer tick, *Ixodes scapularis*, also known as *Ixodes dammini* (Steere *et al.*, 1978). Later, the causative agent was isolated from its tick vector and determined to be a new species of gram-negative spirochetal bacteria (Burgdorfer *et al.*, 1982; Steere *et al.*, 1983) and subsequently named *Borrelia burgdorferi* (Johnson *et al.*, 1984). The early symptoms of Lyme disease include influenza-like symptoms such as fever, fatigue, chills and headaches, as well as erythema migrans, a characteristic bulls-eye rash at the site of the tick bite that is a hallmark of the disease. Antibiotics are generally effective first line treatments in this early stage although there are cases in which persistent infection occurs. Unfortunately, not all cases present with erythema migrans, making Lyme disease difficult to diagnose since the disease shares symptoms with other more common diseases. Left untreated, it can progress to debilitating

symptoms such as Bell's palsy, meningitis, arrhythmia and chronic arthritis (Schoen, 1991; Burgdorfer, 1991; Cooke and Dattwyler, 1992; Steere, 2001; Wright, 2012).

Despite rising awareness about Lyme borreliosis and its danger to public health, a United States Food and Drug Administration (FDA)-approved recombinant vaccine received poor public response. The vaccine prevented transmission of *B. burgdorferi* from ticks to humans. It was made available to the public in December 1998 but was discontinued in February 2002. Among the suggested reasons for the bad reception of the vaccine were cost effectiveness, the need for multiple booster shots and questions about the possibility of autoimmune reaction (Hayes and Piesman, 2003; Clark and Hu, 2008; Embers and Narasimhan, 2013). Current recommended preventive measures include avoidance of ticks, the use of tick repellant, the use of protective clothing, vegetation removal at high-risk residential areas, or sometimes antimicrobial prophylaxis (Poland, 2001; Hayes and Piesman, 2003; Wormser *et al.*, 2006; Vazquez *et al.*, 2008; Clark and Hu, 2008).

In the years since its discovery, the enzootic life cycle of *B. burgdorferi* has been well-studied, as depicted in Figure 1. The spirochete is transmitted when uninfected deer tick larvae feed on small infected mammals such as mice. The fed larva then molts into an infected nymph which feeds on other (uninfected) mammals, thereby perpetuating the transmission cycle. It is usually in this nymphal stage that humans become inadvertent hosts because the seasonal appearance of blood-seeking nymphs from May to July coincides with the peak of human summer outdoor activities in July. It is interesting to note that the white-tailed deer, *Odocoileus virginianus*, has been linked to the maintenance of *B. burgdorferi* in nature even though it is apparently resistant to infection

by the spirochete. It appears that the deer is simply an important host for *I. scapularis* adults, whereas it is the white-footed mouse, *Peromyscus leucopus*, which has been shown to be the natural reservoir of *B. burgdorferi*. However, other small- and medium-sized mammals such as birds, squirrels, chipmunks, raccoons and opossums can also maintain the spirochetes and are hosts to questing deer ticks (Lane *et al.*, 1991; Fish, 1995; Clark and Hu, 2008; Radolf *et al.*, 2012). The molecular mechanisms behind the fastidious spirochete's ability to adapt to two very distinct environments have been rigorously studied but much remains to be understood in order to eradicate the debilitating disease associated with it.

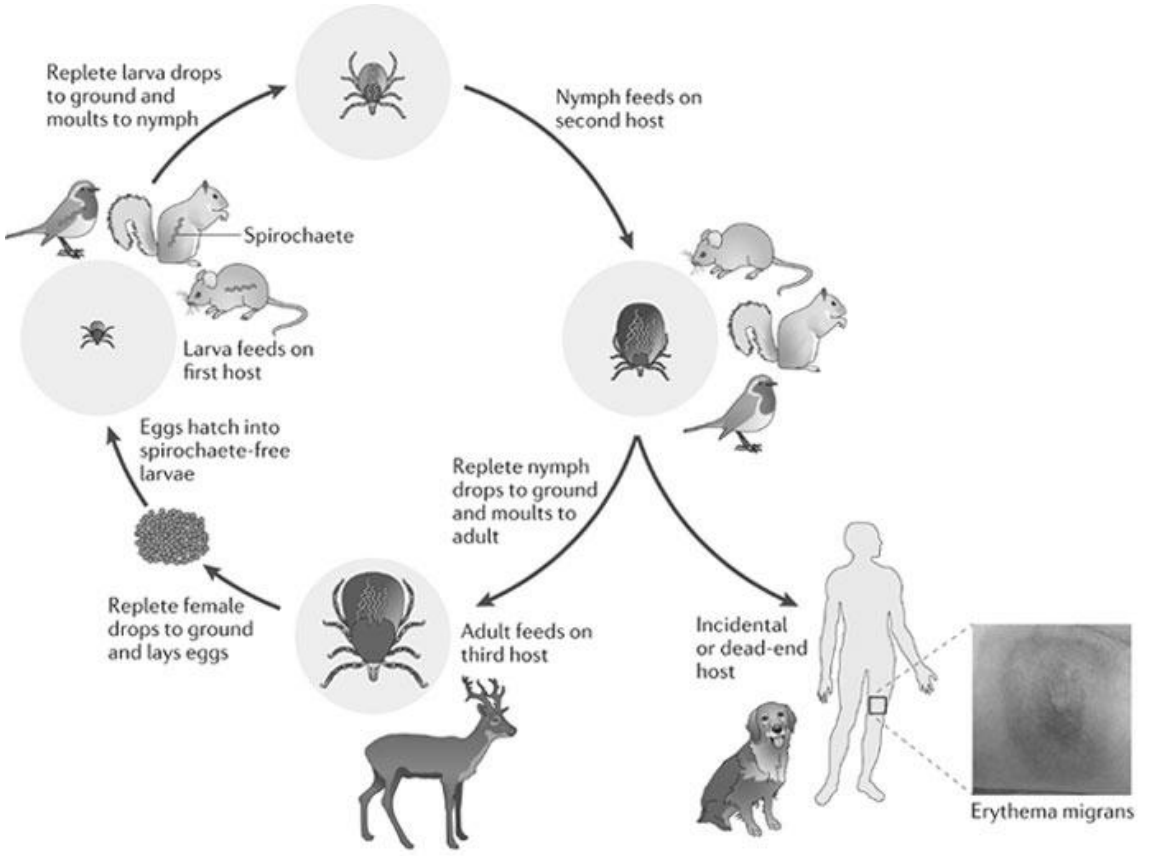

Replete larva drops to ground and moults to nymph
Nymph feeds on second host
Spirochaete
Larva feeds on first host
Eggs hatch into spirochaete-free larvae
Replete nymph drops to ground and moults to adult
Replete female drops to ground and lays eggs
Adult feeds on third host
Incidental or dead-end host
Erythema migrans
Nature Reviews | Microbiology

Figure 1. The life cycle of *Borrelia burgdorferi* and its relation to human infection. Larval and nymphal ticks tend to feed on small- to medium-sized mammals, whereas adults generally prefer large mammals such as deer and also carry out their sexual reproduction on the large hosts (Radolf *et al.*, 2012).

Reciprocal regulation of major membrane lipoproteins

It has been shown that *B. burgdorferi*'s aptitude at surviving so proficiently when moving between invertebrate vector and vertebrate host relies on differential expression of two major outer membrane lipoproteins, outer surface protein A (OspA) and outer surface protein C (OspC). Several groups have observed that OspA is expressed in unfed nymphal ticks whereas OspC is virtually undetectable, but OspA is downregulated during nymphal tick feeding whereas OspC is upregulated (Schwan *et al*., 1995; Montgomery *et al*., 1996; de Silva *et al.*, 1996; Schwan and Piesman, 2000; Gilmore and Piesman, 2000; Fingerle *et al.*, 2002). Figure 2 illustrates the reciprocal regulation of OspA and OspC in the *B. burgdorferi* transmission cycle (Mulay *et al.*, 2009). This reciprocal regulation can be mimicked in the laboratory by incubating *Borrelia* cultures in different temperatures—at 23°C, OspA is upregulated whereas OspC is virtually undetectable, and at 37°C, OspC is upregulated whereas OspA is downregulated (Schwan *et al.*, 1995; Yang *et al.*, 2000). Naturally, the next step was for investigators to elucidate the molecular mechanism behind this important differential gene expression. A breakthrough came when the Norgard group showed that OspC was controlled by the alternative sigma factor RpoS (σ^S or σ^{38}), which was in turn controlled by another alternative sigma factor, RpoN (σ^N or σ^{54}) (Yang *et al.*, 2000; Hübner *et al.*, 2001; Yang *et al.*, 2003a). OspA was subsequently shown to be required for tick infection (Yang *et al.*, 2004; Pal *et al.*, 2004b) and conversely, OspC was required for mammalian infection (Grimm *et al.*, 2004; Pal *et al.*, 2004a).

The Rrp2-RpoN-RpoS pathway

Sigma factors accord specificity to what would otherwise be indiscriminate and weak gene transcription by bacterial RNA polymerase (RNAP). Sigma factors are divided into the σ^{70} family and the σ^{54} family. RpoN (σ^{N} or σ^{54}) and its orthologs are the only members of the σ^{54} family whereas all the other sigma factors are lumped into the σ^{70} family. While the σ^{70} family sigma factors recognize canonical -35/-10 promoter sequences, σ^{54} recognizes a unique promoter with conserved GG and GC sequences at the -24 and -12 regions respectively. The number of sigma factors from the σ^{70} family varies according to the microbe (for example, *Streptomyces coelicolor* has 63 whereas *Escherichia coli* has six), but microbes tend to only have one σ^{54}-type sigma factor, if any at all. RNAPs are composed of the subunits $\alpha_2\beta\beta'\omega$ and it is the β and β' subunits that form an association with a sigma factor to create the functional RNAP holoenzyme (Merrick, 1993; Barrios *et al.*, 1999; Buck, *et al.*, 2000; Studholme and Buck, 2000b; Gruber and Gross, 2003; Ghosh *et al.*, 2010; Österberg *et al.*, 2011; Bush and Matthew, 2012; Lee *et al.*, 2012).

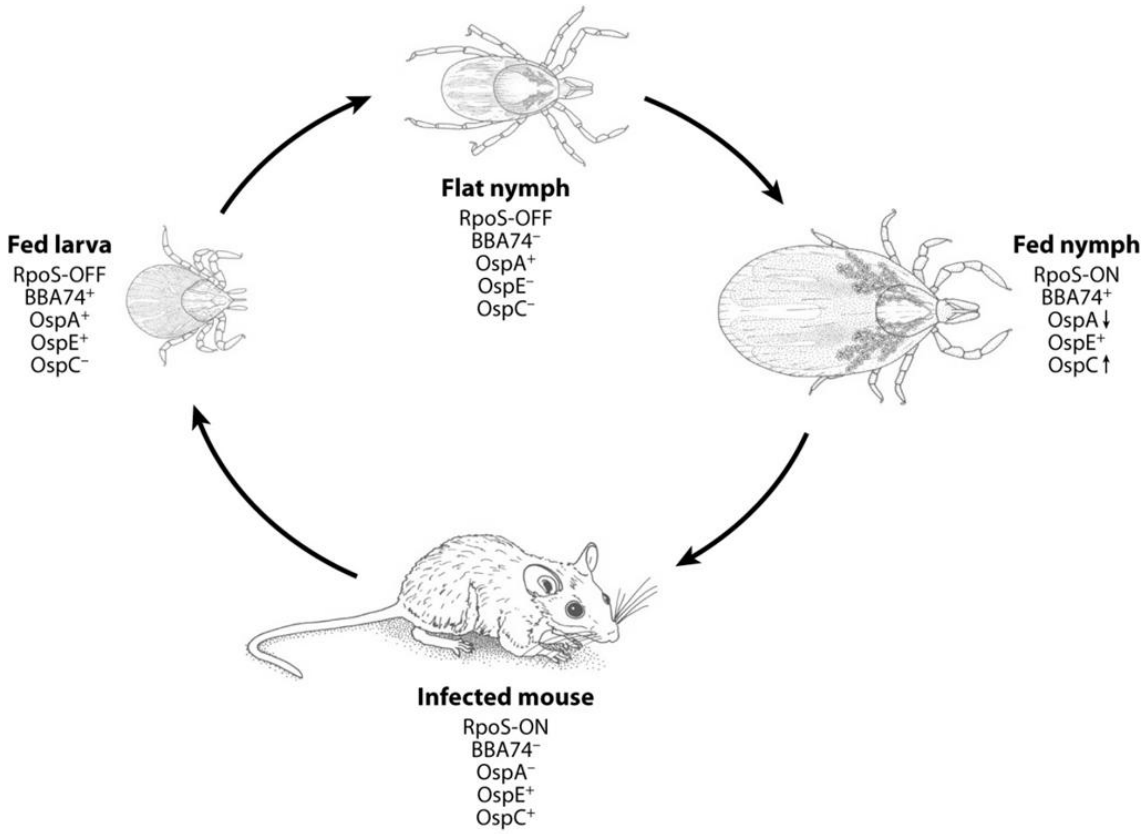
Based on *B. burgdorferi*'s published full genomic sequence, it appears that besides RpoD (σ^{70}, known also as the "housekeeping" sigma factor because of its role in transcription initiation of most genes in all bacteria), the spirochete encodes only two alternative sigma factors in its genome—RpoS and RpoN (Fraser *et al.*, 1997). RpoS is a widely distributed sigma factor associated with stress adaptation in other pathogenic bacteria such as *E. coli* (Hengge-Aronis, 1993), *Salmonella spp.* (Fang *et al.*, 1992), and *Pseudomonas aeruginosa* (Suh *et al.*, 1999) but variably associated with virulence (Dong and Schellhorn, 2010). As such, it was assumed that RpoS would follow a similar pattern

in *B. burgdorferi* and regulate the general stress response but this was disproved by the Radolf group when they found that RpoS was not essential in spirochete survival during environmental stresses (Caimano *et al.*, 2004). However, they and the Norgard group did find that RpoS controls other genes that are required for virulence such as OspC and decorin binding protein A (DbpA) and was essential for mammalian infection, being upregulated in mice but downregulated in ticks (Figure 2) (Yang *et al.*, 2000; Hübner *et al.*, 2001; Yang *et al.*, 2003; Caimano *et al.*, 2004; Fisher *et al.*, 2005; Yang *et al.*, 2005; Samuels, 2011)**.** It was shown that RpoS expression in mammals and ticks could be mimicked *in vitro*, with growth at 23°C mirroring tick conditions and growth at 37°C mirroring mammalian conditions (Yang *et al*., 2000).

The other sigma factor encoded in the *B. burgdorferi* genome, RpoN, is also commonly found in other gram-negative pathogens such as *E. coli*, *P. aeruginosa* and *Helicobacter pylori*, where it has been implicated in the control of several pathways including nitrogen assimilation (for which the 'N' in RpoN and σ^N stand for), formate metabolism and flagellin synthesis (Totten *et al.*, 1990; Merrick, 1993; Reitzer and Schneider, 2001; Smith *et al.*, 2009). RpoN's singular trademark of recognizing -24/-12 promoter sequences instead of the usual -35/-10 clued the Norgard group in on the novel possibility of RpoN-dependent transcription of *rpoS* when a study reported the possible presence of the -24/-12 consensus sequence immediately upstream of the *rpoS* gene in *B. burgdorferi* (Studholme and Buck, 2000a). The Norgard group subsequently showed that not only was *rpoS* transcription dependent on RpoN, it was also directly controlled by RpoN (Smith *et al.*, 2007; Burtnick *et al*., 2007). To date, evidence of RpoN-dependent

RpoS transcription has only been published for *B. burgdorferi* and no other bacterial system.

Unlike the σ^{70} family, RpoN requires the aid of an enhancer-binding protein (EBP) with ATPase activity in order to initiate gene transcription. RpoN-dependent EBPs generally bind relatively far upstream (85-150 bp) of the transcriptional start site causing DNA to loop and isomerizing the closed complex of DNA bound to the RpoN-RNA polymerase holoenzyme into an open complex (Merrick, 1993; Morett and Segovia, 1993; Buck, *et al.*, 2000; Gruber and Gross, 2003; Österberg *et al.*, 2011; Shingler, 2011). Accordingly, the work of Yang *et al*. pointed to Rrp2, one of only two putative two-component response regulators and the only predicted EBP in *B. burgdorferi* (Yang *et al.*, 2003b; Fraser *et al.*, 1997), as the RpoN-dependent EBP activating the RpoN-RpoS pathway. The Gherardini group corroborated the finding, but reported the unexpected discovery that Rrp2 did not appear to require binding to predicted enhancer sequences upstream of RpoN-dependent genes in order to initiate transcription. Figure 3a illustrates their proposed pathway model (Burtnick *et al.*, 2007). Blevins *et al*. confirmed the results, finding that *B. burgdorferi* mutants with a minimal RpoN-dependent *rpoS* promoter that did not include the predicted enhancer elements were enough to cause infectivity in mice (Blevins *et al.*, 2009).



Samuels DS. 2011.
Annu. Rev. Microbiol. 65:479–99

Figure 2. Differential expression of OspA and OspC in a reciprocal manner corresponding to RpoS expression pattern (Samuels, 2011). BBA74 and OspE are not addressed in this dissertation.

BosR, another activator of the Rrp2-RpoN-RpoS pathway

B. burgdorferi further confounded researchers when they discovered that not only did the spirochete possess an EBP with unorthodox qualities in Rrp2, it now appeared that there was also a second transcriptional activator for the same novel pathway activated by Rrp2. Although BB0647—known as BosR for *Borrelia* oxidative stress regulator protein—has been scrutinized as far back as 2003 (Boylan *et al.*, 2003), it was not until 2009 and 2010 that the Norgard and Skare groups drew a possible link between BosR and the Rrp2-RpoN-RpoS pathway in two separate papers. The Skare group saw that the increased expression of OspC and decorin binding protein A (DbpA) correlated with the increased expression of BosR in their IPTG-induced conditional mutant (Hyde *et al.*, 2010). The Norgard group reported a similar result with their *bosR* deletion strain and further found that RpoS was also abrogated in the absence of BosR. Importantly, they also discovered that BosR is required for transmission and infectivity in mice and that the absence of BosR correlated with the absence of RpoS, but not of Rrp2 (Ouyang *et al.*, 2009).

BosR was initially designated as ferric uptake regulation protein (Fur) homolog when the full *B. burgdorferi* annotated genome was published (Fraser *et al.*, 1997). However, a study published in 2000 challenged the naming of the protein when it suggested that *B. burgdorferi* does not require iron, one of the very few microbes to possess that trait (Posey and Gherardini, 2000; Andrews *et al.*, 2003). It is well-documented that Fur and its homologs have been found in a wide variety of gram-negative as well as gram-positive bacterial species, most notably in pathogenic species such as *Salmonella typhimurium*, *Yersinia pestis* and *Listeria monocytogenes*. As its

name suggests, Fur controls iron homeostasis but it also functions as a global regulator of genes that are both iron-related and non-iron-related such as genes involved in flagellum assembly and colonization (Escolar *et al.*, 1999; Carpenter *et al.*, 2009). The Gherardini group published the first study on BB0647, suggesting that it appeared to be more similar to the peroxide stress response regulator (PerR), a member of the Fur family of transcriptional regulator proteins, in *Bacillus subtilis* based on a 50.7% sequence homology, but found that it did not share the same functions. They proposed that while PerR is a transcriptional repressor, it appeared that its *B. burgdorferi* homolog is a transcriptional activator, and thereafter named the protein BosR. They found that BosR activates transcription of *napA* which is an oxidative stress regulator gene (Boylan *et al.*, 2003). However, Katona *et al* referred to BosR as Fur in their 2004 paper, and argued that it functioned as a repressor when exposed to peroxide stress.

Using *in vitro* DNA footprinting studies, Ouyang *et al.* suggested that BosR binds to three regions very close to the *rpoS* promoter which they termed Binding Site (BS) 1, BS2 and BS3. The *rpoS* -24/-12 promoter (P*rpoS*) is part of the BS2 sequence (Figure 3c). It appeared that BosR bound with much higher affinity to BS2 compared to BS1 and BS3 and that there was a consensus sequence of TAAATTAAAT in BS2, similar to the AT-rich consensus sequences of Fur and PerR-binding boxes (Ouyang *et al.*, 2011). However, prior to that study, Blevins *et al.* had indirectly shown that BS1 and the TAAATTAAAT portion of BS2 directly in front of P*rpoS* (Figure 3c) are not required for BosR-induction of *rpoS* transcription because BS3 together with what was left of BS2 was sufficient to rescue RpoS protein expression as well as the expression of two RpoS-dependent proteins, OspC and DbpA, in an *rpoS* deletion mutant of *B. burgdorferi*

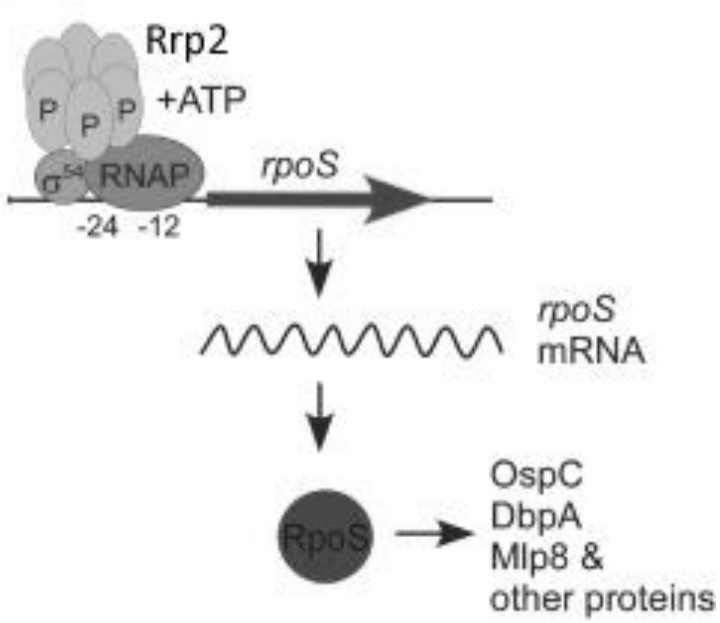
(*rpoS*-, Lane 2) as seen in Figure 4, albeit with decreased levels as compared to wild-type strain 297 (Lane 1) (Blevins *et al.*, 2009). Nonetheless, the minimal P*rpos*-complemented mutant strain (*rpoS*-/pJSB298, Lane 4) displayed similar protein expression levels as the fully-complemented mutant strain (*rpoS*-/pJSB259, Lane 3), suggesting that the decrease was probably due to other factors such as transcriptional stability. Importantly, the *rpoS* deletion strain complemented with a minimal P*rpoS* containing a point mutation in the -24/-12 region (Lane 5) could not rescue the expression of the three proteins. Ouyang *et al* themselves remarked that their finding of BosR binding tightest to BS2 as compared to BS1 and BS3 could be "physiologically irrelevant." It must be pointed out that BS3 was intact in the Blevins *et al* complement strains, implying a compensatory role or even that BS3 is the most relevant physiological BosR binding site. Interestingly, BS3 is located downstream of the *rpoS* transcriptional start site which is highly unusual in the bacterial world since transcriptional regulators generally bind to upstream regions. However, there have been certain exceptions to this rule such as that of the transcriptional activator RutR in *E. coli* (Shimada *et al.*, 2008) and that of an unidentified regulator in *Azotobacter vinelandii* (Mitra *et al.*, 2005). BosR's mode of action still remains to be elucidated especially *in vivo* although a proposed model of *rpoS* transcription is shown in Figure 3b (Burtnick *et al.*, 2007).

An intriguing new possible trait of BosR came to light fairly recently when an unpublished study by Haijun Xu, a postdoctoral researcher in the Yang lab, revealed that although the expression of BosR protein in cultured *B. burgdorferi* mimicked the differential regulation of tick and mammalian conditions, it appeared that its mRNA levels were similar in both conditions, indicating that there may be regulation of BosR at

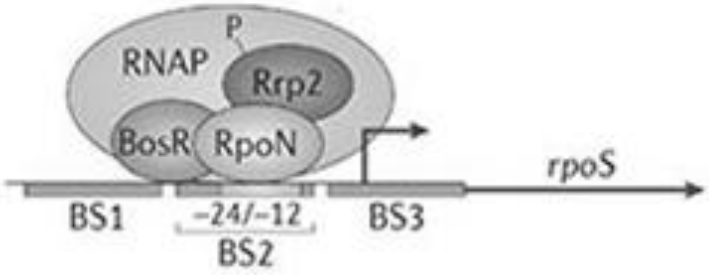
a post-transcriptional level (Xu and Yang, unpublished data). This led us to hypothesize that a protease could be involved in the regulation of BosR, a suggestion again as-yet unseen in other bacteria, assuming the homology of BosR to Fur and PerR hints at functional and regulatory similarities. Although both Fur and PerR are acknowledged as important transcriptional regulators and are essential for virulence in some bacteria such as *P. aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Vibrio cholerae* (Escolar *et al.*, 1999; Horsburgh *et al.*, 2001; Rea *et al.*, 2004; Carpenter *et al.*, 2009), very little research has been done on the regulation of the two proteins themselves.

What little is currently known suggests that Fur and PerR can auto-regulate their gene expression in microbes such as *Campylobacter jejuni* and *Vibrio vulnificus* (Chan *et al.*, 1995; Lee *et al.*, 2003; Lee *et al.*, 2007), and Fur is regulated by RpoS in *V. vulnificus* (Lee *et al*., 2003) and may be reciprocally regulated in *E. coli* with the Crl protein (Lelong *et al.*, 2007). Since mRNA levels of *bosR* at room temperature and 37°C cultures are similar, it appears unlikely that *bosR* is regulated at the transcriptional level. Since BosR is required for the activation of the Rrp2-RpoN-RpoS pathway, it is also unlikely that RpoS regulates the expression of BosR. Because of these observations, our focus shifted to exploring the regulation of BosR. We theorized that BosR is post-transcriptionally regulated by a protease.

(a)



(b)



(c)

AAGCTTTGGCCTTGCCGATTTAATTTACAATCAATTACAAAAAAAGTAAATAATTCAAAA

BS1

AATACTGCCCCTAAACTCAAAATTTTATATCCTATTTAGTTTAAAACCATTTTTAAATTA

BS2

AAT**TGGCACAGTTTTTGCA**TGAAAATTAAGTAGTAAAAACTTAATCACAATATTCAAGAA

(-24/-12) *

AGGGGAGAAAATATAATAACT**ATG**AACATATTTAGTAATGAGGATTTAAACATATATTTA

BS3

AAATCAGTAAG

Figure 3. Proposed model of Rrp2-RpoN-RpoS pathway (a) before and (b) after the *in vitro* characterization of BosR's role in the pathway (Burtnick *et al.*, 2007). (c) Sequence of Ouyang *et al.*'s proposed BosR binding sites on P*rpoS* (Ouyang *et al.*, 2011). The dotted line boxes denote binding sites 1, 2 and 3 (BS1, BS2 and BS3) and the underlined sequences are the proposed binding sequences. The -24/-12 sequence as well as the translational start site (ATG) are in bolded font, and the transcriptional start site is marked with an asterisk (*).

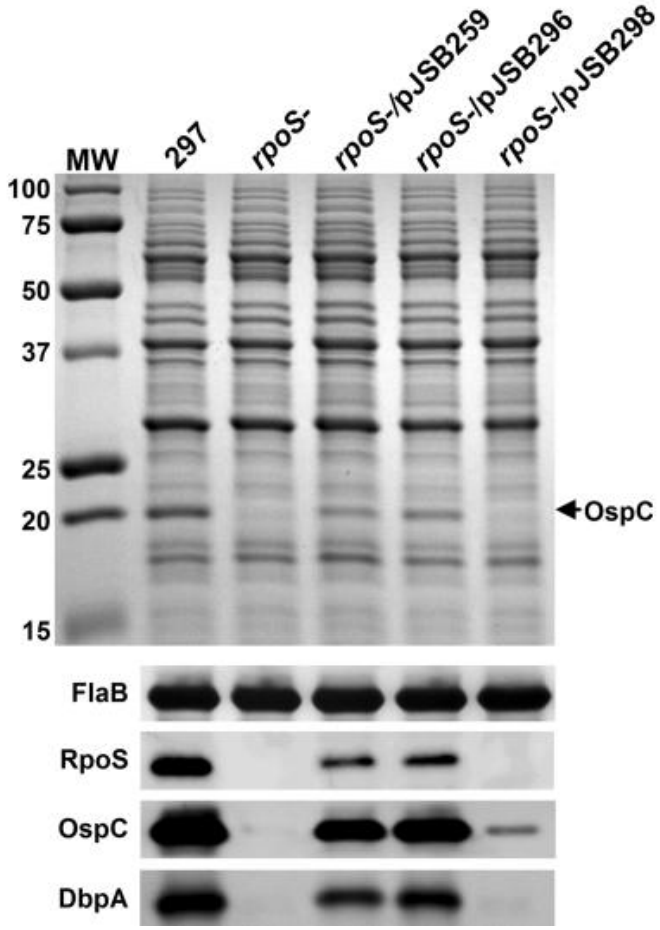

MW
297
rpoS-
rpoS-/pJSB259
rpoS-/pJSB296
rpoS-/pJSB298
100
75
50
37
25
20
15
OspC
FlaB
RpoS
OspC
DbpA

Figure 4. Upper panel shows a Coomassie stain of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the OspC band pointed out and lower panel shows a Western immunoblot with FlaB as loading control. 297 is a wild-type *B. burgdorferi* strain, *rpoS*- is an *rpoS* deletion mutant strain, *rpoS*-/pJSB259 is an *rpoS*- strain fully complemented with the *rpoS* gene and an upstream region that includes BS1, BS2 and BS3, *rpoS*-/pJSB296 is a partially complemented strain that contains the *rpoS* gene complement with an upstream region that includes BS3 and BS2 but with the TAAATTAAAT sequence and *rpoS*-/pJSB298 is a minimal *rpoS*- promoter-complemented strain with a point mutation in the promoter sequence. All complementation was done via transformation with shuttle vectors (Blevins *et al.*, 2009).

MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains of *B. burgdorferi* used were wild-type strain B31-MI and OY10/H3, a *bosR* deletion strain provided by Zhiming Ouyang (Ouyang *et al.*, 2009), which was derived from parental strain B31-MI. B31-MI has no antibiotic marker whereas OY10/H3 has a kanamycin resistance marker. The spirochetes were grown in BSK-Y medium (Table 1 and Pollack *et al.*, 1993), either at room temperature or 37°C. *E. coli* strain Rosetta™ BL21 (DE3) pLysS Competent Cells (Novagen, Billerica, MA) was grown in a medium containing 0.5x Difco™ LB Broth, Miller (Luria-Bertani) from BD Diagnostics (Sparks, MD), 0.5x phosphate-buffered saline (PBS) (Table 1), 0.2% glucose and 35 µg/mL chloramphenicol at 37°C with vigorous shaking.

Plasmid DNA purification

We used the QIAGEN (Valencia, CA) Plasmid Mini Kit or Sigma-Aldrich (St. Loius, MO) GenElute™ Plasmid Maxiprep Kit, depending on the amount of DNA required and bacterial culture volume, as per manufacturer's instructions. The concentration and purity of isolated DNA was determined using the NanoDrop 2000c from Thermo Scientific (Wilmington, DE).

RNA purification

B. burgdorferi cells were grown to stationary phase and 5 x 10^8 spirochetes were then collected by centrifugation at 5,000 x *g*, 4°C, 20 minutes. The cells were washed once in 0.9% (w/v) sodium chloride before being lysed in 1 mL TRIzol® Reagent

(Invitrogen, Grand Island, NY) with repeated pipetting and incubation at room temperature for five minutes to allow for complete dissociation of nucleoprotein complexes. Chloroform was added in a 1:5 ratio of chloroform to TRIzol® Reagent and after vigorous shaking, the mixture was centrifuged at the following conditions: 8,000 x *g*, 4°C, 15 minutes. After a two to three minute incubation at room temperature, the aqueous phase was saved and the following steps were adapted based on the protocol provided by QIAGEN in their RNeasy Mini Kit, and all centrifugation was done at 8,000 x *g* and room temperature. A roughly equivolume amount of 70% ethanol was added with vortexing before transfer to an RNeasy Mini spin column and centrifugation for one minute. After a wash with Buffer RW1, 10 µL of DNase I (New England Biolabs, Ipswich, MA) in 70 µL Buffer RDD was added directly onto the spin column membrane and incubated for 15 minutes at room temperature. The Buffer RW1 wash step was then repeated and another two wash steps using Buffer RPE were performed. After drying the membrane via a one minute centrifugation, the RNA was eluted using RNase-free double distilled water and the concentration and purity were determined using a NanoDrop 2000c (Thermo Scientific).

Polymerase chain reaction (PCR)

Single-stranded cDNA was reverse transcribed from purified RNA using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis System (Invitrogen) as per the manufacturer's protocol. PCR with double-stranded cDNA as the end product were carried out with Phusion® High-Fidelity PCR Kit (Thermo Scientific) based on kit

instructions at a temperature roughly in between the melting temperatures of the forward and reverse primers. Quantitative PCR (qPCR) was done using RT^2 SYBR® Green PCR Master Mix (New England Biolabs), according to the supplied manual. The machines used were MyCycler™ Personal Thermal Cycler (Bio-Rad, Hercules, CA) and for qPCR, ABI Prism 7000 Sequence Detector (Applied Biosystems, Grand Island, NY).

SDS-PAGE and Western blot

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), samples were diluted 1:1 with Laemmli Sample Buffer (Bio-Rad) and boiled for 10 minutes then electrophoresed using 12% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) in Running Buffer at 7mA constant current per gel for 45 minutes then 20mA per gel for 90 minutes. The gel was then stained with Coomassie Dye Solution overnight and destained with Destaining Solution overnight. For immunoblotting, the gel was not stained with Coomassie blue. A piece of nitrocellulose membrane (Bio-Rad) with 0.2µM pores and Transfer Buffer were used for the immunoblot which was run at 80V and 4°C for two hours. The membrane was blocked with PBS-Tween for 30 minutes before overnight incubation with primary mouse polyclonal antibody. Excess primary antibody was then rinsed off twice with 1x PBS-Tween for five minutes each time and goat anti-mouse immunoglobulin G (secondary antibody) was added. The membrane was incubated with secondary antibody for three to four hours then rinsed with Tris-NaCl (pH 7.6) twice for ten minutes each time. The dilutions of antibodies used were 1:4000 dilution of BosR antibody, 1:50 dilution of FlaB antibody (loading control) and 1:1000 dilution of secondary antibody. Developing solution was added to the membrane and

when bands were visualized, the reaction was stopped with distilled water. All antibody incubation, rinse and membrane development steps were done at 4°C on a rocker to prevent protein degradation as well as localized reactions. Table 1 details the exact composition of all reagents used.

Generation of *B. burgdorferi* cell-free extract

We generated cell-free extract (CFE) from 4 x 10^{10} B31-MI cells grown to stationary phase in BSK-Y medium at room temperature (RT-CFE) and 37°C (37-CFE). We centrifuged the cells at 5,000 x *g* for 20 minutes at 4°C, washing three times with sterile Potassium Phosphate Buffer (KP) (Table 1). On the last wash, we concentrated the spirochetes in fresh KP 1000-fold from the original culture volume. Bacterial suspensions were aliquoted into Lysing Matrix B tubes (MP Biomedicals, Solon, OH) – which have beads that are specialized for tissue or cell homogenization – then mounted in a FastPrep®-24 instrument (MP Biomedicals). The machine was run at its lowest setting for ten seconds, after which the tubes were allowed to rest for five minutes on ice. This step was repeated at least five times before centrifuging the tubes at highest speed for 20 minutes at 4°C. The supernatant, which was the cell-free extract (CFE), was saved and stored at -80°C.

Purification of recombinant BosR

We purified recombinant BosR (rBosR) from *E. coli* using a technique adapted from Ouyang *et al*. in their 2009b paper. Plasmid pOY21, generously provided by Zhiming Ouyang, was transformed into *E. coli* strain Rosetta™ BL21 (DE3) (Novagen).

The pOY21 plasmid contained the *bosR* gene in a pPROEX-HTB vector, and the resulting protein is His_6-tagged at the N-terminus. The pPROEX-HTB vector has an ampicillin-resistance gene so accordingly, 135 µg/mL of ampicillin was added to the growth medium described in the earlier section on bacterial culture conditions. After 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction of protein expression, BosR was purified using the Ni-NTA Agarose resin system from QIAGEN using a protocol adapted from the manufacturer's instructions. Two hours after IPTG induction, the *E. coli* cells were pelleted at 5,000 x *g* for 15 minutes at 4°C. The supernatant was discarded and the cells were resuspended in Buffer A at a 1:100 ratio compared to the original volume of the cell culture. 17.4 µg/mL phenylmethanesulfonylfluoride (PMSF), a serine protease inhibitor, was added to the suspension and the bacterial cells were then lysed using the FastPrep®-24 instrument (MP Biomedicals) as described in the preceding section. A slim glass column was capped at the bottom with a rubber cork containing a flexible capillary tube. The tube was then filled with Ni-NTA Agarose (QIAGEN) and the beads were allowed to settle and compact. CFE from the lysis step was added to the Ni-NTA column and the flow-through collected in 2-3 mL fractions. Recombinant BosR was then eluted using 15 mL each of solutions 1, 2 and 3 which were added consecutively and also collected in fractions of 2-3 mL. 50 µL of each fraction was taken for SDS-PAGE to determine which contained the purified rBosR. Those fractions were mixed together in a 50 mm flat width regenerated cellulose dialysis tube (Fisherbrand, Pittsburgh, PA) and put into 4 L of storage buffer together with a magnetic stirrer set at high speed for 6 hours. The storage buffer was then discarded and the dialysis repeated for another 12 hours. The Ni-NTA elution and subsequent dialysis were carried out at

4°C. The dialyzed rBosR was analyzed using SDS-PAGE to check its purity and the protein was stored at -80°C. The exact composition of the buffers and solutions used are detailed in Table 1.

Determination of protein concentration

We ascertained the protein concentration of the CFEs using the Coomassie (Bradford) Protein Assay Reagent from Thermo Scientific according to manufacturer's instructions. Firstly, 30 µL each of CFE and bovine serum albumin (BSA) solutions of known concentrations (0.1 g/mL, 0.25 g/mL, 0.5 g/mL and 1.0 g/mL) were added to 1.5 mL of room temperature reagent respectively and light absorbance at 595 nm was determined using an Evolution 160 UV-Vis Spectrophotometer (Thermo Scientific). A standard curve of absorbance versus BSA concentration was then plotted and the protein concentration of the CFE was determined based on the curve. If the light absorbance of the CFE was higher than the light absorbance of the highest concentration of BSA, 15 µL or 3 µL of CFE was used for the assay instead, and protein concentration calculated accordingly.

Protein degradation assay

Recombinant BosR was incubated with RT-CFE and 37-CFE at room temperature using a 1:100 ratio of protein to CFE, and the mixture was analyzed at several time points using Western blot technique. The time points were: immediately after mixing rBosR with CFE, and 2 hours, 4 hours, 6 hours, 12 hours, 24 hours and 48 hours after mixing. It

is important to note that the amount of rBosR used in the experiment is very likely much higher than physiologically seen in *B. burgdorferi*.

Construction of luciferase reporter plasmids

We began with pJD48, a promoterless luciferase reporter plasmid that contains a kanamycin resistance marker (Blevins *et al*., 2007). The kanamycin resistance marker was replaced with a gentamycin resistance gene via restriction digests and ligations. The replacement was done because *B. burgdorferi* strain OY10/H3 contains a kanamycin resistance marker and hence electrotransformation with reporter plasmids containing the same marker prevents selection for true transformants. Desired DNA fragments were inserted also using restriction digests and ligations. Restriction digest enzymes and buffer were bought from Fermentas (Vilnius, Lithuania) whereas ligase enzymes and buffer were from Promega (Madison, WI). The protocols used were provided by manufacturers. The inserts from Figure 5b and Figure 5c were cut from a plasmid that contained the desired product (Haijun Xu, unpublished data) and the insert from Figure 5d was cloned using *B. burgdorferi* B31-MI template DNA whereas the inserts from Figure 5e and Figure 5f were cloned with *E. coli* TOP10 DNA as a template. The primers used in the cloning are detailed in Table 2.

Electrotransformation of *B. burgdorferi*

We adapted our protocol from Hyde *et al.*'s 2005 paper. For every sample to be transformed, a stationary phase culture of 7.5 x 10^9 spirochetes was washed twice with 0.9% (w/v) sodium chloride and three times with Electroporation Solution (EPS) (Table 1). For each wash step, 8 mL of solution was used and the cells were centrifuged at 4,000

x *g* to 5000 x *g* for 20 minutes at 4°C. After the final wash step, the cells were resuspended in 50 µL of EPS buffer and incubated on ice for 1 minute with 20 µg of the desired plasmid DNA in a 0.2 cm electroporation cuvette (Bio-Rad Gene Pulser). The mixture was then electroporated using Gene Pulser Xcell™ Microbial System (Bio-Rad) at 2.5 kV, 25 µF and 200 e at a time constant of between 4 and 6 milliseconds. Immediately after electroporation, 1 mL of BSK-Y was added before transfer into another 35 mL of BSK-Y. After overnight incubation at 37°C, antibiotics (0.4 mg/mL gentamycin and 1 mg/mL kanamycin, as appropriate) and 600 µL of phenol red (for stronger medium color visualization) were added. Aliquots of 180 µL of the culture were placed into a 96-well cell culture plate. The plate was then incubated at 37°C and observed over a period of seven to 14 days. A change in color of the medium from red to yellow indicated bacterial cell proliferation (positive clones), which was verified using dark-field microscopy. Wells identified as having growing borrelial cells were sub-cultured in a 1:5,000 ratio of culture to fresh media containing appropriate antibiotics and when cell growth reached stationary phase, plasmid DNA was harvested to determine true transformants.

Confirmation of true *Borrelia* transformant clones

The tests we conducted were chemical transformation of *E. coli* and DNA sequencing of the plasmid DNA isolated from successful *E. coli* transformants, using appropriate primers flanking the desired gene. Plasmid DNA isolated from *B. burgdorferi* transformant clones were chemically transformed into *E. coli* TOP10 calcium chloride-treated competent cells using a well-known 42°C heat-shock method.

Chemotransformation of *E. coli* with pure plasmid DNA diluted to the same concentration as the DNA purified from *B. burgdorferi* served as a positive control. *E. coli* chemotransformation of plasmid DNA from true *B. burgdorferi* transformants produces a similar number of colonies to *E. coli* transformed with pure plasmid DNA. For those that passed the test, plasmid DNA was isolated from the *E. coli* hosts and subjected to sequence confirmation at the DNA Sequencing Core Facility of Indiana Univesity. The chemical transformation of *E. coli* was used as an additional confirmation step.

Luciferase reporter assay

Approximately 1 x 10^9 spirochetes grown to stationary phase were used for each reaction. The cells were washed twice with sterile 0.9% NaCl solution at 5,000 x *g*, 10 minutes, 4°C. The cells were then lysed with vigorous vortexing in a solution containing 1x Cell Culture Lysis Reagent (Promega) and 0.125% lysozyme from chicken egg white (USB, Cleveland, OH), and supplemented with 5 mg/mL BSA (Blevins *et al.*, 2007). Luciferase Assay Reagent (Promega) and the measurement of light produced was carried out using Promega's protocol for single-tube luminometers on a TD-20/20 Luminometer (Turner Design, Sunnyvale, CA).

Page(s)	Reagent	Component	Concentration
20	**Barbour-Stoenner-Kelly medium (BSK-Y)** (pH 7.5, filtered using 0.2µM Millipore Stericup® or Corning® vacuum filtration systems)	HEPES	25.2 mM
		Bacto™ Neopeptone (BD)	0.5% (w/v)
		D-(+)-Glucose	27.8 mM
		Sodium pyruvate	7.3 mM
		Sodium citrate	0.07% (w/v)
		Bacto™ TC Yeastolate (BD)	0.25% (w/v)
		N-acetylglucosamine	1.8 mM
		Sodium bicarbonate	26.2 Mm
		Probumin, universal grade (Millipore)	5% (w/v)
		CMRL 1066 (US Biological)	0.98% (w/v)
		Rabbit serum, heat inactivated at 56°C for 45 minutes (Equi-tech, Inc)	6.4% (v/v)
20	**1x Phosphate-buffered saline (PBS)** (pH 7.4)	Sodium chloride	137 mM
		Potassium chloride	2.7 mM
		Sodium phosphate, dibasic	10 mM
		Potassium phosphate, monobasic	1.8 mM
22	**Running Buffer**	Sodium dodecyl sulfate (SDS)	3.5 mM
		Tris-base	25 mM
		Glycine	192 mM
22	**Coomassie Dye Solution**	Coomassie Brilliant Blue R-250	0.86 mM
		Glacial acetic acid	14.3% (v/v)
		Methanol	14.3% (v/v)
22	**Destaining Solution**	Glacial acetic acid	10% (v/v)
		Methanol	10% (v/v)

Continued on Page 30

22, 23	**Transfer Buffer**	Glycine Tris base Methanol	38.7 mM 23.1 mM 20 % (v/v)
23	**PBS-Tween**	1x PBS Tween-20	99.5% (v/v) 0.5% (v/v)
23	**Tris-NaCl** (pH 7.6)	Tris base NaCl	50 mM 200 mM
23	**Developing Solution** (Tris-NaCl was the solvent used)	4-chloro-1-naphthol in methanol, 16.8 mM Hydrogen peroxide	16.67% (v/v) 0.1% (v/v)
24	**Buffer A**	Sodium phosphate, dibasic Sodium phosphate, monobasic NaCl β-mercaptoethanol	20 mM 20 mM 200 mM 100 µM
N/A	**Buffer B**	Sodium phosphate, dibasic Sodium phosphate, monobasic NaCl β-mercaptoethanol Imidazole	10 mM 10 mM 100 mM 50 µM 200 mM
25	**Solution 1**	Buffer A Buffer B Imidazole	98% (v/v) 2% (v/v) 20 mM
25	**Solution 2**	Buffer A Buffer B Imidazole	90% (v/v) 10% (v/v) 50 mM

Continued on Page 31

25	**Solution 3**	Buffer B Imidazole	85% (v/v) 500 mM
25	**Storage Buffer** (pH 7.8)	Potassium phosphate, dibasic Potassium phosphate, monobasic Ethylenediaminetetraacetic acid (EDTA) Glycerol	45 mM 5 mM 0.1 mM 35% (v/v)
25	**Potassium Phosphate Buffer** (pH 7.8)	Potassium phosphate, dibasic Potassium phosphate, monobasic	45.5 mM 4.5 mM
27	**Electroporation Solution (EPS)** (filtered using 0.2µM Millipore Stericup® or Corning® vacuum filtration systems)	Sucrose Glycerol	271.7 mM 15% (v/v)

Table 1. Composition of reagents used in our studies. All reagents used double distilled water as a solvent, unless otherwise stated.


(a)
no promoter
SD
native NCR
Bb luc
(b)
Bb min BS2
Bb rpoS -24/-12
SD
rpoS NCR
Bb luc
TTTTAAATTAAATTGGCACAGTTTTTGCATGGAAATTAAGTAGTAAAAAC
Bb min BS2
Bb rpoS -24/-12
TTAATCACAATATTCAAGAAAGGGGAGAAAATATAATAACTATG
luc
SD
(c)
Bb min BS2
Bb rpoS -24/-12
SD
flaB NCR
Bb luc
TTTTAAATTAAATTGGCACAGTTTTTGCATGGAAATTAAGTAAGGATTTGC
Bb min BS2
Bb rpoS -24/-12
CAAAGTCAGAAATTTAAATTTTATCATGGAGGAATGATAT ATG
luc
SD
(d)
Bb rpoS -24/-12
SD
rpoS NCR
Bb luc
TTTTGGCACAGTTTTTGCATGGAAATTAAGTAGTAAAAAC
Bb rpoS -24/-12
TTAATCACAATATTCAAGAAAGGGGAGAAAATATAATAACTATG
luc
SD
(e)
Ec glnALG -24/-12
SD
Ec glnALG NCR
Bb luc
TTTTGGCACAGATTTCGCTTTATCTTTTTTACGGCGACACGGCCAAAATAATTGCA
Ec glnALG -24/-12
GATTTCGTTACCACGACGACCATGACCAATCCAGGAGAGTTAAAGTATG
luc
SD
(f)
Bb rpoS -24/-12
SD
Ec glnALG NCR
Bb luc
TTTTGGCACAGTTTTTGCATTATCTTTTTTACGGCGACACGGCCAAAATAATTGCA
Bb rpoS -24/-12
GATTTCGTTACCACGACGACCATGACCAATCCAGGAGAGTTAAAGTATG
luc
SD

Figure 5. Illustrations and sequences of various promoter elements fused to luciferase reporter plasmid shuttle vectors. Bb refers to *B. burgdorferi*, min BS2 refers to the BS2 TAAATTAAAT consensus sequence, SD refers to the Shine-Dalgarno box of the various promoter elements, NCR refers to the region between the -24/-12 element and the translational start site, *Ec* refers to *E. coli* and *luc* refers to the luciferase gene. The NCR of *flaB* was chosen because FlaB is ubiquitously expressed in *B. burgdorferi* and also because it is not RpoN-dependent. The foreign sequence we chose was from *gln*ALG in *E. coli*, which also has an RpoN-dependent promoter (Reitzer and Schneider, 2001). The plasmids will be referred to as 5a to 5f respectively throughout the rest of this dissertation.

Insert	Direction	Primer Sequence
For Figure 5d	Forward	5'- AGATCTTGGCACAGTTTTTGCAT -3'
	Reverse	5'- CATATGTATTATATTTTCTCCCC -3'
For Figure 5e	Forward	5'- AGATCTTGGCACAGATTTCGCTT -3'
	Reverse	5'- CATATGACTTTAACTCTCCTGGA -3'
For Figure 5f	Forward	5'- AGATCTTGGCACAGTTTTTGCATTATCTTTTTTACGGCGACAC -3'
	Reverse	5'- CATATGACTTTAACTCTCCTGGA -3'

Table 2. Primer sequences for cloning of desired DNA fragments, as explained in the "Materials and Methods" section entitled "Construction of luciferase reporter plasmids."

RESULTS

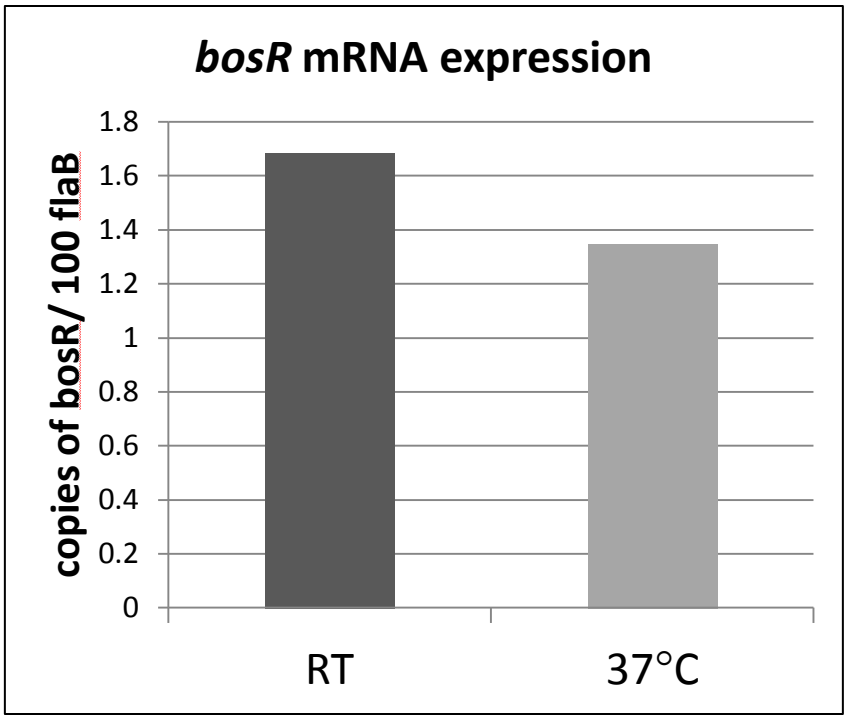
The degradation of BosR

As suggested by Haijun Xu, a previous postdoctoral scholar in the Yang lab, while the mRNA transcript levels of BosR are not significantly different between the two culture conditions of room temperature and 37°C, the BosR protein can only be detected at 37°C but not room temperature (Figure 6). We hypothesized that a protease may be active at 23°C so we conducted a room-temperature protein degradation assay by mixing purified rBosR with CFE of B31-MI cultured to stationary phase at room temperature (RT-CFE) and 37°C (37-CFE) in a 1:2 ratio of rBosR to CFE. The Coomassie-stained SDS-PAGEs in Figure 7a show that rBosR is completely degraded after 48 hours when incubated with RT-CFE. Notice that in the 48-hour SDS-PAGE (Figure 7a, right panel), the BSA band appears to retain its color intensity.

Since BosR is not present in such a high amount under physiological conditions, we scaled down the ratio of rBosR to CFE from 1:2 to 1:100 to better reflect *in vivo* conditions and also to narrow down the time frame for BosR degradation. The data we generated from the immunoblots in this experiment confirmed the data from SDS-PAGEs. Figure 7b shows the Western blot analysis which suggests that RT-CFE degrades rBosR within 12 hours, whereas 37-CFE does not degrade rBosR. The slight decrease in rBosR level at 24 and 48 hours in the 37°C group probably reflects the instability of the low amount of BosR used in the experiment when left at room temperature in an inconducive environment for a prolonged period. Figure 7c sharpens the time frame for BosR degradation, showing that rBosR begins to be degraded after 2 hours and is nearly completely degraded at 12 hours. Taken together, the SDS-PAGEs

and immunoblots suggest that BosR is degraded by a protease that is only active when *B. burgdorferi* is cultured at room temperature, and the protease exhibits some specificity with regards to its substrate.

(a)


bosR mRNA expression
copies of bosR/ 100 flaB
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
RT
37°C

(b)

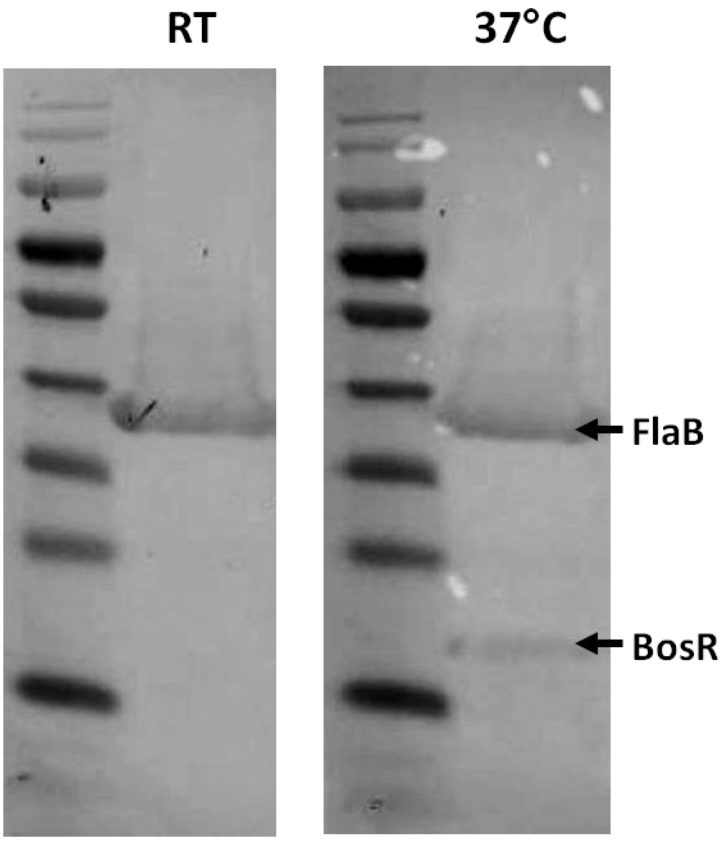
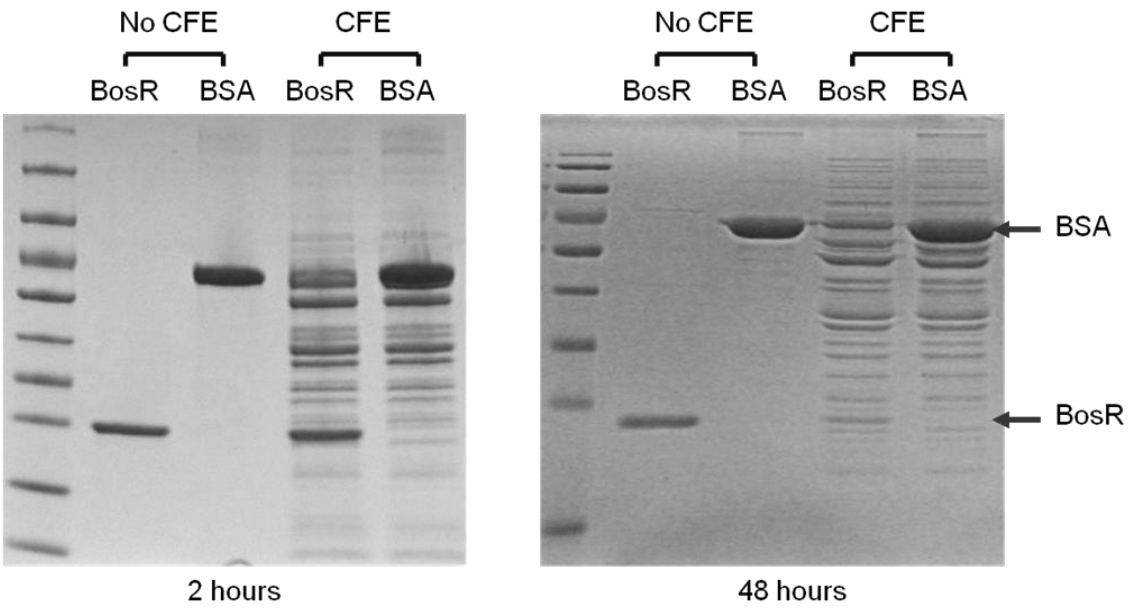
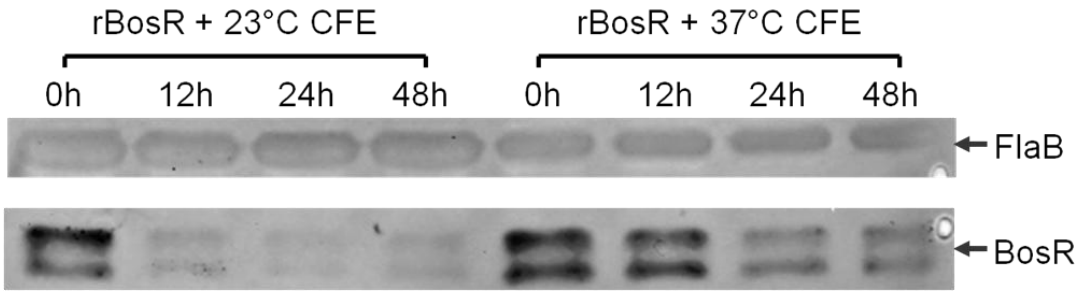

RT
37°C
FlaB
BosR

Figure 6. Expression levels of *bosR* mRNA (a) and BosR protein (b) in *B. burgdorferi* wild-type strain B31-MI at room temperature (RT) and 37°C. RT cultures mimic tick vector physiological conditions whereas 37°C cultures mimic mammalian host physiological conditions. The protein ladder used in the immunoblots in (b) was Fisher BioReagents EZ-Run Prestained Rec Protein Ladder.

(a)


No CFE
CFE
BosR
BSA
BosR
BSA
No CFE
CFE
BosR
BSA
BosR
BSA
BSA
BosR
2 hours
48 hours

(b)


rBosR + 23°C CFE
rBosR + 37°C CFE
0h
12h
24h
48h
0h
12h
24h
48h
FlaB
BosR

(c)

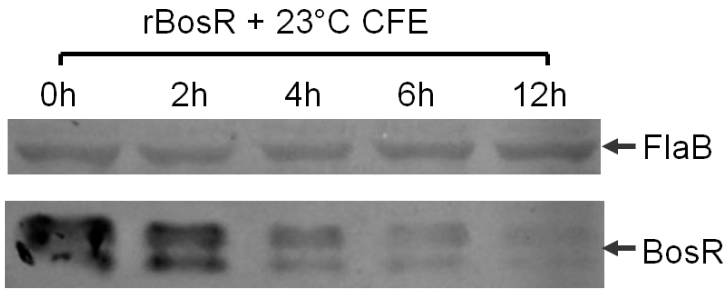

rBosR + 23°C CFE
0h
2h
4h
6h
12h
FlaB
BosR

Figure 7. Time-course experiments of BosR degradation by B31-MI CFE. (a) Coomassie-stained SDS-PAGEs. The protein ladder used was Fisher BioReagents® EZ-Run® Prestained *Rec* Protein Ladder. (b) and (c) Immunoblots of rBosR using FlaB as a loading control. The appearance of two bands when blotting for rBosR is due to the purification of rBosR always yielding two bands despite several different attempts at protein isolation. We have not yet been able to identify the cause but speculate that it may be due to dimerization of rBosR. The antibody to rBosR is based on the isolated protein.

Borrelia transformations with luciferase reporter plasmid constructs

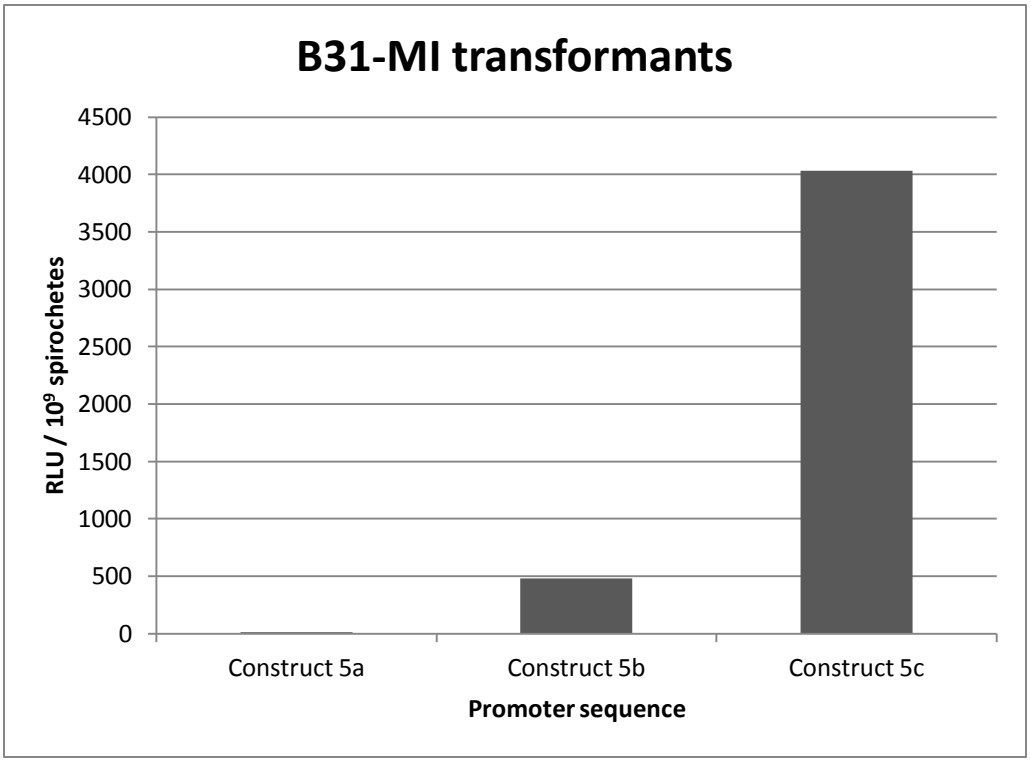
Although Blevins *et al.* and Ouyang *et al.* have both demonstrated that BosR is a DNA-binding transcriptional activator of P*rpoS in vitro*, to date there has not been any study confirming those studies *in vivo*. To address that lack of knowledge, we electrotransformed the luciferase reporter plasmid constructs from Figure 5 into *B. burgdorferi bosR* mutant strain OY10/H3 and wild-type strain B31-MI, the parental strain of OY10/H3. The *bosR* mutant strain was generously provided by Zhiming Ouyang. Because transformation of *B. burgdorferi* is notoriously difficult, we unfortunately were unable to generate all the transformants that we needed. The successful transformants were: constructs from Figure 5a-c and e-f in B31-MI, and constructs from Figure 5a and c in OY10/H3.

Contribution of the putative BosR binding site to *rpoS* expression

The results shown in Figure 8a suggest that the absence of BS3 does not abolish *rpoS* transcription, as evidenced by high luciferase activity of the 5b wild-type strain compared to the negative control, and that BS2 is sufficient for *rpoS* induction, as evidenced by high luciferase activity of the 5c wild-type strain as compared to negative control. The much higher luciferase activity of the 5c wild-type strain as compared to the 5b wild-type strain could be due to regulation of the *flaB* non-coding region or another form of *rpoS* regulation and these will be addressed in the "Discussion" section. Figure 8b shows that BosR is most likely required for transcriptional activation of P*rpoS* since the *bosR* null mutant transformed with the 5c plasmid registered much lower luciferase

activity compared to its wild-type counterpart. This confirms the studies of Blevins *et al.* and Ouyang *et al.* that demonstrated BosR binding to P*rpoS* in separate *in vitro* assays.

(a)


B31-MI transformants
4500
4000
3500
3000
2500
2000
1500
1000
500
0
RLU / 10⁹ spirochetes
Construct 5a
Construct 5b
Construct 5c
Promoter sequence

(b)

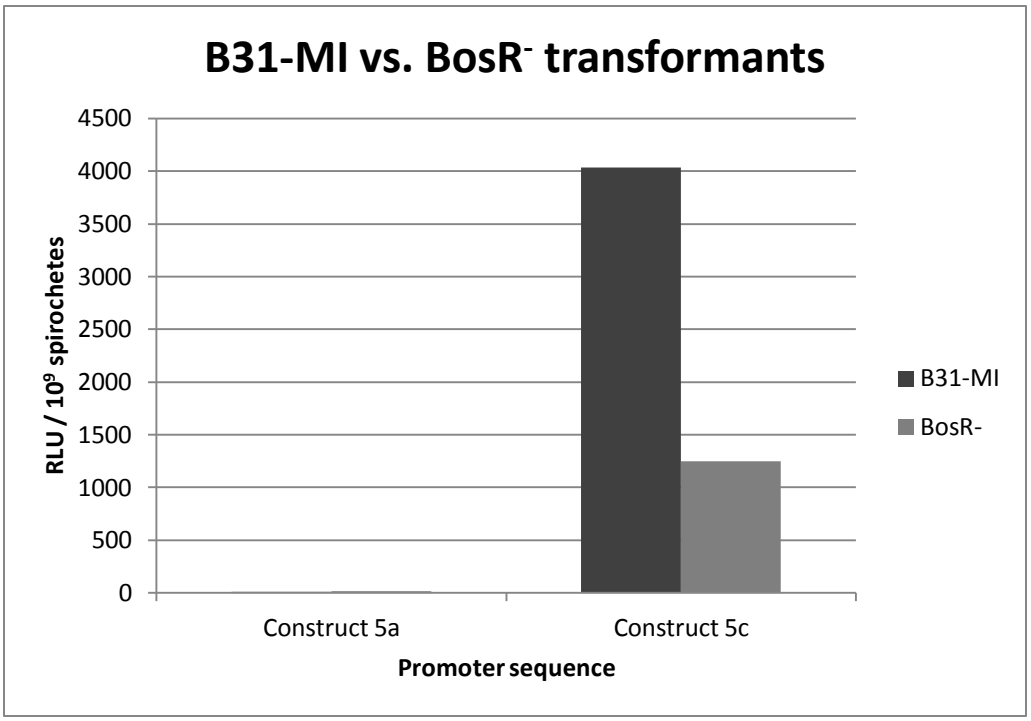

B31-MI vs. BosR⁻ transformants
4500
4000
3500
3000
2500
2000
1500
1000
500
0
RLU / 10⁹ spirochetes
Construct 5a
Construct 5c
Promoter sequence
B31-MI
BosR-

Figure 8 (a) and (b). Luciferase activity of different *B. burgdorferi* transformants. WT refers to the wild-type strain B31-MI and BosR- refers to the *bosR* deletion strain OY10/H3. The plasmid constructs are explained in Figure 5. Two clones of each transformant were used and the experiments were repeated twice.

DISCUSSION

The ability of *B. burgdorferi* to thrive in two completely different environments has fascinated researchers ever since the identification of the spirochete as the etiological agent of Lyme disease. Many studies on this perseverant microbe have focused on the molecular mechanisms behind this skill. After the elucidation of the unusual Rrp2-RpoN-RpoS pathway as being the mechanistic cascade required for mammalian infection (Yang *et al.*, 2003b; Radolf *et al.*, 2012), researchers were surprised again when it emerged that there was another required transcriptional activator separate from Rrp2—a protein known by its controversial name of BosR (Boylan *et al.*, 2003; Ouyang *et al.*, 2011). Despite the importance of BosR in this pathway, no study has yet been published on the regulation of this essential protein. Our studies have now paved the way for the illumination of a novel mechanism of BosR regulation in *B. burgdorferi*. Our data show that an as-yet-unidentified protease is likely responsible for the regulation of BosR and hence, the adaptive abilities of *B. burgdorferi* under disparate vector-host conditions. The annotated *B. burgdorferi* genome alludes to the existence of 21 proteases, so the next step to take is to characterize the protease in question and identify it. Since Fur and PerR (the homologs of BosR) have been shown to be autoregulatory in other bacteria (Chan *et al.*, 1995; Lee *et al.*, 2003; Lee *et al.*, 2007), this possibility should be explored in addition to protease identification.

Since the publication of *in vitro* studies that implicated a DNA-binding regulatory role of BosR in *rpoS* transcriptional activation, there has not been any publication confirming the results in *B. burgdorferi* cultures. We decided to fill that gap in knowledge by undertaking the *in vivo* studies using a luciferase reporter assay adapted to

the *B. burgdorferi* system (Blevins, *et al.*, 2007). The data in Figure 8 confirm the *in vitro* studies and lend weight to a possible compensatory effect of the BosR binding sites for each other. Alternatively, none of the binding sites may be required for BosR-dependent *rpoS* transcription, with BosR instead interacting with another member of the Rrp2-RpoN-RpoS pathway such as the Rrp2 protein or RpoN protein, or even the RNA polymerase itself to initiate *rpoS* transcription. This alternative is possible because of the existence of Rrp2 as another transcriptional activator of the pathway.

As mentioned in the "Results" section, the *flaB* NCR may be regulated by other factors and as such, using a foreign NCR will strengthen this study. As a point of interest, there has been rather extensive research in several bacterial systems into the control of the *rpoS* gene as well as its protein product that shows that the gene and protein are regulated transcriptionally, post-transcriptionally as well as post-translationally. For example, RpoS is controlled at all three stages in *E. coli*, at the transcriptional level in *Pseudomonas* spp. (Venturi, 2003), at the post-transcriptional level in *B. burgdorferi* (Lybecker and Samuels, 2007) and *S. typhimurium* (Brown and Elliott, 1996). In the future, if and when construction of the full range of *Borrelia* transformants mentioned in this dissertation are completed, it will be possible to definitively prove whether or not BosR even requires promoter binding in order to initiate *rpoS* transcription. Given that *B. burgdorferi* has already surprised researchers several times, it would not be a stretch to imagine that this steadfast spirochete has a few more tricks up its metaphorical sleeve.

REFERENCES

1. 2012. Reported Cases of Lyme Disease by Year, United States, 2002-2011. Accessed March 1, 2013. http://www.cdc.gov/lyme/stats/chartstables/casesbyyear.html)

2. Andrews SC, Robinson AK, Rodríguez-Quiñones F. 2003. Bacterial iron homeostasis. FEMS Microbiology Reviews 27: 215-237.

3. Bacon RM, Kugeler, KJ and Mead, PS. 2008. Surveillance for Lyme Disease -- United States, 1992-2006. Morbidity and Mortality Weekly Report Surveillance Summaries (Centers for Disease Control and Prevention) 57: 1-9.

4. Barrios H, Valderrama B, Morett E. 1999. Compilation and analysis of σ54-dependent promoter sequences. Nucleic Acids Research 27: 4305-4313.

5. Blevins JS, Revel AT, Smith AH, Bachlani GN, Norgard MV. 2007. Adaptation of a luciferase gene reporter and *lac* expression system to *Borrelia burgdorferi*. Applied and Environmental Microbiology 73: 1501-1513.

6. Blevins JS, Xu H, He M, Norgard MV, Reitzer L, Yang XF. 2009. Rrp2, a σ54-dependent transcriptional activator of *Borrelia burgdorferi*, activates *rpoS* in an enhancer-independent manner. Journal of Bacteriology 191: 2902-2905.

7. Boylan JA, Posey JE, Gherardini FC. 2003. Borrelia oxidative stress response regulator, BosR: A distinctive Zn-dependent transcriptional activator. Proceedings of the National Academy of Sciences 100: 11684-11689.

8. Brown L, Elliott T. 1996. Efficient translation of the RpoS sigma factor in *Salmonella typhimurium* requires host factor I, an RNA-binding protein encoded by the *hfq* gene. Journal of Bacteriology 178: 3763-3770.

9. Buck M, Gallegos M-T, Studholme DJ, Guo Y, Gralla JD. 2000. The bacterial enhancer-dependent σ54(σN) transcription factor. Journal of Bacteriology 182: 4129-4136.

10. Burgdorfer W. 1991. Lyme borreliosis: Ten years after discovery of the etiologic agent, *Borrelia burgdorferi*. Infection 19: 257-262.

11. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. 1982. Lyme disease--a tick-borne spirochetosis? Science 216: 1317-1319.

12. Burtnick MN, Downey JS, Brett PJ, Boylan JA, Frye JG, Hoover TR, Gherardini FC. 2007. Insights into the complex regulation of *rpoS* in *Borrelia burgdorferi*. Molecular Microbiology 65: 277-293.

13. Bush M, Dixon R. 2012. The role of bacterial enhancer binding proteins as specialized activators of σ54-dependent transcription. Microbiology and Molecular Biology Reviews 76: 497-529.

14. Caimano MJ, Eggers CH, Hazlett KRO, Radolf JD. 2004. RpoS is not central to the general stress response in *Borrelia burgdorferi* but does control expression of one or more essential virulence determinants. Infection and Immunity 72: 6433-6445.

15. Carpenter BM, Whitmire JM, Merrell DS. 2009. This is not your mother's repressor: the complex role of Fur in pathogenesis. Infection and Immunity 77: 2590-2601.

16. Chan VL, Louie H, Bingham HL. 1995. Cloning and transcription regulation of the ferric uptake regulatory gene of *Campylobacter jejuni* TGH9011. Gene 164: 25-31.

17. Clark RP, Hu LT. 2008. Prevention of Lyme disease and other tick-borne infections. Infectious Disease Clinics of North America 22: 381-396.

18. Cooke WD, Dattwyler RJ. 1992. Complications of Lyme borreliosis. Annual Review of Medicine 43: 93-103.

19. de Silva AM, Telford SR, Brunet LR, Barthold SW, Fikrig E. 1996. *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. The Journal of Experimental Medicine 183: 271-275.

20. Dong T, Schellhorn HE. 2010. Role of RpoS in virulence of pathogens. Infection and Immunity 78: 887-897.

21. Embers ME, Narasimhan S. 2013. Vaccination against Lyme disease: past, present, and future. Frontiers in Cellular and Infection Microbiology 3: 1-16.

22. Escolar L, Pérez-Martín J, de Lorenzo V. 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. Journal of Bacteriology 181: 6223-6229.

23. Fang FC, Libby SJ, Buchmeier NA, Loewen PC, Switala J, Harwood J, Guiney DG. 1992. The alternative sigma factor katF (rpoS) regulates *Salmonella* virulence. Proceedings of the National Academy of Sciences 89: 11978-11982.

24. Fingerle V, Rauser S, Hammer B, Kahl O, Heimerl C, Schulte-Spechtel U, Gern L, Wilske B. 2002. Dynamics of dissemination and outer surface protein expression of different European *Borrelia burgdorferi sensu lato* strains in artificially infected *Ixodes ricinus* nymphs. Journal of Clinical Microbiology 40: 1456-1463.

25. Fish D. 1995. Environmental risk and prevention of Lyme disease. The American Journal of Medicine 98: 2S-9S.

26. Fisher MA, Grimm D, Henion AK, Elias AF, Stewart PE, Rosa PA, Gherardini FC. 2005. *Borrelia burgdorferi* σ54 is required for mammalian infection and vector transmission but not for tick colonization. Proceedings of the National Academy of Sciences of the United States of America 102: 5162-5167.

27. Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O, Ketchum KA, Dodson R, Hickey EK, Gwinn M, Dougherty B, Tomb JF, Fleischmann RD, Richardson D, Peterson J, Kerlavage AR, Quackenbush J, Salzberg S, Hanson M, van Vugt R, Palmer N, Adams MD, Gocayne J, Weidman J, Utterback T, Watthey L, McDonald L, Artiach P, Bowman C, Garland S, Fuji C, Cotton MD, Horst K, Roberts K, Hatch B, Smith HO, Venter JC. 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature 390: 580-586.

28. Ghosh T, Bose D, Zhang X. 2010. Mechanisms for activating bacterial RNA polymerase. FEMS Microbiology Reviews 34: 611-627.

29. Gilmore RD, Piesman J. 2000. Inhibition of *Borrelia burgdorferi* migration from the midgut to the salivary glands following feeding by ticks on OspC-immunized mice. Infection and Immunity 68: 411-414.

30. Grimm D, Tilly K, Byram R, Stewart PE, Krum JG, Bueschel DM, Schwan TG, Policastro PF, Elias AF, Rosa PA. 2004. Outer-surface protein C of the Lyme disease spirochete: A protein induced in ticks for infection of mammals. Proceedings of the National Academy of Sciences of the United States of America 101: 3142-3147.

31. Gruber TM, Gross CA. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annual Review of Microbiology 57: 441-466.

32. Hayes EB, Piesman J. 2003. How can we prevent Lyme disease? New England Journal of Medicine 348: 2424-2430.

33. Hengge-Aronis R. 1993. Survival of hunger and stress: The role of rpoS in early stationary phase gene regulation in *E. coli*. Cell 72: 165-168.

34. Horsburgh MJ, Ingham E, Foster SJ. 2001. In Staphylococcus aureus, Fur Is an Interactive Regulator with PerR, Contributes to Virulence, and Is Necessary for Oxidative Stress Resistance through Positive Regulation of Catalase and Iron Homeostasis. Journal of Bacteriology 183: 468-475.

35. Hübner A, Yang X, Nolen DM, Popova TG, Cabello FC, Norgard MV. 2001. Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN–RpoS regulatory pathway. Proceedings of the National Academy of Sciences 98: 12724-12729.

36. Hyde JA, Weening EH, Skare JT. 2005. Genetic transformation of *Borrelia burgdorferi*. Current Protocols in Microbiology, John Wiley & Sons, Inc.

37. Hyde JA, Shaw DK, Smith R, Trzeciakowski JP, Skare JT. 2010. Characterization of a conditional *bosR* mutant in *Borrelia burgdorferi*. Infection and Immunity 78: 265-274.

38. Johnson RC, Schmid, GP, Hyde, FW, Steigerwalt, AG, Brenner, DJ. 1984. *Borrelia burgdorferi* sp. nov.: Etiologic agent of Lyme disease. International Journal of Systematic Bacteriology 34: 496-497.

39. Katona LI, Tokarz R, Kuhlow CJ, Benach J, Benach JL. 2004. The Fur homologue in *Borrelia burgdorferi*. Journal of Bacteriology 186: 6443-6456.

40. Lane RS, Piesman J, Burgdorfer W. 1991. Lyme borreliosis: Relation of its causative agent to its vectors and hosts in North America and Europe. Annual Review of Entomology 36: 587-609.

41. Lee DJ, Minchin SD, Busby SJW. 2012. Activating transcription in bacteria. Annual Review of Microbiology 66: 125-152.

42. Lee H-J, Bang SH, Lee K-H, Park S-J. 2007. Positive regulation of *fur* gene expression via direct interaction of Fur in a pathogenic bacterium, *Vibrio vulnificus*. Journal of Bacteriology 189: 2629-2636.

43. Lee H-J, Park K-J, Lee AY, Park SG, Park BC, Lee K-H, Park S-J. 2003. Regulation of *fur* expression by RpoS and Fur in *Vibrio vulnificus*. Journal of Bacteriology 185: 5891-5896.

44. Lelong C, Rolland M, Louwagie M, Garin J, Geiselmann J. 2007. Mutual regulation of Crl and Fur in *Escherichia coli* W3110. Molecular & Cellular Proteomics 6: 660-668.

45. Lybecker MC, Samuels DS. 2007. Temperature-induced regulation of RpoS by a small RNA in *Borrelia burgdorferi*. Molecular Microbiology 64: 1075-1089.

46. Merrick MJ. 1993. In a class of its own — the RNA polymerase sigma factor σ54 (σN). Molecular Microbiology 10: 903-909.

47. Mitra R, Das HK, Dixit A. 2005. Identification of a positive transcription regulatory element within the coding region of the *nifLA* operon in *Azotobacter vinelandii*. Applied and Environmental Microbiology 71: 3716-3724.

48. Montgomery RR, Malawista SE, Feen KJ, Bockenstedt LK. 1996. Direct demonstration of antigenic substitution of *Borrelia burgdorferi* ex vivo: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. The Journal of Experimental Medicine 183: 261-269.

49. Morett E, Segovia L. 1993. The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. Journal of Bacteriology 175: 6067-6074.

50. Mulay VB, Caimano MJ, Iyer R, Dunham-Ems S, Liveris D, Petzke MM, Schwartz I, Radolf JD. 2009. *Borrelia burgdorferi* bba74 Is expressed exclusively during tick feeding and is regulated by both arthropod- and mammalian host-specific signals. Journal of Bacteriology 191: 2783-2794.

51. Österberg S, del Peso-Santos T, Shingler V. 2011. Regulation of alternative sigma factor use. Annual Review of Microbiology 65: 37-55.

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

53. Ouyang Z, Kumar M, Kariu T, Haq S, Goldberg M, Pal U, Norgard MV. 2009. BosR (BB0647) governs virulence expression in *Borrelia burgdorferi*. Molecular Microbiology 74: 1331-1343.

54. Pal U, Yang X, Chen M, Bockenstedt LK, Anderson JF, Flavell RA, Norgard MV, Fikrig E. 2004a. OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. The Journal of Clinical Investigation 113: 220-230.

55. Pal U, Li X, Wang T, Montgomery RR, Ramamoorthi N, deSilva AM, Bao F, Yang XF, Pypaert M, Pradhan D, Kantor FS, Telford S, Anderson JF, Fikrig E. 2004b. TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. Cell 119: 457-468.

56. Poland GA. 2001. Prevention of Lyme disease: A review of the evidence. Mayo Clinic Proceedings 76: 713-724.

57. Pollack RJ, Telford SR, Spielman A. 1993. Standardization of medium for culturing Lyme disease spirochetes. Journal of Clinical Microbiology 31: 1251-1255.

58. Posey JE, Gherardini FC. 2000. Lack of a role for iron in the Lyme disease pathogen. Science 288: 1651-1653.

59. Radolf JD, Caimano MJ, Stevenson B, Hu LT. 2012. Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. Nature Reviews Microbiology 10: 87-99.

60. Rea RB, Gahan CGM, Hill C. 2004. Disruption of putative regulatory loci in *Listeria monocytogenes* demonstrates a significant role for Fur and PerR in virulence. Infection and Immunity 72: 717-727.

61. Reitzer L, Schneider BL. 2001. Metabolic context and possible physiological themes of σ54-dependent genes in *Escherichia coli*. Microbiology and Molecular Biology Reviews 65: 422-444.

62. Samuels DS. 2011. Gene regulation in *Borrelia burgdorferi*. Annual Review of Microbiology 65: 479-499.

63. Samuels DS, Radolf JD. 2009. Who is the BosR around here anyway? Molecular Microbiology 74: 1295-1299.

64. Schoen RT. 1991. Pathogenesis, diagnosis, manifestations, and treatment of Lyme disease. Current Opinion in Rheumatology 3: 610-616.

65. Schwan TG, Piesman J. 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. Journal of Clinical Microbiology 38: 382-388.

66. Schwan TG, Piesman J, Golde WT, Dolan MC, Rosa PA. 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proceedings of the National Academy of Sciences 92: 2909-2913.

67. Shimada T, Ishihama A, Busby SJW, Grainger DC. 2008. The *Escherichia coli* RutR transcription factor binds at targets within genes as well as intergenic regions. Nucleic Acids Research 36: 3950-3955.

68. Shingler V. 2011. Signal sensory systems that impact σ54-dependent transcription. FEMS Microbiology Reviews 35: 425-440.

69. Smith TG, Pereira L, Hoover TR. 2009. *Helicobacter pylori* FlhB processing-deficient variants affect flagellar assembly but not flagellar gene expression. Microbiology 155: 1170-1180.

70. Smith AH, Blevins JS, Bachlani GN, Yang XF, Norgard MV. 2007. Evidence that RpoS (σS) in *Borrelia burgdorferi* Is controlled directly by RpoN (σ54/σN). Journal of Bacteriology 189: 2139-2144.

71. Steere AC. 2001. Lyme disease. New England Journal of Medicine 345: 115-125.

72. Steere AC, Broderick TF, Malawista SE. 1978. Erythema chronicum migrans and Lyme arthritis: epidemiologic evidence for a tick vector. American Journal of Epidemiology 108: 312-321.

73. Steere AC, Coburn J, Glickstein L. 2004. The emergence of Lyme disease. The Journal of Clinical Investigation 113: 1093-1101.

74. Steere AC, Malawista SE, Snydman DR, Shope RE, Andiman WA, Ross MR, Steele FM. 1977. An epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis & Rheumatism 20: 7-17.

75. Steere AC, Grodzicki RL, Kornblatt AN, Craft JE, Barbour AG, Burgdorfer W, Schmid GP, Johnson E, Malawista SE. 1983. The spirochetal etiology of Lyme disease. New England Journal of Medicine 308: 733-740.

76. Studholme DJ, Buck M. 2000a. Novel roles of σN in small genomes. Microbiology 146: 4-5.

77. Studholme DJ, Buck M. 2000b. The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. FEMS Microbiology Letters 186: 1-9.

78. Suh S-J, Silo-Suh L, Woods DE, Hassett DJ, West SEH, Ohman DE. 1999. Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. Journal of Bacteriology 181: 3890-3897.

79. Totten PA, Lara JC, Lory S. 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. Journal of Bacteriology 172: 389-396.

80. Vazquez M, Muehlenbein C, Cartter M, Hayes EB, Ertel S-H, Shapiro ED. 2008. Effectiveness of personal protective measures to prevent Lyme disease. Emerging Infectious Diseases 14: 210-216.

81. Venturi V. 2003. Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? Molecular Microbiology 49: 1-9.

82. Wormser GP, Dattwyler, RJ, Shapiro, ED, Halperin, JJ, Steere, AC, Klempner, MS, Krause, PJ, Bakken, JS, Strle, F, Stanek, G, Bockenstedt, L, Fish, D, Dumler, JS, Nadelman, RB. 2006. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clinical Infectious Diseases 43: 1089-1134.

83. Wright WF, Riedel DJ, Talwani R, Gilliam BL. 2012. Diagnosis and management of Lyme disease. American Family Physician 85: 1086-1093.

84. Yang XF, Alani SM, Norgard MV. 2003b. The response regulator Rrp2 is essential for the expression of major membrane lipoproteins in *Borrelia burgdorferi*. Proceedings of the National Academy of Sciences 100: 11001-11006.

85. Yang XF, Hübner A, Popova TG, Hagman KE, Norgard MV. 2003a. Regulation of expression of the paralogous Mlp family in *Borrelia burgdorferi*. Infection and Immunity 71: 5012-5020.

86. Yang XF, Pal U, Alani SM, Fikrig E, Norgard MV. 2004. Essential role for OspA/B in the life cycle of the Lyme disease spirochete. The Journal of Experimental Medicine 199: 641-648.

87. Yang XF, Goldberg MS, Popova TG, Schoeler GB, Wikel SK, Hagman KE, Norgard MV. 2000. Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. Molecular Microbiology 37: 1470-1479.

88. Yang XF, Lybecker MC, Pal U, Alani SM, Blevins J, Revel AT, Samuels DS, Norgard MV. 2005. Analysis of the *ospC* regulatory element controlled by the RpoN-RpoS regulatory pathway in *Borrelia burgdorferi*. Journal of Bacteriology 187: 4822-4829.

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