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**A MOLECULAR GENETIC INVESTIGATION OF THE REGULATION OF OUTER
SURFACE PROTEIN C IN *BORRELIA BURGENDORFERI*: IDENTIFICATION AND
CHARACTERIZATION OF THE NOVEL REGULATORY PROTEIN BBD18**

By

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Dissertation

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for the degree of

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in Biomedical Sciences

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A Molecular Genetic Investigation of the Regulation of Outer Surface Protein C in *Borrelia burgdorferi*: Identification and Characterization of the Novel Regulatory Protein BBD18

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Lyme disease, caused by *Borrelia burgdorferi*, is the most prevalent arthropod-borne disease in the U.S. In nature, *B. burgdorferi* is maintained in an enzootic cycle between *Ixodes* ticks and mammalian hosts. In order to cause infection in mammals, *B. burgdorferi* spirochetes within a feeding tick sense environmental changes and subsequently alter their gene expression and protein profiles. As part of this adaptation, spirochetes within the tick midgut downregulate outer surface protein (Osp) A and upregulate OspC. Although OspC is an essential *B. burgdorferi* virulence factor needed for early mammalian infection, it is also a target for the mammalian acquired immune response, and thus, OspC is repressed soon after *B. burgdorferi* establishes infection. It has been shown that the central Rrp2/RpoN/RpoS pathway is essential for the upregulation of OspC and other genes important for mammalian infection. However, many key aspects in the strict regulation of OspC remain undefined. In order to investigate the complex regulatory mechanisms controlling OspC expression, we adapted the *lacZ* reporter system from *Escherichia coli* for use in *B. burgdorferi*. Using this *lacZ* system and other molecular genetic approaches, we identified BBD18 as a novel factor that has the potential to negatively regulate OspC. Expression of BBD18 in *B. burgdorferi* repressed transcription of *ospC* and abrogated infection in mice. We determined that BBD18 likely interfaces with the central Rrp2/RpoN/RpoS pathway and exerts its effect on OspC through the repression of RpoS. Structural modeling indicated that BBD18 has a putative DNA-binding motif, and site-directed mutagenesis within this domain abrogated BBD18's ability to repress OspC and prevent infection of mice. We propose that the BBD18 protein acts in concert with other regulatory factors to precisely control gene regulation in *B. burgdorferi* during the enzootic cycle.

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CHAPTER ONE: INTRODUCTION

The Biology of *Borrelia burgdorferi*

Tick-borne spirochetes in the genus *Borrelia* fall into two major groups, one containing the causative agents of Lyme borreliosis (referred to as Lyme disease in the United States), which are collectively referred to as *Borrelia burgdorferi* sensu lato (s.l.), and the other containing the spirochetes responsible for relapsing fever. The *B. burgdorferi* s. l. complex includes more than 20 species, but three species predominate as the human pathogens: *B. burgdorferi* sensu stricto (s.s.) in the United States and Western Europe, and *Borrelia garinii* and *Borrelia afzelii* in Eurasia (1). Hereafter, *B. burgdorferi* is used to refer to *B. burgdorferi* s.l.

Borrelia species are neither Gram-negative nor Gram-positive. Although Borreliae have a double membrane, they are distinguished from Gram-negative bacteria by the absence of lipopolysaccharide (LPS) (2). A thin outer membrane surrounds the protoplasmic cylinder, which includes a peptidoglycan layer and the cytoplasmic membrane, enclosing the cellular cytoplasm. The flagella, which wrap around the cytoplasmic cylinder multiple times within the periplasmic space, are attached at each pole and give spirochetes their characteristic helical shape and motility (Figure 1-1) (3)

B. burgdorferi spirochetes are transmitted to mammalian hosts by hard-bodied ticks of the genus *Ixodes* (see below) and do not survive in nature outside of these environments (4). Due to the nature of this life style, combined with a relatively small genome (see below), *B. burgdorferi* have few biosynthetic capabilities and require amino acids, nucleotides, and fatty acids from their hosts for

Figure 1-1: Structure and Morphology of *Borrelia burgdorferi* (5)

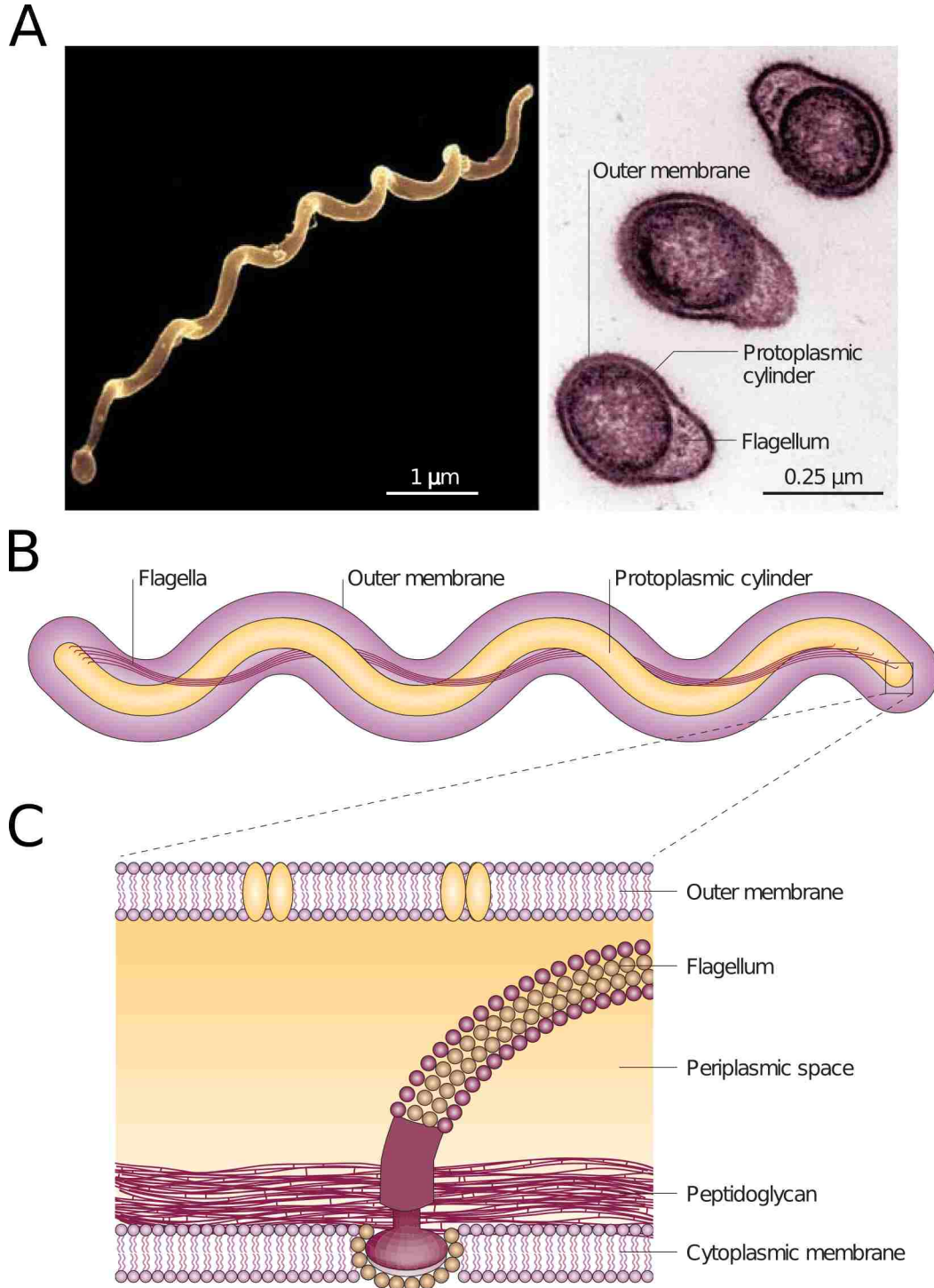


Figure 1-1 legend: **A.** Scanning (left) and transmission (right) electron micrographs of *Borrelia burgdorferi*. The helical shape of *Borrelia* (visible in the scanning electron micrograph) is imparted by the periplasmic flagella, which can be seen in the cross-sectional view of the spirochete in the transmission electron micrograph. Micrographs provided by David Dorward and Elizabeth Fischer, Rocky Mountain Laboratories, NIAID, NIH. Left-hand panel is reproduced with permission from Nature 11 Dec 1997 (cover image) © Macmillan Magazines Ltd. **B.** Diagram of the spirochete. Flagellar insertion points are located near the termini of the spirochete. Bundles of flagella wind around the flexible, rod-shaped protoplasmic cylinder of *Borrelia* and overlap in the middle. The outer membrane constrains the flagellar bundles within the periplasm. **C.** Detailed diagram of flagella. Each flagellum is inserted into the cytoplasmic membrane and extends through the cell wall into the periplasm. Flagella are multi-component, complex structures. Spirochete motility results from coordinated rotation of the flagella. Part C is modified with permission from (6) © (2002) Annual Reviews. This figure was reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (5) © 2005.

growth. Additionally, *B. burgdorferi* only utilizes a limited number of carbon sources and the fermentation of glucose to lactic acid is the primary source of energy for these obligate extracellular parasites (2, 7).

The Life Cycle of *B. burgdorferi*

B. burgdorferi spirochetes exist in an enzootic cycle between hard bodied tick vectors and vertebrate hosts (Figure 1-2) (4). The primary tick vector for the transmission of *B. burgdorferi* s.s. is *Ixodes scapularis* in the eastern and midwestern portions of the United States, and *I. pacificus* in the western U.S; *I. ricinus* and *I. persulcatus* are primarily responsible for transmission of *B. burgdorferi* s. l. in Europe and Asia, respectively (8).

Ixodes ticks undergo a three-stage life cycle: larva, nymph, and adult, with one blood meal per stage. *B. burgdorferi* is not transmitted from an infected adult to eggs (transovarial transmission) so larval ticks are not infected with *B. burgdorferi* when they hatch. Thus, larval ticks acquire *B. burgdorferi* by feeding on an infected reservoir host. Although larval *Ixodes* ticks feed on many different animals, the main reservoir for *B. burgdorferi* s.s. in the northeastern U.S. is the white-footed mouse, *Peromyscus leucopus* (9); several species of rodents, small mammals, and migratory birds act as the reservoirs for *B. burgdorferi* s.l. strains in Europe. Once larval ticks acquire *B. burgdorferi*, the spirochetes are maintained throughout subsequent tick stages (transstadial transmission). After molting to the nymphal stage, ticks transmit spirochetes to the animal that provides their next blood meal. Humans act as incidental hosts for infected nymphs and are generally thought of as dead-end hosts and not part of the enzootic cycle of *B. burgdorferi*. Adult *Ixodes* ticks are not traditionally important in the maintenance of *B. burgdorferi*, as they predominantly

Figure 1-2: The enzootic cycle of *B. burgdorferi* (10)

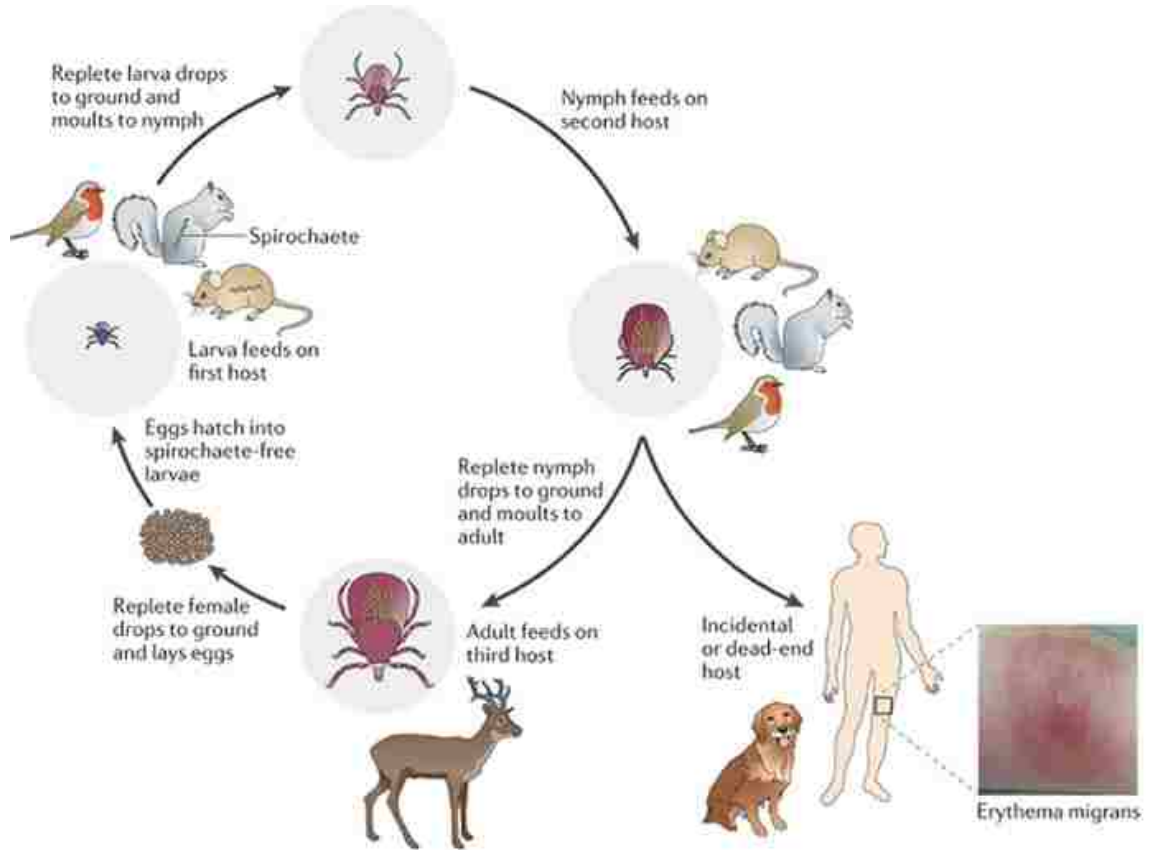


Figure 1-2 legend: Graphical representation of the infectious cycle of *B. burgdorferi*.
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feed on incompetent hosts for *B. burgdorferi*, such as deer. However, since adult ticks mate on these larger animals (10), they are important for the maintenance of the tick population and thus indirectly for the enzootic cycle of *B. burgdorferi*.

Epidemiology

Lyme disease is the most common arthropod-borne disease in the U.S. The incidence of Lyme disease has increased dramatically since the Centers for Disease Control and Prevention began surveillance in 1982, with approximately 32,000 confirmed and probable cases in the U.S. for 2011. Since *B. burgdorferi* is transmitted by *Ixodes* ticks, most cases of Lyme disease occur during early spring to summer, when outdoor activities coincide with nymphal ticks searching for their blood meal. *Ixodes* ticks typically feed for 3-7 days, depending on the life stage; transmission of spirochetes generally occurs after ticks have been attached for 48 hours or longer and rarely, if ever, occurs during the first 24 hours (11, 12). Upon initiation of feeding, *B. burgdorferi* within the tick midgut undergo rapid replication and subsequently migrate to the tick salivary glands, a process that takes ~48 hours, accounting for the delay in transmission of *B. burgdorferi* (13, 14). Thus, the chance of acquiring Lyme disease is greatly reduced if ticks are removed shortly after attachment.

Lyme disease

Lyme disease was first recognized in the U.S. in 1977 when an epidemic of childhood arthritis occurred in Lyme, Connecticut (15). It was subsequently discovered that an antibiotic-sensitive organism transmitted by *Ixodes* ticks was responsible for the disease (16). In 1982, Burgdorfer and colleagues successfully isolated the etiological agent of Lyme disease, which was named *Borrelia*

burgdorferi, from the midgut of *I. scapularis* ticks collected from Shelter Island, NY (4).

During tick feeding, *B. burgdorferi* are deposited into the bite site. Although these tick-inoculated spirochetes can cause chronic infection in a wide variety of mammals, the disease manifestation depends on the recipient animal. The reservoir hosts remain persistently infected without showing any overt symptoms. All laboratory strains of mice (*Mus musculus*) are susceptible to infection, but the development of clinical symptoms in immunocompetent mice is highly strain specific.

In humans, Lyme disease is a multi-system, multi-stage disorder that can affect the skin, joints, heart, and nervous system and is generally subdivided into three stages (17). During stage 1, 70-80% of patients develop a localized inflammatory reaction in the skin called erythema migrans (EM) around the site of the tick bite. Stage 1 manifestations can also include mild influenza-like symptoms, occurring with or without the characteristic “bull’s eye” EM rash (18). Within weeks to months, *B. burgdorferi* will disseminate through a transient blood-borne phase, and thus, symptoms associated with stage 2 Lyme disease, which include arthritis, carditis, and neuropathies, revolve around the organ(s) that spirochetes have colonized. If untreated, Lyme disease can progress to stage 3. Patients with this late-persistent disease suffer from chronic arthritis and neuroborreliosis, which can manifest as meningoencephalitis, facial paralysis (Bell’s palsy), or radiculoneuritis. A skin condition known as acrodermatitis chronica atrophicans (ACA) is a common manifestation of *B. burgdorferi* infection in Europe but rarely occurs in the U.S. The prevalence of particular symptoms varies between the geographic locations, with

arthritis more common in the U.S. and neurological symptoms and skin disorders (e.g. ACA) more common in Europe (17), presumably reflecting distinct clinical manifestations that result from infection with different *B. burgdorferi* s.l. genospecies (19).

The *B. burgdorferi* genome does not encode any known toxins or the delivery system required to secrete them (2, 20) and thus, Lyme disease is thought to be mediated by the mammalian inflammatory response elicited by the spirochetes. Several studies have associated the variation in severity and symptoms of Lyme disease with a dysregulation in the balance of pro- and anti-inflammatory responses (21-24). Still, the symptoms of Lyme disease respond to antibiotic therapy in most patients, suggesting that disease manifestations are the direct results of spirochetal infection. A 2-4 week course of antibiotics is generally effective for stage 1 and 2 Lyme disease, although some patients develop chronic symptoms that are unresponsive to antibiotic therapy, suggesting the possibility of infection-induced autoimmunity (17).

The *B. burgdorferi* genome

Spirochetes of the genus *Borrelia* are unique in that they possess the most complex genomes of all characterized bacteria. More than 20 coexisting linear and circular genetic elements have been identified in the *B. burgdorferi* type strain B31 (Figure 1-3). This includes a small (~910 kilobase pairs, kbp) linear chromosome, 12 linear plasmids ranging in size from five to 56 kbp, and nine circular plasmids ranging from nine to 32 kbp (2, 20). Additional plasmids have been described in other *B. burgdorferi* isolates (25, 26). In total, these plasmids comprise 610 kbp and account for ~40% of the genetic content of *B. burgdorferi* (20).

Figure 1-3: The Unique Genome of *Borrelia burgdorferi* B31

Linear chromosome-910kb

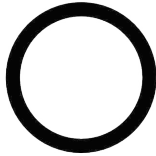


Circular plasmids

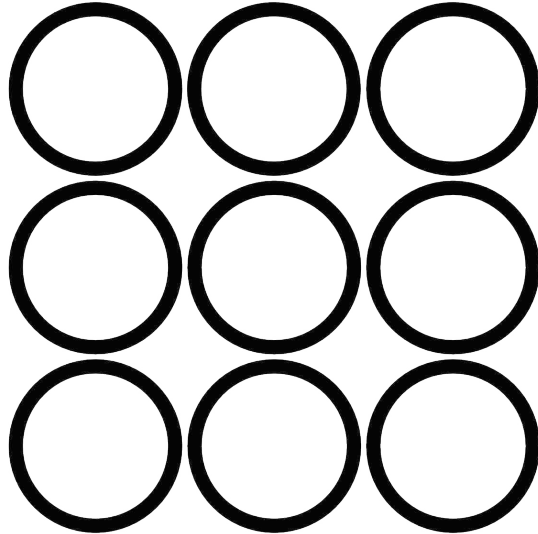
cp9



cp26



cp32 (1-9)



Linear plasmids

lp5



lp17



lp21



lp25



lp28-1



lp28-2



lp28-3



lp28-4



lp36



lp38



lp54



lp56



Figure 1-3 legend: Graphical representation of the segmented genome of *B. burgdorferi* (not to scale). Numbers represents the approximate size of the circular plasmid (cp) or linear plasmid (lp) in kilobase pairs (kbp). This figure closely resembles a figure from reference (27).

The chromosome encodes the majority of housekeeping genes and is fairly well conserved across the *Borrelia* genus (28). More than half of the predicted chromosomal open reading frames (ORFs) were assigned functions based on homology to genes in other organisms (2). In contrast, when originally annotated, only 8% of plasmid-encoded ORFs had homologs in other bacteria and less than 6% of those had an assigned function (2, 20). Although these numbers have increased somewhat with the addition of many new bacterial genome sequences, the highly segmented genome structure has remained unique to *Borrelia* (26).

Much of the content of these plasmids contains non-coding DNA and pseudogenes. In addition, there is high degree of redundancy in the plasmid DNA, with a large number of paralogous gene families distributed throughout the plasmids, and large stretches of essentially identical DNA within the nine-32 kbp circular plasmids (20, 26). Still, a large proportion of plasmid-encoded genes have been shown to be differentially regulated in response to environmental cues that distinguish various stages of the infectious cycle (29-37), suggesting a role for plasmid-encoded genes during the infectious cycle of *B. burgdorferi*.

Although linear chromosomes and plasmids have been described in very few bacteria, the replication initiation mechanisms for the *B. burgdorferi* linear DNA elements appear to be similar to standard circular bacterial chromosomes and plasmids. Picardeau and colleagues mapped the chromosomal origin of replication to a central location and showed that replication proceeds symmetrically and bi-directionally from that region (38). Origins of replication were subsequently identified for lp17, lp25, lp28-4, and lp54, supporting the idea that both the linear chromosome and linear plasmids use similar mechanisms for bi-directional

replication (39). The *B. burgdorferi* linear DNA molecules have covalently closed hairpin ends referred to as telomeres. At each end, the two DNA strands are connected and form a near-perfect AT-rich inverted repeat (40-43). Bi-directional replication is thought to create a head-to-head and tail-to-tail circular dimer, which then must be resolved into two linear DNA molecules (44). An enzyme called telomere resolvase cleaves the duplex DNA in the telomeric regions and regenerates the covalently closed hairpin loops in the progeny molecules (45). Synthetic telomeres have been a key tool in the investigation of the mechanisms of telomere resolution (44). In addition, truncated versions of lp17 created by the insertion of these synthetic telomeres have been used to ascribe functions to some lp17-encoded genes, including the genes necessary for autonomous replication (46).

The putative origins of replication for *B. burgdorferi* circular and linear DNA molecules are all located within the vicinity of a homolog of *parA*, an ATPase that is involved in plasmid partitioning. Furthermore, this gene clusters with other members of a paralogous gene family thought to encode proteins involved in plasmid replication and partitioning. Indeed, autonomously replicating shuttle vectors have been developed using regions from cp9 (47), cp32-3 (48), lp25, lp28-1 (49), cp26 (50), and lp38 (51) that contain members of several paralogous gene families and the putative origin of replication for each native plasmid. These shuttle vectors are incompatible with the native *B. burgdorferi* plasmids from which they were derived, indicating a role for members of the paralogous gene families in the maintenance and compatibility of the many co-existing DNA elements in *B. burgdorferi* (48, 52). In addition, shuttle vectors derived from linear *B. burgdorferi* plasmids lp25 and lp28-1 exist in a circular form, indicating that replication and

partitioning mechanisms are conserved, regardless of DNA form (49). Beaurepaire and Chaconas reported that the transcription of genes varied, depending on whether the gene was located on a linear or a circular plasmid, and suggested that the different topologies present in the *B. burgdorferi* genome might play a role in the complex genetic regulation that the spirochetes undergo as they moves from vector to host and vice versa (53).

B. burgdorferi linear and circular plasmids vary in their stability during *in vitro* growth and some plasmids are frequently lost in a few generations. The fact that most plasmids can be lost indicates that they are not required for bacterial growth *in vitro* (54-56). However, the loss of certain plasmids has been associated with a loss of infectivity in mice (see below) (54, 57-61). Thus, it is necessary to demonstrate that the plasmid content of all relevant *B. burgdorferi* strains are identical to those with which they are being compared in molecular genetic studies.

Certain *B. burgdorferi* plasmids, like cp26 and lp54, are rarely if ever lost in natural isolates. The only copy of the *B. burgdorferi* *resT* gene, encoding the telomere resolvase (see above), is located on cp26 (2, 20, 45), providing a simple explanation for its universal retention. However, *resT* is not the only gene encoded on cp26 that makes it an essential plasmid, as a cp26-derived shuttle vector harboring a copy of *resT* was still unable to displace the native cp26 in *B. burgdorferi* (50). Further studies showed that an incompatible plasmid containing *bbb26*, *bbb27*, and *resT* was able to displace cp26, indicating one or both of these cp26-encoded genes, in addition to *resT*, is required for spirochete viability (62). Of note, *bbb26* and *bbb27* refer to the 26th and 27th genes encoded on cp26, which is designated by the third 'b.' For clarity in naming genes, each *B. burgdorferi* plasmid was given an

arbitrary letter, with the letter 'a' designating genes on lp54, 'c' genes are located on cp9, and so forth.

The gene encoding outer surface protein (Osp) C, which is not essential for *in vitro* growth but is required during early stages of infection in the mammal (see below), is also located on cp26 (63, 64). At least, two other genes encoded on cp26, *guaA* and *guaB*, which are involved in the purine salvage pathway (65), are essential for mammalian infectivity and provide a growth advantage for *B. burgdorferi* within ticks (66).

Although lp54 is usually very stable during *in vitro* growth, it has been lost from at least one highly attenuated strain (67). lp54 encodes many genes that are differentially regulated in response to environmental cues like temperature and pH, suggesting a role for lp54 during the *B. burgdorferi* infectious cycle. The *ospAB* operon is located on lp54. The inverse relationship of OspA and OspC during tick colonization and transmission was the first observation of differentially regulated genes in *B. burgdorferi* (see below) (14). Other genes encoded on lp54 that likely contribute to the *B. burgdorferi* enzootic cycle include *bba52* ((68), *lp6.6* (69), *bba64* (70), *cspA* (71-73), and the *dbpAB* operon (74, 75).

The presence of lp25, lp28-1, and lp36 in *B. burgdorferi* has been directly correlated with infectivity in mice (57, 60, 76). Through molecular genetic approaches, the genes encoded on each of these plasmids that contribute to *B. burgdorferi* infectivity have been identified. Infectivity of mice was restored when spirochetes lacking lp25 were complemented with the *pncA* gene, which encodes a nicotinamidase, suggesting *pncA* is the only gene carried on lp25 that is required for mouse infectivity (59). An additional gene on lp25, *bbe02*, encodes a restriction-

modification system and is responsible for decreased transformation efficiency (77). Inactivating *bbe02* in low passage clones results in increased transformation frequency with shuttle vectors and the resulting clones are infectious in mice (78, 79). An additional restriction-modification system is located on lp56, and spirochetes lacking lp56 exhibit increased transformation efficiency but retain their infectivity in mice (77, 79). One gene encoded on lp25, *bbe16* or *bptA*, is essential for persistence in the tick vector and presumably encodes a surface-exposed lipoprotein that helps anchor spirochetes to the tick midgut (80).

The correlation of lp28-1 and infectivity in mice is attributed to the presence of a mechanism of antigenic variation similar to the variable major protein (*vmp*) system in *B. hermsii*, which causes relapsing fever. In *B. burgdorferi*, this locus is designated as the Vmp-like sequence or *vls* locus, which consists of an expressed *vlsE* region and 15 upstream silent *vls* cassettes (81). The antigenically variable VlsE protein allows *B. burgdorferi* to maintain persistent infection in spite of the host's adaptive immune response to the spirochete (82).

Spirochetes lacking lp36 are non-infectious in mice by needle inoculation and exhibit low infectivity by tick bite, with limited dissemination to internal organs. Infectivity was partially restored when *bbk17*, which encodes a putative adenine deaminase, was expressed on a shuttle vector in lp36-deficient spirochetes (76). Seshu and colleagues reported that inactivating *bbk32*, encoding a fibronectin-binding adhesin, attenuated the infectivity of spirochetes in mice (83). It is unclear whether additional lp36-encoded genes also contribute to mammalian infectivity or persistence in the enzootic cycle of *B. burgdorferi*.

Genetic Regulation

The environmental changes that accompany the tick blood meal trigger a dramatic change in gene expression and the protein profile of *B. burgdorferi* within the tick midgut. *In vitro* changes in pH, temperature, and cell density have been used to induce changes in gene expression, which are thought to parallel the adaptation of *B. burgdorferi* *in vivo*. Direct analysis of *in vivo* gene expression or protein profiles in *B. burgdorferi* is not easily achieved due to the low numbers of spirochetes that can be isolated from infected animals. However, Akins *et al.* developed a technique in which spirochetes are grown to a high density in culture medium within a dialysis membrane chamber (DMC) implanted in the peritoneal cavity of a rat (84), which allows direct analysis of spirochete proteins and RNA (30, 34, 85, 86). Studies using this technique have determined that host-specific factors invoke additional changes in gene expression that are not evident when *B. burgdorferi* are grown under varying culture conditions *in vitro* that mimic tick feeding (30, 34). Using both *in vitro*-grown and host-adapted spirochetes grown within DMCs, the mechanisms that *B. burgdorferi* utilizes to adapt throughout the enzootic cycle have begun to be elucidated.

Differential Regulation of the *B. burgdorferi* Osps

The dramatic alteration in *B. burgdorferi* gene expression that accompanies tick feeding was first recognized with the reciprocal synthesis of the outer surface (lipo) proteins A and C (OspA and OspC, respectively) (87). In unfed nymphal *I. scapularis* ticks infected with *B. burgdorferi*, spirochetes located in the tick midgut express OspA, but not OspC. During tick feeding, the proportion of OspA-positive

spirochetes decreases as spirochetes begin to upregulate OspC, preparing for transmission to the mammalian host (14, 87).

It has been reported that OspA is required for the colonization of the tick midgut (88), presumably through its binding to a tick midgut receptor termed TROSPA (89). However, Battisti *et al.* subsequently demonstrated that the defect in tick colonization of spirochetes lacking the *ospAB* operon was more likely due to immune clearance by antibodies in the incoming blood meal, suggesting a primary function of OspA as a shield for conserved outer surface proteins (90). This is also the premise behind the OspA vaccine for Lyme disease, in which antibodies made against recombinant OspA, a protein not normally produced during natural infection, prevent the transmission of *B. burgdorferi* from the tick to the vaccinated host (91).

The role of OspC during early mammalian infection has been less straightforward. It is well documented that OspC is required for transmission to, and establishing infection within, the mammalian host (92-97). However, the specific functions of OspC are not fully understood.

Early studies reported that OspC is involved in the dissemination of spirochetes from the tick midgut to the salivary glands (93, 98). However, Grimm and colleagues reported that spirochetes lacking OspC migrated to the tick salivary glands efficiently but were unable to infect mice, suggesting that OspC is not essential for the dissemination to the salivary glands but rather provides a critical function during early infection in mammals (94-97). In addition, patients infected with *B. burgdorferi* often develop an early antibody response to OspC, indicating that spirochetes expressing OspC are transmitted from vector to host (99).

However, OspC on the surface of *B. burgdorferi* is transient, as it represents a target for neutralizing antibodies, and thus, spirochetes downregulate *ospC* to avoid being cleared by the host's adaptive immune response (82, 100, 101).

The strict timing of *ospC* expression coincides with the host's innate immune response and suggests that OspC may play a role in avoiding clearance by the innate immune system. There have been several reports implicating myeloid differentiation marker 88 (MyD88) in mammalian innate immunity to *B. burgdorferi* (102-104). MyD88 is a common adapter molecule required by many receptors and pathways that trigger the innate immune response to microbial invasion. Stewart and colleagues hypothesized that OspC might be important in evasion of MyD88-dependent innate immunity, but spirochetes lacking *ospC* were unable to infect MyD88-deficient mice, suggesting that the role of OspC is not solely to avoid clearance by MyD88-dependent immunity (95). Sarkar *et al.* also showed that *B. burgdorferi* resistance to antimicrobial peptides was not dependent on the presence of OspC. Although the primary function of OspC remains mysterious, the essential nature of OspC is quite clear.

It has been shown that variation in culture conditions to which mimic the environmental changes experienced within a feeding tick, such as an increase in temperature and/or a decrease in pH, induce the expression of OspC. In contrast, the downregulation of OspA has only been seen in spirochetes within feeding ticks or grown within DMCs and is not evident *in vitro*. Regardless, the upregulation of OspC and the downregulation of OspA are both dependent on the alternative sigma factor RpoS (34, 105-108), which acts as a central 'gatekeeper,' regulating the

inverse relationship of *B. burgdorferi* genes involved in mammalian infection and in persistence of spirochetes in the tick vector (34).

The RpoN/RpoS regulon

Considering the extensive transcriptional changes that the spirochete undergoes throughout the infectious cycle, the *B. burgdorferi* genome does not encode many commonly known genetic regulators, but genes for two alternative sigma factors RpoN and RpoS are located on the chromosome (2). In many bacteria, alternative sigma factors function as master regulators by coordinating the RNA polymerase holoenzyme to specific genes required for the adaptation to environmental or physiological stress. In *E. coli*, RpoS is required for the global response to such stresses as nutrient deprivation, high osmolarity, and acidic pH (109). However, Caimano *et al.* demonstrated that the *B. burgdorferi* RpoS is not involved in the stress response but controls virulence expression during the enzootic cycle (85).

E. coli RpoS levels are under strict control through multiple mechanisms that regulate *rpoS* transcription, translation, and protein degradation. In *B. burgdorferi*, RpoS, which is necessary for mammalian infection (85) and transmission during tick feeding (110), is also tightly regulated. Yang *et al.* first demonstrated that RpoS was OspC-like in that it is induced by conditions that mimic those within a feeding tick (see above), suggesting that *ospC* might be controlled through RpoS (105). This hypothesis was later confirmed (106-108). In a seminal study, Hübner and colleagues demonstrated that *B. burgdorferi* RpoN regulates *rpoS* transcription through the distinct -24/-12 promoter (106). These data established the novel dual

sigma factor cascade in the regulation of important virulence factors during the *B. burgdorferi* enzootic cycle.

RpoN-dependent transcription requires the activity of an enhancer binding protein, adding another level of regulation. In *B. burgdorferi*, a response regulator Rrp2 coordinates with RpoN to induce *rpoS* transcription (111). However, Rrp2 must be phosphorylated to activate RpoN-dependent *rpoS* gene expression. The *hk2* gene, which encodes a sensor kinase, is located just upstream of *rrp2* and was implicated in activating Rrp2 and thus the RpoN/RpoS cascade. However, disruption of *hk2* had no effect on RpoN/RpoS or infectivity in mice, suggesting Hk2 was not responsible for activation of Rrp2 (112). Subsequent studies demonstrated that acetyl phosphate could bypass the need for sensor kinases and directly phosphorylate Rrp2 to induce the RpoN/RpoS cascade (86).

Levels of acetyl phosphate within *B. burgdorferi* are controlled by two enzymes, AckA and Pta, where AckA forms acetyl-phosphate from acetate and Pta degrades it in the process of making acetyl-CoA. Recently, it was shown that *bb0184*, which encodes carbon storage regulator A (CsrA), can affect the RpoN/RpoS regulon through its effect on acetyl phosphate levels, presumably by repressing Pta. In *E. coli*, CsrA is an RNA binding protein that regulates gene expression post-transcriptionally. CsrA induction in *B. burgdorferi* is correlated with activation of Rrp2 and upregulation of RpoS-dependent genes, further suggesting a role for CsrA in the complicated regulatory cascade during the transmission of *B. burgdorferi* from tick to mammal (113, 114). However, the mechanisms by which CsrA is activated remain unclear. In addition, multiple other *B. burgdorferi* factors have been implicated in regulating the Rrp2/RpoN/RpoS pathway.

BB0647, originally annotated as a homolog of Ferric uptake regulator (Fur) (2), was renamed Borrelia oxidative stress regulator (BosR) after it was shown that *B. burgdorferi* does not use iron (115) and that BB0647 activated the oxidative stress response in *B. burgdorferi* (116). Two parallel studies determined that BosR was required for efficient RpoS production and thus the upregulation of *ospC* and other genes necessary for mammalian infectivity (37, 117). This is presumably the reason the *bosR* mutant is not infectious in mice (37, 117, 118), as spirochetes lacking RpoS or OspC are non-infectious in mice (85, 94-97). BosR affects the transcription of *rpoS*, but levels of *rrp2* and *rpoN* levels were not altered in the BosR mutant, indicating that BosR specifically targets *rpoS* and not the entire Rrp2/RpoN/RpoS cascade (37, 117, 118). It has been reported that there are three putative BosR binding sites that could potentially affect *rpoS* transcription; two sites are located upstream of the RpoN-dependent -24/-12 *rpoS* promoter, and one is located within the *rpoS* ORF (118). However, Ouyang *et al.* only explored BosR binding to synthetic probes and not activation of *rpoS* transcription in *B. burgdorferi* (118). In addition, a previous study restored mouse infectivity to spirochetes lacking *rpoS* using *rpoS* expressed from a minimal *rpoS* promoter that only contained the -24/-12 regions (119) and lacked the two sites to which BosR bound with greater affinity (118), suggesting that the putative BosR binding sites were not necessary for mammalian infection. Still, these mice were infected by needle inoculation, and it is unclear if *rpoS* expressed from this minimal promoter would be infectious in mice through natural transmission by tick bite.

In *E. coli*, several small non-coding RNAs (sRNAs) bind to the *rpoS* transcript and regulate its translation. Lybecker and Samuels identified an sRNA in *B.*

burgdorferi that is complementary to the upstream region of *rpoS* mRNA, which they named DsrA after its similarity to the sRNA of the same name in *E. coli* (120). In the canonical RpoN-dependent pathway, *rpoS* is induced in response to an increase in temperature, decrease in pH and/or an increase in cell density. However, Lybecker and Samuels identified a longer *rpoS* transcript transcribed at low cell density, which is not dependent on RpoN. Furthermore, only this longer *rpoS* transcript contains the sequence to which DsrA is complementary (120). In *E. coli*, DsrA base pairs with the upstream region of the *rpoS* transcript, which leads to a conformational change that frees the Shine-Dalgarno sequence, allowing translation to occur, and it is thought that DsrA in *B. burgdorferi* functions by a similar mechanism to regulate *rpoS* translation (120).

The activity of sRNAs in *E. coli* requires the RNA chaperone Hfq. *B. burgdorferi* seems similar in this regard as Lybecker and colleagues identified a *B. burgdorferi* homolog of the *E. coli* Hfq. They determined that the *B. burgdorferi* Hfq bound DsrA and the *rpoS* transcript (121). Interestingly, neither the long *rpoS* transcript nor DsrA are required for infectivity in mice by needle inoculation (119, 122), but the *hfq* mutant is non-infectious in mice (121). These data suggest that Hfq likely has other roles in the cell besides its interaction with DsrA and the long *rpoS* transcript. In addition, the infectivity of the DsrA mutant by needle inoculation suggests that DsrA and the long *rpoS* transcript might function exclusively in the tick vector as spirochetes prepare for transmission to the mammalian host (122).

Lastly, there have been some conflicting results as to whether Rrp2 and RpoN have functions other than their requirements for expression of RpoS. Whereas Fisher *et al.* described groups of genes that were dependent on RpoN, RpoS, or both

(110), Ouyang *et al.* reported considerable overlap in genes that were regulated by these two alternative sigma factors (35). However, Ouyang and colleagues also reported that a subset of genes regulated by Rrp2 were independent of RpoN and RpoS (35). Both of these studies used microarray analysis as their primary technique to identify differentially regulated genes and it is well documented that changes in culture conditions affect *B. burgdorferi* gene expression, which could account for the discrepancies between the studies.

Since Lyme disease was first recognized in the U.S. three decades ago, there has been an impressive gain of knowledge on the pathogens that cause Lyme disease, but many aspects of the biology of *B. burgdorferi* remain mysterious. Undoubtedly, future studies will further elucidate how *B. burgdorferi* adapts to changing environments to survive and persist in its enzootic cycle.

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CHAPTER TWO

A lacZ reporter system for use in Borrelia burgdorferi

Forward

This chapter is adapted from Hayes BM, Jewett MW, Rosa PA. 2010. A *lacZ* reporter system for use in *Borrelia burgdorferi*. Applied and Environmental Microbiology 76:7407-7412. Copyright © 2010, American Society for Microbiology. All rights reserved.

Abstract

Regulation of gene expression is critical for the ability of *Borrelia burgdorferi* to adapt to different environments during its natural infectious cycle. Reporter genes have been used successfully to study gene regulation in multiple organisms. Here, I have introduced a *lacZ* gene into *B. burgdorferi* and demonstrate that *B. burgdorferi* produces a protein with detectable β -galactosidase activity in both liquid and solid media when *lacZ* is expressed from a constitutive promoter. Furthermore, when *lacZ* is expressed from the *ospC* promoter, β -galactosidase activity is detected only in *B. burgdorferi* clones that express *ospC*. Using this *ospC* promoter-*lacZ* fusion, I show that *ospC* promoter activity varies on a colony level in *B. burgdorferi*, which has not been previously demonstrated. These data demonstrate that *lacZ* can be used to accurately monitor endogenous gene expression in *B. burgdorferi*. The addition of *lacZ* to the repertoire of genetic tools available for use in *B. burgdorferi* has contributed to a better understanding of how *B. burgdorferi* gene expression is regulated during the infectious cycle.

Introduction

Borrelia burgdorferi sensu lato, the pathogens that cause Lyme disease (1), alternate between two distinct environments, an arthropod vector and a vertebrate host. As *B. burgdorferi* moves from one milieu to the other, its ability to adapt and survive requires dramatic changes in gene expression. Many studies have shown that different *B. burgdorferi* gene products are upregulated or downregulated at specific times throughout the infectious cycle (2, 3) and in response to host and environmental signals (4-8). Although it is clear that *B. burgdorferi* alters gene expression to adapt to different environments, the genetic tools for studying gene regulation in *B. burgdorferi* are limited.

Within the last two decades, the complete genomic sequence of *B. burgdorferi* strain B31 was published (9, 10), and techniques for basic genetic manipulation of *B. burgdorferi* became available (11-17). A chloramphenicol acetyltransferase (CAT) gene was the first reporter gene fused to *B. burgdorferi* promoters for analysis of promoter strength (18). The development of luciferase (19) and multiple fluorescent proteins (15, 20, 21) as reporter systems in *B. burgdorferi* followed. Although these systems have value, there are limitations with each (see discussion). β -galactosidase, encoded by *lacZ*, has been used extensively as a convenient reporter gene in *Escherichia coli* and is still applicable to a broad range of organisms, both prokaryotic and eukaryotic, but has not yet been used in *B. burgdorferi*. β -galactosidase activity can be monitored easily and quickly by simple colorimetric assays in both liquid and solid media, neither of which require expensive or specialized equipment. Additionally, a wide variety of substrates for β -galactosidase allow for different levels of sensitivity in either *in vitro* or *in vivo* detection formats

(22). Having *lacZ* available as a genetic tool for *B. burgdorferi* would enhance investigation of the complex regulatory events that are integral to the spirochete's infectious cycle. To this end, I developed *lacZ* as a reporter gene in *B. burgdorferi* and demonstrated its utility.

Materials and Methods

All chemicals and materials were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Bacterial Strains and Growth Conditions.

Plasmids (Table 2-1) for cloning were transformed into electrocompetent or chemically competent Top10 *E. coli* (Invitrogen, Carlsbad, CA) and final constructs transformed into a Δlac *E. coli* strain, MC4100 *lamB- zjb::Tn10* (kindly provided by John Carlson, Rocky Mountain Laboratories, Hamilton, MT). *E. coli* was plated on LB agar (10 g/L Tryptone, 5 g/L NaCl, 5 g/L Yeast extract, and 15 g/L agar) with the appropriate antibiotic (spectinomycin at 300 $\mu\text{g}/\text{mL}$, kanamycin at 30 $\mu\text{g}/\text{mL}$, or gentamicin at 5 $\mu\text{g}/\text{mL}$). Liquid cultures were grown in LB broth supplemented with the appropriate antibiotics (spectinomycin or kanamycin at 100 $\mu\text{g}/\text{mL}$ or gentamicin at 10 $\mu\text{g}/\text{mL}$).

BSKII medium (23) for culture of *B. burgdorferi* was made with CMRL 1060 lacking phenol red (US Biologicals, Swampscott, MA). *B. burgdorferi* strain B31 clones A34 (24) or B312 (25) were grown to mid log phase ($\sim 5 \times 10^7$ cell/ml). Spirochetes were enumerated using dark-field microscopy and a Petroff-Hausser counting chamber and prepared for electroporation as described previously (11). Transformed A34 and B312 were selected using gentamicin at a concentration

Table 2-1: Plasmids used in Chapter Two

Plasmid	Resistance	Comments; source or reference
pPBMB101	kan ^R	plasmid with an <i>E. coli lacZ</i> gene, used as template to amplify <i>lacZEc</i> ; (26)
pCR8/GW/TOPO	spec ^R	Gateway PCR entry vector; Invitrogen
pCR2.1/TOPO	kan ^R , amp ^R	PCR cloning vector; Invitrogen
pBSV2G	gent ^R	<i>B. burgdorferi</i> shuttle vector; (13)
pBSV2G_dvB2	gent ^R	altered pBSV2G, Gateway destination vector; James A. Carroll (unpublished)
pBH-lacZEc	gent ^R	<i>E. coli lacZ</i> gene (<i>lacZEc</i>) cloned into pBSV2G_dvB2; this work
pBHflaBp-lacZEc	gent ^R	pBH-lacZEc with the <i>flaB</i> promoter cloned upstream of <i>lacZEc</i> ; this work
pBH-lacZBb	gent ^R	<i>B. burgdorferi</i> codon-optimized <i>lacZ</i> gene (<i>lacZBb</i>) cloned into pBSV2G; this work
pBHflaBp-lacZBb	gent ^R	pBH-lacZBb with the <i>flaB</i> promoter cloned upstream of <i>lacZBb</i> ; this work
pBHospCp-lacZBb	gent ^R	pBH-lacZBb with the <i>ospC</i> promoter cloned upstream of <i>lacZBb</i> ; this work

of 40µg/mL. All *B. burgdorferi* cultures were grown at 35°C and plates were incubated under 2.5% CO₂.

Plasmid construction.

The *E. coli lacZ* gene (*lacZEc*), the multiple cloning site (MCS), and the transcriptional terminator were amplified from pPBMB101 (Table 2-1) (26) using primers 1 and 2 (Table 2-2). The PCR product was cloned into the Gateway® entrance vector pCR8/GW/TOPO (Table 2-1) (Invitrogen) and confirmed by direct sequencing with primers 1-10 (Table 2-2). pBSV2G_dvB2 (Table 2-1), kindly provided by James A. Carroll (Rocky Mountain Laboratories, Hamilton, MT), is an altered form of the *B. burgdorferi* shuttle vector pBSV2G, which has *attR1* and *attR2* sequences surrounding a chloramphenicol resistance cassette and the counter-selectable gene, *ccdB*, making it a suitable destination vector for Gateway® cloning. The insert containing *lacZEc*, the MCS, and the transcriptional terminator was transferred from pCR8 to pBSV2G_dvB2 using the Clonase II enzyme (Invitrogen), creating pBH-lacZEc (Table 2-1 and Figure 2-1A).

The *flaB* promoter was amplified from *B. burgdorferi* genomic DNA using primers 13 and 14 (Table 2-2). The PCR fragment was cloned into pCR2.1-TOPO (Invitrogen) and sequenced to confirm the insert. The *flaB* promoter was excised with BamHI and XhoI and ligated into appropriately digested pBH-lacZEc, creating pBHflaBp-lacZEc (Table 2-1 and Figure 2-1A). BamHI and XhoI were used for creating promoter-*lacZ* fusions in pPBMB101, which has a ribosome binding site (RBS) between the XhoI site and the *lacZ* start site (26).

pBH-lacZEc and pBHflaBp-lacZEc were transformed into electrocompetent *B. burgdorferi* (11). Spirochetes were allowed to recover overnight in BSK II medium

Table 2-2: Primers and Probes used in Chapter Two

	Name	Sequence (5'-3') ^a
1	Long <i>lacZ</i> 5' (NotI)	AAGGAAAAAAGCGGCCGCTCTAGAGGATCCCGGGTCGACTAGTCTGCAGCTCGAGT
2	Long <i>lacZ</i> 3' (NotI)	AAGGAAAAAAGCGGCCGAGATCTTACTCAGGAGAGCGTTCACCGACAAACAACAG
3	<i>lacZ</i> 430	TGATGAAAGCTGGCTACAGGAAGG
4	<i>lacZ</i> 908	GTCACACTACGTCTGAACGTCGAA
5	<i>lacZ</i> 1525	GGCCACCGATATTATTTGCCCGAT
6	<i>lacZ</i> 2053	GGATGTCGCTCCACAAGGTAACA
7	<i>lacZ</i> 2593	TATCAGCCGGAAAACCTACCGGAT
8	<i>lacZ</i> 3121	GCTGTTTTGGCGGATGAGAGAAGA
9	<i>lacZ</i> 1533 5'	ATATTATTTGCCCGATGTACGCGCGCGTGGATGAA
10	<i>lacZ</i> 1538 3'	TTCATCCACGCGCGGTACATCGGGCAAAT
11	<i>lacZBb</i> Int-1 Forward	TGGTGTAATGGAAGATGGGTTGG
12	<i>lacZBb</i> Mid Reverse	GATAGACCATTTAGGAACAGCAGG
13	<i>flaB</i> promoter 5' (BamHI)	CCCGCGGATCCCTGTCGCCTCTTGTGGCTTC
14	<i>flaB</i> promoter 3' (XhoI)	CCCCGCTCGAGCATATATCATTCCTCCATGA
15	<i>ospC</i> promoter 5' (BamHI)	CCCGCGGATCCAATTAACCTTTTTTTATTAAGTA
16	<i>ospC</i> promoter 3' (XhoI)	CCCCGCTCGAGTAATTTGTGCCTCCTTTTTATTTAT
Taqman primers/probes^b		
17	<i>flaB</i> forward	TCTTTTCTCTGGTGAGGGAGCT
18	<i>flaB</i> reverse	TCCTTCCTGTTGAACACCCTCT
19	<i>flaB</i> probe	AAACTGCTCAGGCTGCACCGGTTC
20	<i>lacZBb</i> forward	TTCTCTTGGAGGATTTGCTAAAT
21	<i>lacZBb</i> reverse	ATCCCAAACAAATCCACCTT
22	<i>lacZBb</i> probe	TGGCAAGCATTTCAGACAATATCCAAG
23	<i>ospC</i> forward	ACGGATTCTAATGCGGTTTTACTT
24	<i>ospC</i> reverse	CAATAGCTTTAGCAGCAATTTTCATCT
25	<i>ospC</i> probe	CTGTGAAAGAGGTTGAAGCGTTGCTGTCAT

^a underlined bases denote restriction sites

^b Taqman probes were labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) at the 5' and 3' ends, respectively

without antibiotic selection and then plated in solid BSK medium supplemented with gentamicin. Individual colonies were screened by PCR for *lacZ_{Ec}* and the gentamicin cassette.

lacZ optimization.

E. coli lacZ codon usage in *B. burgdorferi* was analyzed using the Graphical Codon Usage Analyzer version 2.0 (www.gcua.schoedl.de). More than one-third of the codons in the *E. coli lacZ* gene, *lacZ_{Ec}*, are considered rare (used less than 20% of the time) in *B. burgdorferi*. A codon-optimized version of *lacZ*, *lacZ_{Bb}*, which uses less than 1% of *B. burgdorferi* rare codons, was synthesized by GenScript Corporation (Piscataway, NJ). A sequence alignment of the codon optimized *lacZ_{Bb}* gene with the original *lacZ_{Ec}* gene is provided in Figure 2-2. The synthesized gene was preceded by the same MCS and RBS as the original *lacZ_{Ec}*. *lacZ_{Bb}* was cloned into pBSV2G (13) with XbaI and KpnI, producing pBH-*lacZ_{Bb}* (Table 2-1 and Figure 2-1B). The *flaB* promoter was added as described above to create pBH*flaB*p-*lacZ_{Bb}* (Table 2-1 and Figure 2-1B). The intergenic region between *guaA* and *ospC*, which includes the *ospC* promoter and regulatory operator sequences (27-30), was amplified from *B. burgdorferi* genomic DNA with primers 15 and 16 (Table 2-2), cloned into pCR2.1, sequenced, and digested with BamHI and XhoI to ligate into pBH-*lacZ_{Bb}*, creating pBH*ospC*p-*lacZ_{Bb}* (Table 2-1). Constructs were transformed into electrocompetent *B. burgdorferi* (11) and Δ *lac E. coli*.

SDS-PAGE and Immunoblotting

β -galactosidase expression in *E. coli* and *B. burgdorferi* was assessed using SDS-PAGE as previously described (31). Briefly, *E. coli* and *B. burgdorferi* were harvested and washed twice in HEPES-NaCl (HN) buffer (50mM each, pH 7.6).

Figure 2-1: Schematic diagram of the *lacZ* shuttle vectors

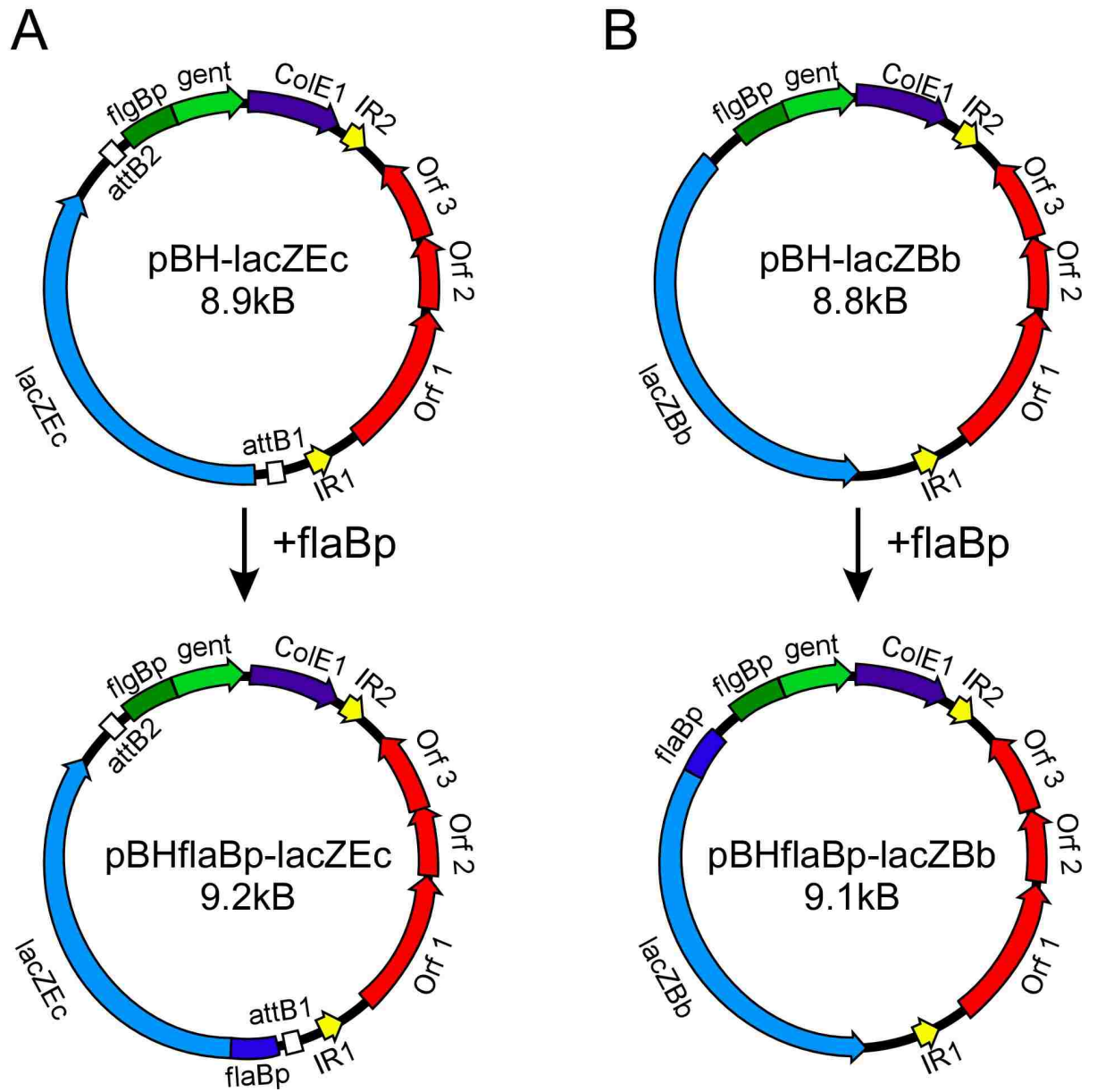


Figure 2-1 legend: The *E. coli lacZ* gene was cloned into pBSV2G_dvB2, creating pBH-lacZEc (A, top). A *B. burgdorferi* codon-optimized *lacZ* gene, *lacZBb*, was cloned directly into pBSV2G, creating pBH-lacZBb (B, top). The *flaB* promoter from *B. burgdorferi* (*flaBp*) was added to each construct, yielding pBHflaBp-lacZEc (A, bottom) and pBHflaBp-lacZBb (B, bottom), respectively.

Figure 2-2: Nucleotide alignment of *lacZBb* and *lacZEc*

<i>lacZBb</i> (1)	ATGATTTGATCCTGTTGTTTTACAAAGAAGAGATTGGGAAAACCTGGAGTTACACAAATAAATAGATTAGCTGCTCATCCTCCTTTTGCTTCTGGAGA
<i>lacZEc</i> (1)	ATGATAGATCCCGTCGTTTTACAACTCGTACTGGGAAAACCTGGCGTTACCCAATTAATCGCCTTGACGACATCCCCCTTTGCGCAGCTGGCGT
<i>lacZBb</i> (100)	AATTCGAAGAAGCTAGAACAGATAGACCTTCTCAACAATTGAGATCACCTAATGGAGAATGGAGATTGCTTGGTTTCTGTTCTGAACTGTTCCCT
<i>lacZEc</i> (100)	AATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGGCAGCTGAATGGCGAATGGCGCTTTGCTGTTTCCGGTACCAGAAGCGGTGCCG
<i>lacZBb</i> (199)	GAATCTGGTTAGAAATGTGATTTACCTGAAGCTGATACAGTTGCTGTTCTCTTAATGGCAAATGCATGGATATGATGCTCCTATATACAAATGTT
<i>lacZEc</i> (199)	GAAAGCTGGTGGAGTGCATCTTCTGAGGCCGATACCTGCTGTCGTCGTCCTCAAACCTGGCAGATGCACGGTTACGATGCCCTTACACCAACGTA
<i>lacZBb</i> (298)	ACATATCCTATAACAGTTAATCCTCCTTTTGTCTTACAGAAAACCTACAGGATGTTATTCTCTTACATTTAATGTTGATGAATCTGGTTACAAAGAA
<i>lacZEc</i> (298)	ACCTATCCCATTACGGTCAATCCGCCGTTTGTCCACGGAGAATCCGACGGTGTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAA
<i>lacZBb</i> (397)	GGACAAACAAGAAATAATTTTGATGGAGTTAATTCCTGCTTTTCATCTTTGGTGTAAATGGAAAGTGGGTTGGATATGGACAAGATCAAGACTTCCCTCT
<i>lacZEc</i> (397)	GGCCAGACGCGAATTAATTTTGATGGCGTTAACTCGCGGTTTTCATCTGTGGTGCACACGGCGCTGGGTCCGTTACGGCCAGGACAGTCTGTTGCCGCTC
<i>lacZBb</i> (496)	GAATTTGATTTATCTGCTTTCCTTAGAGCTGGAGAAAATAGATTAGCTGTTATGGTTTAAAGATGGTCTGATGGATCTTATTTAGAAGATCAAGATATG
<i>lacZEc</i> (496)	GAATTTGACCTGAGCGCATTTTACCGCCGGAGAAAACCGCTTCGCGGTGATGGTGTGCTGCTGGTGGAGTGACGGCAGTTATCTGGAAAGATCAGGATATG
<i>lacZBb</i> (595)	TGGAGAATGTCTGGAAATTTTAGAGATGTTCTTACTTCATAAACTACAACACAAAATTTCTGATTTTCATGTTGCTACAAGATTTAATGATGATTTTC
<i>lacZEc</i> (595)	TGGCGGATGAGCGGCATTTCCGTGACGTCCTGTTGCTGCATAAACCGACTACACAAAATCAGCGATTTCCATGTTGCCACTCGCTTTAATGATGATTTTC
<i>lacZBb</i> (694)	TCAAAGCTGTTTGAAGCTGAAGTTCAAATGTGTGGAGAACTTAGAGATTATCTTAGAGTTACAGTTTCTTTATGGCAAGGTGAAACACAAGTTGCT
<i>lacZEc</i> (694)	AGCCCGCTTACTGGAGGCTGAAGTTCAGATGTGCGCGAGTTGCGTGACTACCTACGGTAACAGTTTCTTTATGGCAGGTTGAAACCGAGGTCCGCT
<i>lacZBb</i> (793)	TCTGGAAACAGCTCCTTTTGGAGGAGAAAATAAGATGAAAGAGGAGGATATGCTGATAGAGTTACACTTAGACTTAAATGTTGAAATCCTAAACTTTGG
<i>lacZEc</i> (793)	AGCGCACCCGCTTTCGCGGTGAAATTAATCGATGAGCGTGGTGGTTATGCCGATCGGTCACACTACGCTGAAACCTGAAACCCGAAACTGTGG
<i>lacZBb</i> (892)	TCTGCTGAAATACCTAATCTTTATAGAGCTGTTGTTGAACTTCATACAGCTGGAACACTTATTGAAAGCTGAAGCATGTGATGTTGGATTTAGAGAA
<i>lacZEc</i> (892)	AGCGCGAAATCCCGAATCTCTATCGTGGGTGTTGAACTTCACACCGGATGTTGAAAGCTGATGTTGAAAGCTGATGTTGAAAGCTGATGTTGAAAGCT
<i>lacZBb</i> (991)	GTTAGAAATAGAAAATGGACTTCTTCTTCTTAATGGAAAACCTCTTCTTATTAGAGGATTAATAGACATGAACATCATCCTCTTCATGGACAAGTTATG
<i>lacZEc</i> (991)	GTGGCGATGAAAATGCTGCTGCTGCTGAACCGCAAGCCGTTGCTGATTCGAGGCGTTAACCGTACAGGACATCATCCTCTGCATGCTCAGGTCATG
<i>lacZBb</i> (1090)	GATGAACAAACAATGGTACAAGATATCTTCTTATGAAACAAAATAATTTAATGCTGTAGATGTTCTCATTATCCTAATCATCCTCTTTGGTATACA
<i>lacZEc</i> (1090)	GATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAAACACTTTAACCCCTGCGCTGTTCCGATTAATCCGAACCATCCGCTGTGTACAG
<i>lacZBb</i> (1189)	CTTTGTGATAGATATGGACTTATGTTGTTGATGAAGCTAATATTGAAACACATGGAATGGTTCCTATGAATAGATTAACAGATGATCCTAGATGGTTA
<i>lacZEc</i> (1189)	CTGTGCGACCGCTACGGCTGTATGTGGTGGATGAAGCAATATTGAAACCCAGGATGGTGCATTAATGATGATGATGATGATGATGATGATGATGATGAT
<i>lacZBb</i> (1288)	CCTGCTATGTCTGAAAGAGTTACAAGAATGGTCAAAGAGATAGAATCATCCTCTGTTATTATTGGTCTTTAGGAAATGAATCTGGACATGGAGCT
<i>lacZEc</i> (1288)	CCGCGATGAGCGAACCCGTAACCGGAATGGTGCAGCGATGTAACCTGCAATCAGCCGATGTGATCATCTGGTCCGTTAGGGAAATGAATCAGGCCACCGGCT
<i>lacZBb</i> (1387)	AATCATGATGCTTTATATAGATGGATTAATCTGTTGATCCTTCAAGACCAGTTCAATATGAAGGTGGAGGCTGATACAACAGCTACAGATATTATT
<i>lacZEc</i> (1387)	AATCAGCAGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCGCGCGTGCAGTATGAAGCGCGGAGCCGACACCAGGCCACCGATATTATT
<i>lacZBb</i> (1486)	TGCTTATGATGCTAGAGTTGATGAAGTCAACCTTTTCTGCTGTTCTTAAATGGTCTATCAAGAAATGGTTATCTTTACCTGGAGAAACAAGACCT
<i>lacZEc</i> (1486)	TGCCGATGATGCGCGCGTGGATGAAGACCAGCCCTTCCGCGTGTGCCGAAATGGTCCATCAAAAATGGTTCGCTACCTGGAGAGACCGGCCG
<i>lacZBb</i> (1585)	CTTATCTTTGTAATATGCTCATGCTATGGGAAATCTCTTGGAGGATTTGCTAAATATTGGCAAGCATTACAGCAATATCCAAGACTTCAAGGTGGA
<i>lacZEc</i> (1585)	AGCTCCTTTGCGAATACGCCACCGGATGGTAACTCTTGGAGGATTTGCTAAATATTGGCAAGCATTACAGCAATATCCAAGACTTCAAGGTGGA
<i>lacZBb</i> (1684)	TTTGTGTTGGGATGGTGTGATCAATCTTTAATAAATATGATGAAATGGAAATCCTTGGTCTGCTTATGGAGGAGATTTGGAGTACACCTAATGAT
<i>lacZEc</i> (1684)	TTCTGCTGGGACTGGTGGATCAGTCTGATTAATATGATGAAACCGCAACCCGTTGGTCCGCTTACCGCGGTGATTTGGCGGATACGCCAACCGAT
<i>lacZBb</i> (1783)	AGACAATTTGTATGAATGGATTAGTTTTTGTGATAGAACCTCATCCTGCTCTTACAGAAAGCTAAAATCAACAACAATTTTCCAAATTTAGACTT
<i>lacZEc</i> (1783)	CGCCAGTTCTGTATGAACGCTGTTGTTGCGCAGCGACCGCGATCCAGCGCTGACGGAAAGCAAAAACACAGCAGCAGTTTTTCCAGTTCCGTTTA
<i>lacZBb</i> (1882)	TCTGGACAAACAATAGAAGTTACATCTGAATATCTTTTAGACATCTGATATGAACCTTCTTATTGGATGGTGGCTTTAGATGAAAACCTTTAGCT
<i>lacZEc</i> (1882)	TCCGGCAAAACCATCGAAGTGACAGCGAATACCTGTTCCGTCATAGCGATAACAGGCTCCTGCACTGGATGGTGGCGCTGGATGGTAAGCCGCTGGCA
<i>lacZBb</i> (1981)	TCTGGAGAAGTTCTTTAGATGTTGCTCCTCAAGGAAAACAATTAATGAAATTAACCTGAAATTAACCTCAACCTGAATCTGCTGGCAATATGTTAACA
<i>lacZEc</i> (1981)	AGCGGTGAAGTGCCTCTGGATGTCGCTCCACAGATGAACAGTTGATGAACTGCTGAACTACCGGACCGGAGCGCGGCAACTCTGGCGAGATPACA
<i>lacZBb</i> (2080)	GTTAGAGTTGTTCAACCTAATGCTACAGCTTGGCTGAAGCTGGACATATTTCTGCTTGGCAACAATGGAGATTAGCTGAAAATTTATCTGTTACATTA
<i>lacZEc</i> (2080)	GTACGCTAGTGCAACCGAACCGCAGCCGATGGTCAAGCGCGGACATCAGCGCTGGCAGCAGTGGCGTCTGGCGGAAAACCTCAGTGTGACGCTC
<i>lacZBb</i> (2179)	CCTGCTGCTTCTCATGCTATTCCTCATTTAACAACATCTGAATGGATTTTGTATAGAACTTGGAAATAAAGATGGCAATTTAATAGACAATCTGGA
<i>lacZEc</i> (2179)	CCGCGCGCTCCACGCCATCCCGCATCTGACACCAGCGAATGGATTTTGCATCGAGCTGGTAATAAGCGTTGGCAATTTAACCGCCAGCTCAGGC
<i>lacZBb</i> (2278)	TTTTTATCTCAAATGTGGATTGGAGATAAAAAACAATCTTCTACACCTCTTAGAGATCAATTTACAAGAGCTCCTTTAGATAATGATATTGGAGTTTCT
<i>lacZEc</i> (2278)	TTTTTTTACAGATGTGGATTGGCGATAAAAAACAATCTGTCAGCCGCTGCGGATCAGTTACCCGCTGCACCGCTGGATAACGACATTTGGCGTAACT
<i>lacZBb</i> (2377)	GAAGCTACAAGAATGATCCTAATGCTTGGTGTGAAAGATGAAAGCTGCTGGACATTTATCAAGCTGAAGCTGCTTTATACAATGTACAGCTGATACA
<i>lacZEc</i> (2377)	GAAGCGACCCGATTTGACCTAAGCCCTGGATGAAAGCTGGAAGCTGCGGCCATTTACAGGCCGAGCGCGGCACTTACAGGCCGAGCGGCTTTGCAATGACCGAGATACA
<i>lacZBb</i> (2476)	TTAGCTGATGCTGTTTTAATTAACAACAGCTCATGCTGGCAACATCAAGGAAAACAATTTTATCTCAAGAAAAACATATAGAATAGATGGATCTGGA
<i>lacZEc</i> (2476)	CTTGTGATGCGGCTGCTGATTACGACCGCTCAGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGAAAACCTACCGGATTTGATGGTGGT
<i>lacZBb</i> (2575)	CAATGGCTATAACAGTTGATGTTGAAGTTGCTTCTGATACACCTCATCCTGCTAGAATGGATTAATTTGCAATTAGCTCAAGTTGCTGAAAGAGTT
<i>lacZEc</i> (2575)	CAATGGCGATTACCGTTGATGTTGAAGTGGCGAGCGATACCCGATCCGCGCGGATTTGGCTGAACTGCCAGCTGGCGAGGTAGCAGAGCGGGTA
<i>lacZBb</i> (2674)	AATGGTTAGGATTAGGACCTCAAGAAAATTAATCCTGATAGATTAAACAGCTGCTGTTTGTATAGATGGGATCTTCTCTTTCTGATATGATACACCT
<i>lacZEc</i> (2674)	AATGGCTCGGATTAGGCGCGCAAGAAAATTAATCCTGATAGATTAAACAGCTGCTGTTTGTATAGATGGGATCTTCTCTTTCTGATATGATACACCT
<i>lacZBb</i> (2773)	TATGTTTCTCTTCTGAAAATGGACTTAGATGTGGAAACAAGAACTTAATATGGACCTCATCAATGGAGGGAGATTTCAATTTAATATCTCAAGA
<i>lacZEc</i> (2773)	TACGTTCTCCGAGCGAAAACGGTCTGCGCTGGGACCGCGCAATTTGAATTTATGGCCACACCAAGTGGGCGCGGACTTCCAGTTCAACATCAGGCCG
<i>lacZBb</i> (2872)	TATTTCTCAACAACAATTTAGAAACATCTCATAGACATCTTCTTCTGCTGAAGAAGAAACATGGCTTAAATAGATGGATTTTCAATATGGAATTTGGA
<i>lacZEc</i> (2872)	TACAGTCAACAGCAACTGATGGAACACCGCATCCGCTCTGCTGACCGCGAAGAAAGGCACATGGCTGAAATACGAGGTTTCCATATGGGATTTGGT
<i>lacZBb</i> (2971)	GGAGATGATTTCTTGGTCTCCTTCTGTTCTGCTGAAATTTCAATTTATCTGCTGGAAGATATCATTTATCAATTAGTTTGGTGTCAAAAATAA
<i>lacZEc</i> (2971)	GGCGACGATCCTGGAGCCGCTCAGTATCGCGGAATTTCCAGCTGAGCGCGGCTGCTACCATTTACCAAGTTGGTCTGCTGTCAAAAATAA

Figure 2-2 legend: Nucleotide sequences of the *Borrelia burgdorferi* codon-optimized *lacZBb* gene (top) aligned with the original *lacZEc* gene (bottom) from *Escherichia coli*. Red letters indicate that the codon was changed in *lacZBb*. Parenthetical numbers represent the position within the gene.

Protein lysate from approximately 10^7 bacteria was loaded per lane in a 10% acrylamide gel. Protein gels were stained with Coomassie Brilliant Blue (Bio-Rad) or prepared for immunoblotting.

For Western Blots, proteins were electrophoretically transferred to nitrocellulose membranes (Bio Rad Trans blot Transfer Medium). Membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20) Monoclonal mouse anti- β -galactosidase (Santa Cruz Biotechnology Inc.) at 1:100, monoclonal mouse anti-FlaB (H9724) (32) at 1:250, and polyclonal rabbit anti-OspC (33) at 1:1500 were used to probe membranes. Primary antibodies were diluted in 5% milk in TBST. Horseradish peroxidase-conjugated secondary antibodies that recognize mouse and rabbit immunoglobulins were diluted 1:10,000 and 1:50,000, respectively, in TBST with 5% milk. Immunoreactivity was visualized using the Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) and x-ray film (Labscientific Inc., NJ).

Screening for β -galactosidase activity.

For β -galactosidase assays, 1mL of overnight *E. coli* cultures and 5-10mL of mid-log *B. burgdorferi* cultures were washed twice in HN buffer. Bacterial pellets were resuspended in Z-buffer (36mM NaH_2PO_4 , 67mM NaHPO_4 , 0.1mM MgCl_2 , 2mM MgSO_4 , 2.7ml/L β -mercaptoethanol) at approximately 5×10^8 bacteria/mL before lysis with chloroform and SDS. β -galactosidase assays were performed using a modified version of the Miller protocol (34). Briefly, aliquots (5-20 μ L) of bacterial lysates were added to 96-well plates (Costar, Corning, NY) in triplicate. Z-buffer was added, increasing the volume to 160 μ L. 50 μ L ONPG dissolved in Z-buffer (4mg/mL) was added and the plate incubated at room temperature (RT) for 10-15 minutes.

This incubation time was chosen because it was in the linear range of activity when a kinetic assay was used (Figure 2-3A). After incubation, 90 μ L of stop buffer (1M Na₂CO₃) was added and the absorbance measured at 405nm (Labsystems Multiskan Plus, Fisher Scientific, Pittsburg, PA). Although the standard Miller protocol (34) uses the absorbance at 420nm to quantitate β -galactosidase hydrolysis of ONPG, I used a plate reader with a filter at 405nm. When compared, however, there was not a significant difference in activity when the absorbance was measured at 420 or 405nm (Figure 2-3B).

β -galactosidase activity units, or Miller units (nmoles/minute), were calculated as described before and reported as units per mg protein (35). Background activity (units/mg protein) of bacteria lacking a shuttle vector was subtracted from reported values.

Both *E. coli* and *B. burgdorferi* were grown on plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, Roche Diagnostics, Indianapolis, IN) at 0.08 g/L and gentamicin. Alternatively, \sim 0.5mL X-gal dissolved in dimethyl sulfoxide (DMSO) (20mg/ml) was spread on *B. burgdorferi* plates after colony formation.

Protein Assay.

The concentration of protein in the bacterial lysates used for β -galactosidase assays was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. Dilutions of BSA (New England Biolabs, NEB, Ipswich, MA) were used as standards. 10 μ L of the standards and 5-10 μ L bacterial lysates were added to 96 well plates in triplicate. A standard curve was generated and used to determine protein concentrations of the lysates

Figure 2-3: Optimizing the conditions for liquid β -galactosidase assays

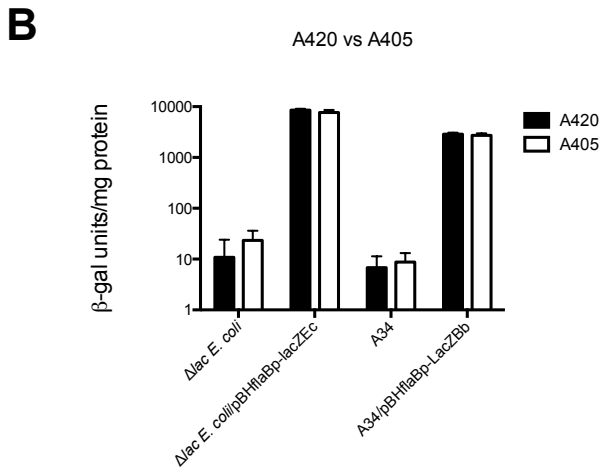
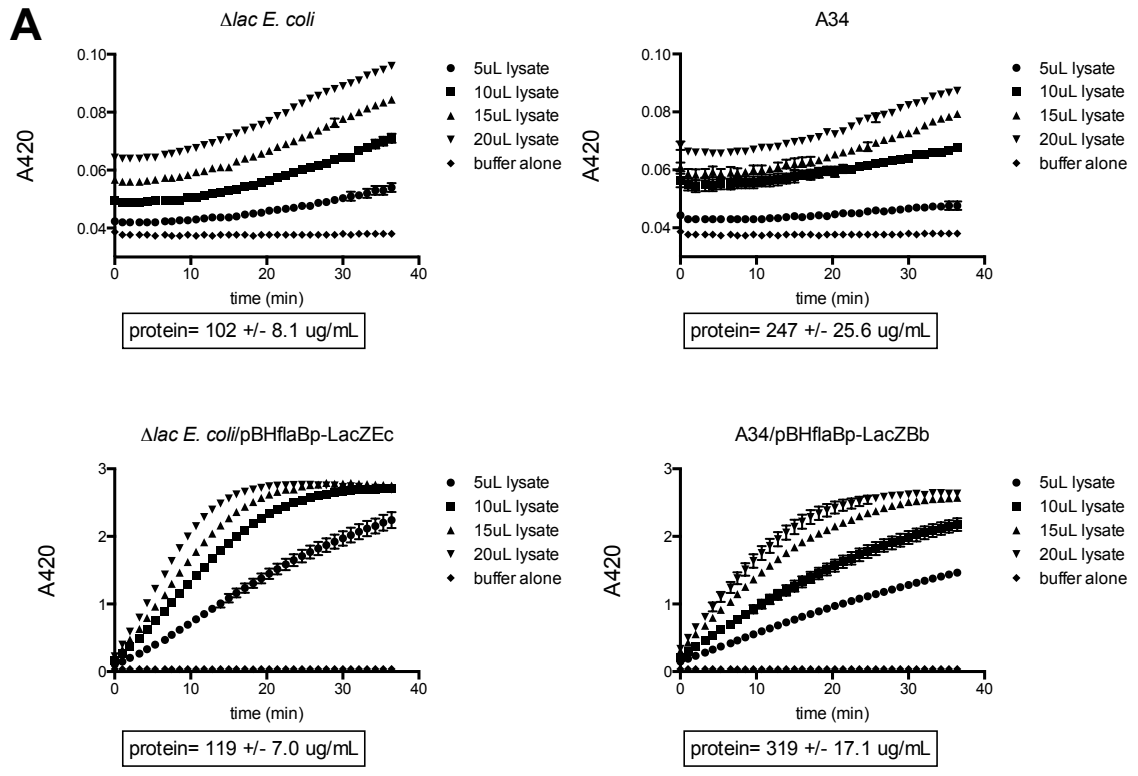


Figure 2-3 legend: **A.** Kinetic β -galactosidase assays. 5, 10, 15, and 20 μ L of whole cell lysates from Δlac *E. coli* with (bottom left) and without pBHflaBp-lacZEc (top left) and *B. burgdorferi* A34 with (bottom right) and without pBHflaBp-lacZBb (top right) were used in a modified version of the Miller protocol (34) to measure β -galactosidase activity. The absorbance at 420 was measured approximately every minute for 35 minutes. Buffer alone was used as a baseline and the protein concentration for each lysate is designated below each X-axis. **B.** β -galactosidase activity measured at 405nm and 420nm. Modified Miller ONPG assays (34) were performed on Δlac *E. coli* and A34 without a shuttle vector or harboring a *lacZ* gene expressed from the *flaB* promoter on a shuttle vector, as indicated. Absorbances were taken at 420nm (black bars) or 405nm (white bars) to determine β -galactosidase units (Miller units), which are reported per mg protein and levels represent mean +/- SD (n=6).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

RNA was harvested from ~30mL of a mid log *B. burgdorferi* culture using a Masterpure RNA Preparation Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's protocol. The integrity of extracted RNA was confirmed on an Agilent 2100 Bioanalyzer using an RNA 6000 Pico Total RNA chip (Agilent Technologies, Foster City, CA) and/or by electrophoresis in an agarose gel. 2-5µg of RNA was used as a template to synthesize cDNA using the High Capacity cDNA Reverse Transcriptase (RT) Kit (Applied Biosystems, Life Technologies, Carlsbad, CA). Quantitative-PCR (qPCR) was performed in triplicate on 100ng cDNA using the Taqman Universal PCR Mastermix (Applied Biosystems) and primer-probe combinations for *lacZBb*, *flaB*, and *ospC* (Table 2-2). Real time qPCR was performed using the ABI Prism 7900 HT Sequence Detection System.

Results

***lacZ* gene constructs.**

To investigate whether *lacZ* could be used as a simple reporter gene in *B. burgdorferi*, I constructed four shuttle vectors, pBH-lacZEc, pBHflaBp-lacZEc, pBH-lacZBb, and pBHflaBp-lacZBb (Table 2-1 and Figure 2-1). These constructs carry an *E. coli lacZ* gene (*lacZEc*) or a *B. burgdorferi* codon-optimized *lacZ* gene (*lacZBb*), with and without the constitutive *B. burgdorferi flaB* promoter. The *lacZEc* gene has 56% G+C base content, while the *B. burgdorferi* genome has only 28% G+C content (10). In order to enhance β -galactosidase synthesis in *B. burgdorferi*, I designed a synthetic *lacZ* gene, *lacZBb*, that reduced the G+C content to better reflect *B. burgdorferi* codon preference. The *flaB* promoter was chosen for initial experiments

because it is constitutively expressed in *B. burgdorferi* and *E. coli* and has been used to drive expression of antibiotic resistance markers in *B. burgdorferi* (13). All constructs were transformed into electrocompetent *B. burgdorferi* A34 and a Δlac *E. coli* strain and assessed for β -galactosidase activity in liquid and solid media.

β -galactosidase activity in bacterial cultures.

In order to assess the β -galactosidase activity in *B. burgdorferi* and *E. coli* transformants carrying the *lacZ* constructs, I adapted the well-established Miller ONPG assay (34) and normalized β -galactosidase activity to the protein content of the bacterial lysates as described by Nielsen *et al.* (35). Both *E. coli* and *B. burgdorferi* harboring pBHflaBp-*lacZ*Ec or pBHflaBp-*lacZ*Bb displayed significant activity when compared to bacteria containing the promoterless *lacZ* constructs (Figure 2-4A). β -galactosidase activity was significantly higher in *B. burgdorferi* lysates containing pBHflaBp-*lacZ*Bb compared to lysates with pBHflaBp-*lacZ*Ec, indicating that codon-optimization of *lacZ* improved activity in *B. burgdorferi* (Figure 2-4A). *B. burgdorferi* harboring pBHflaBp-*lacZ*Bb and pBHflaBp-*lacZ*Ec both produced detectable amounts of β -galactosidase protein when assayed by immunoblot (Figure 2-4B). These results demonstrate that *lacZ* produces a functional β -galactosidase enzyme in *B. burgdorferi* when expressed using the constitutive *flaB* promoter.

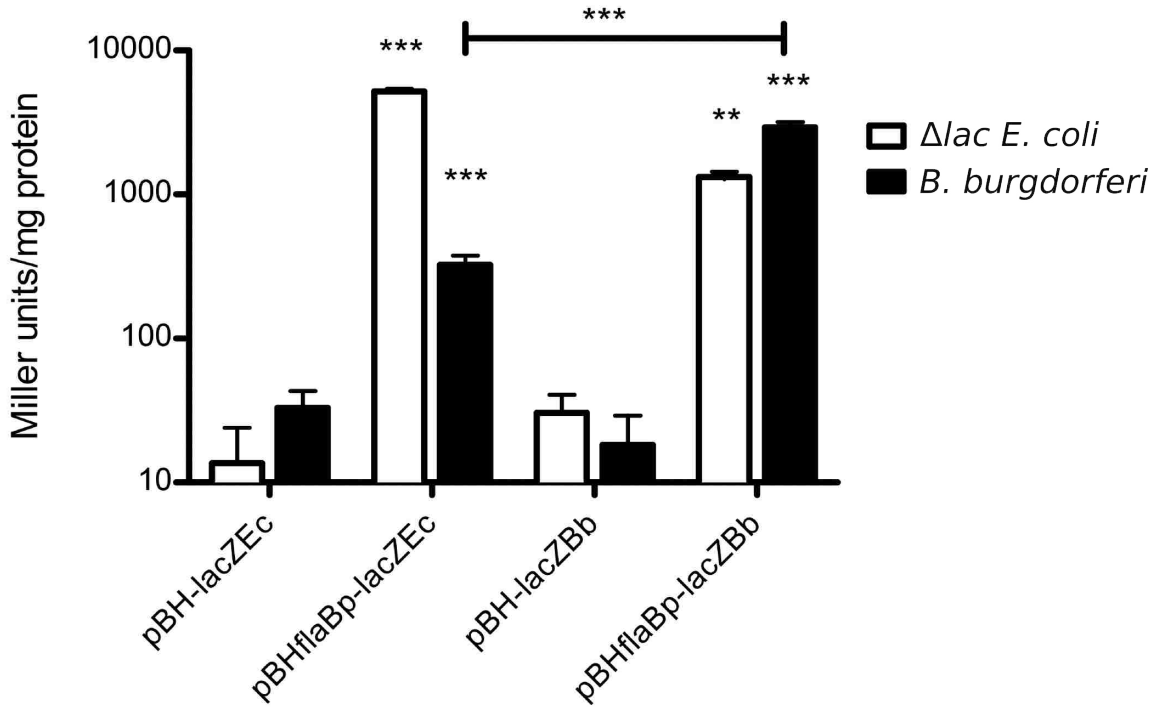
β -galactosidase activity on *B. burgdorferi* plates.

Since β -galactosidase activity could be detected in *B. burgdorferi* lysates, I wanted to determine if *lacZ* could be used as a reporter gene for *B. burgdorferi* colonies in solid medium. Typically, addition of X-gal to solid medium allows for the detection of bacterial colonies that possess an active β -galactosidase enzyme, which

Figure 2-4: β -galactosidase in *E. coli* and *B. burgdorferi* lysates

A

β -galactosidase activity



B

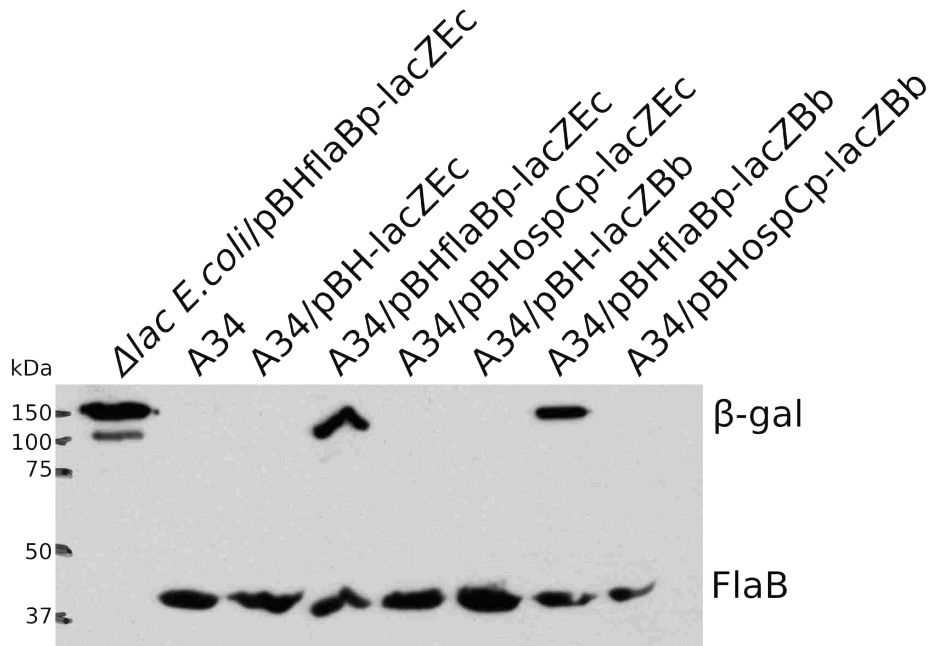
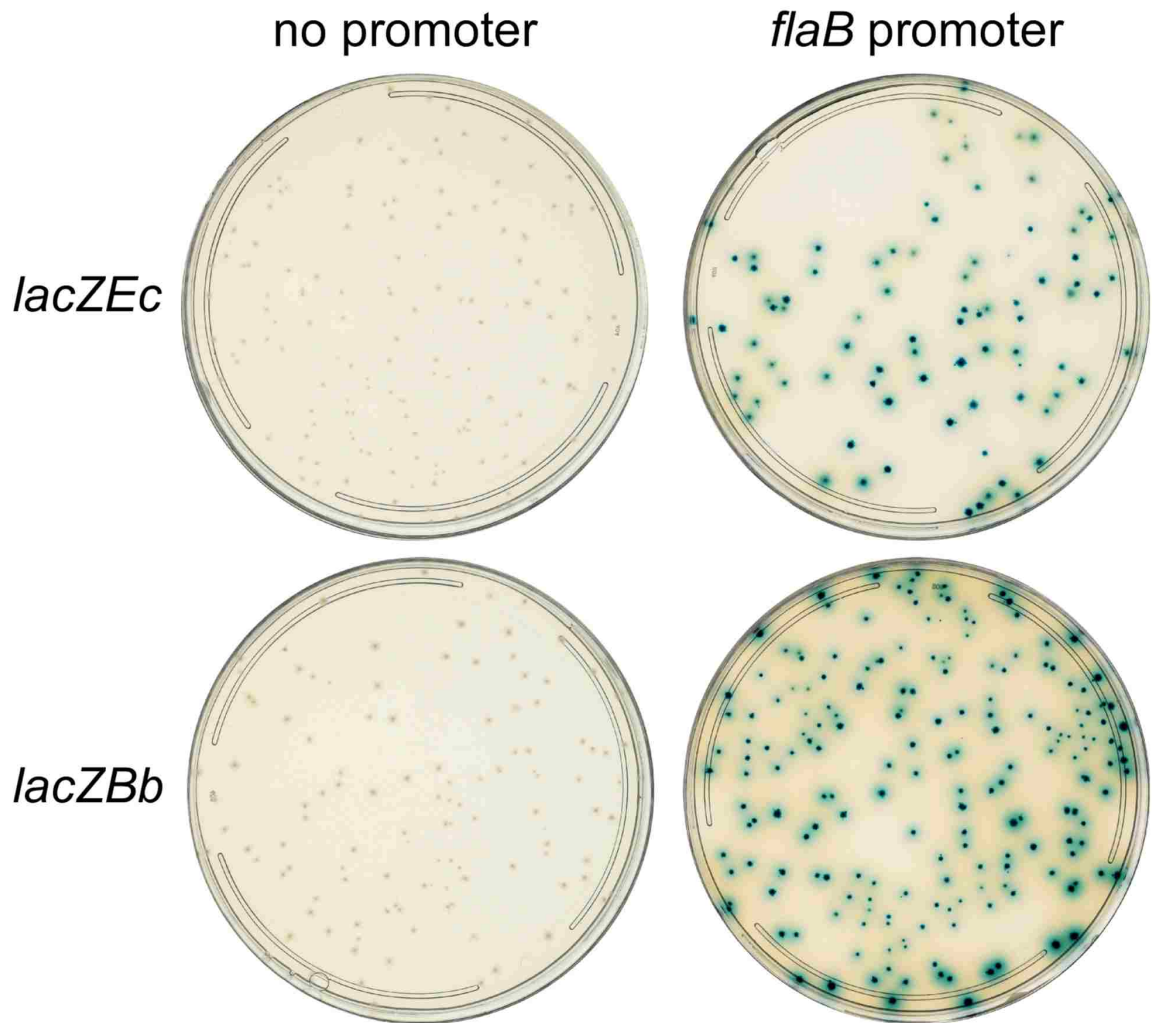


Figure 2-4 legend: **A.** Modified Miller ONPG assays (34) were performed on *Δlac E. coli* (white bars) and *B. burgdorferi* (black bars) harboring pBH-lacZEc, pBHflaBp-lacZEc, pBH-lacZBb, and pBHflaBp-lacZBb, as indicated. β-galactosidase activity units (Miller units) are reported per mg protein and levels represent mean +/- SD (n=3). Background activity of bacteria without the shuttle vector was subtracted from reported values. *** p<0.001 and ** p<0.01 compared to respective promoterless constructs or as indicated based on a Student's two-tailed t-test. **B.** β-galactosidase (β-gal) Immunoblot. *Δlac E coli* containing pBHflaBp-lacZEc, B31-A34 (without plasmid), and A34 clones harboring pBH-lacZEc, pBHflaBp-LacZEc, pBH-lacZBb, and pBHflaBp-lacZBb, as indicated, were probed with antibodies specific for β-gal. Flagellin protein (FlaB) levels were used as a control to normalize for protein loading. Numbers on the left refer to protein size in kDa determined by the mobility of protein standards.

cleaves X-gal into a blue-colored product. Therefore, I used BSK solid medium without phenol red supplemented with X-gal (0.08 g/L) to grow *B. burgdorferi* clones harboring the *lacZ* constructs. Blue color development was observed 2-5 days after colony formation for *B. burgdorferi* colonies harboring either pBHflaBp-lacZec or pBHflaBp-lacZbb, but the incorporation of X-gal into the solid plating medium considerably slowed *B. burgdorferi* colony growth. However, when 2% X-gal in DMSO was spread on *B. burgdorferi* plates after colony formation, color development in colonies harboring the *lacZ* gene driven by the *flaB* promoter was evident within 15 minutes and continued to increase overnight as the substrate diffused throughout the plates (Figure 2-5). Importantly, colonies without a promoter to drive *lacZ* expression remained unchanged (white) even after overnight incubation (Figure 2-5). Furthermore, live spirochetes were recovered from colonies 48 hours post X-gal treatment, indicating X-gal treatment did not hinder bacterial viability. Colonies of *B. burgdorferi* harboring the optimized *lacZ* gene, *lacZbb*, when expressed by the *flaB* promoter, developed a more uniform and greater color intensity than *B. burgdorferi* containing pBHflaBp-lacZec, again suggesting that the optimized gene is better suited for *B. burgdorferi* (Figure 2-5). These results demonstrate that *lacZ* can be used to monitor gene expression of *B. burgdorferi* colonies on plates. Furthermore, results from both liquid and plate assays showed that codon-optimization of the *lacZ* gene (*lacZbb*) improved β -galactosidase activity in *B. burgdorferi*, presumably through increased protein production. Thus, I used only the *B. burgdorferi* optimized *lacZ* gene (*lacZbb*) in further studies.

Figure 2-5: *lacZ* for blue-white selection of *B. burgdorferi* colonies



B. burgdorferi colonies on BSK solid medium without phenol red

Figure 2-5 legend: BSK solid medium without phenol red was used to grow B31-A34 clones harboring pBH-lacZEc (top left), pBHflaBp-lacZEc (top right), pBH-lacZBb (bottom left), and pBHflaBp-lacZBb (bottom right). Approximately 0.5ml of X-gal in DMSO (20mg/ml) was added to plates after colony formation and incubated overnight before photographs were taken.

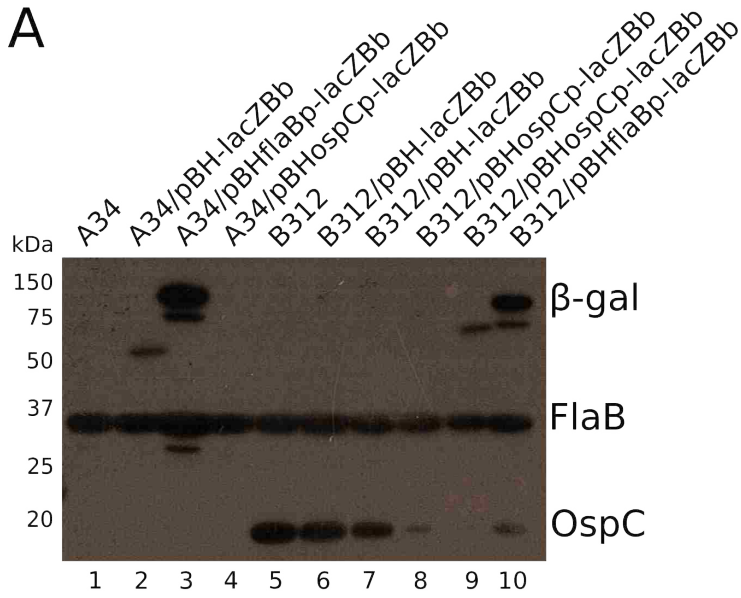
β -galactosidase activity as a reporter for *ospC* expression.

I demonstrated that β -galactosidase protein is produced and shows detectable activity in *B. burgdorferi* by expressing *lacZ* from a constitutive promoter but wanted to confirm that *lacZ* expression reflects the expression of genes that are differentially regulated in *B. burgdorferi* and therefore can be used as a reporter gene for transcriptional activity. For this objective, I utilized two strains of *B. burgdorferi* that differ in their expression of the *ospC* gene: *B. burgdorferi* clone B31-A34 and B312. B31-A34 is derived from non-infectious clone B31-A (36) and does not produce OspC *in vitro* (Figure 2-6A and ref (25)), while B312, a highly attenuated clone of B31 that lacks many plasmids, produces abundant amounts of OspC *in vitro* (Figure 2-6 and ref (25)). In order to determine *lacZ* expression driven by the *ospC* promoter in these two *B. burgdorferi* genetic backgrounds, I cloned the intergenic region between *guaA* and *ospC*, which includes the *ospC* promoter and all *ospC* regulatory regions (27-30), upstream of *lacZBb* in pBH-*lacZBb*, creating pBHospCp-*lacZBb*. I then transformed A34 and B312 with pBHospCp-*lacZBb* and also introduced pBH-*lacZBb* and pBHflaBp-*lacZBb* into B312.

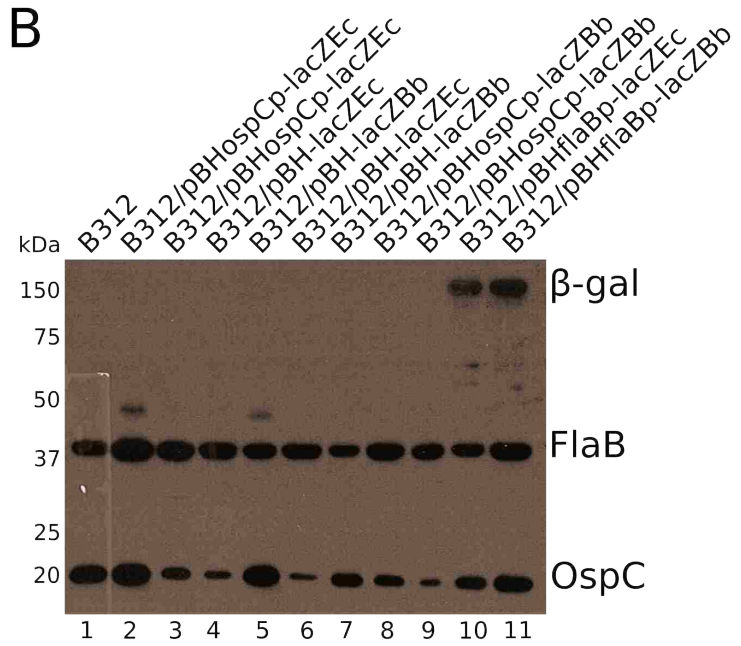
I immediately noticed that transformants of B312 had variable OspC protein levels, and significant variation in OspC protein was detected between different B312 clones (Figure 2-6A) and between independent cultures from the same clone (Figure 2-6B). This observation has not been previously reported. In order to use B312, I first confirmed OspC production in B312 transformants with immunoblot before proceeding with further experiments on those clones. Significant β -galactosidase activity was detected in B312 harboring the pBHospCp-*lacZBb* plasmid compared to clones with the promoterless construct (Figure 2-7A). In

Figure 2-6: Analysis of A34 and B312 protein lysates

A



B



C

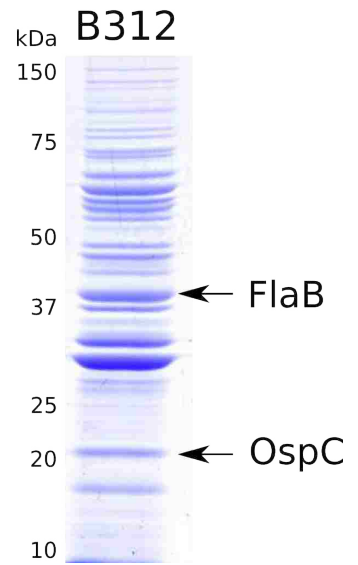


Figure 2-6 legend: **A.** and **B.** Whole cell lysates of A34 and B312 without shuttle vectors and harboring *lacZ* constructs, as indicated, were analyzed by antisera recognizing β -galactosidase (β -gal), FlaB, and OspC. Numbers on the left refer to protein size in kDa determined by the mobility of protein standards. Lysates of B312 with or without shuttle vectors in B are biological replicates of those shown in A and demonstrate the variability in OspC production between samples **C.** B312 whole cell lysate analyzed by SDS-PAGE and Coomassie blue staining. Protein bands corresponding to FlaB and OspC are indicated. Numbers on the left refer to protein size in kDa determined by the mobility of protein standards.

Figure 2-7: *lacZ* expression and β -galactosidase activity as a reporter of *ospC* expression

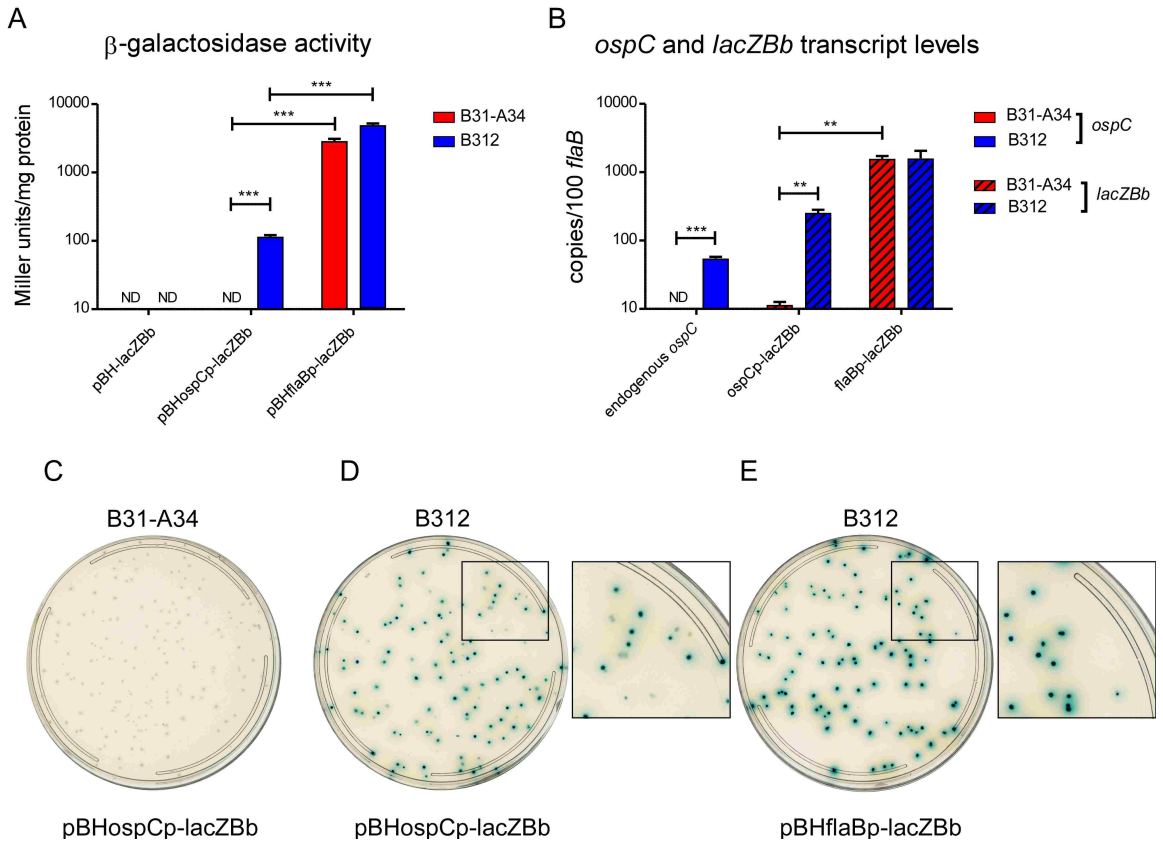


Figure 2-7 legend: **A.** β -galactosidase activity in *B. burgdorferi* B31-A34 (red bars) and B312 (blue bars) harboring pBH-lacZBb, pBHospCp-lacZBb, and pBHflaBp-lacZBb, as indicated. Units are reported per mg protein and levels represent mean \pm SD (n=3). The background activity of bacteria lacking a shuttle vector was subtracted from reported values. ND indicates that no significant activity was detected above background (without plasmid) levels. *** p<0.001 and ** p<0.01 compared to respective promoterless constructs or as indicated based on a Student's two-tailed t-test. **B.** Transcript levels of *ospC* (solid bars) and *lacZBb* (hatched bars) in A34 (red) and B312 (blue) clones harboring *lacZBb*, as indicated, were measured by qRT-PCR with primer/probe sets specific for *ospC* and *lacZBb*, respectively, and normalized to the chromosomal *flaB* gene transcript. *ospC* and *lacZBb* transcript levels are reported per 100 *flaB* copies and values represent mean \pm SD (n=3). cDNA from a RT-PCR reaction without reverse transcriptase was used to control for background detection and was subtracted from reported values. **C-E.** BSK solid medium without phenol red was used to grow B31-A34 harboring pBHospCp-lacZBb (C), B312 harboring pBHospCp-lacZBb (D), or B312 harboring pBHflaBp-lacZBb (E). Approximately 0.5ml of X-gal in DMSO (20mg/ml) was added to plates after colony formation and allowed to incubate overnight before photographs were taken. Magnified insets have been added to D and E to highlight differences between B312 colonies harboring pBHospCp-lacZBb or pBHflaBp-lacZBb.

contrast, β -galactosidase activity was not detected in B31-A34 when the *ospC* promoter was used to express *lacZBb* (Figure 2-7A), consistent with the lack of OspC protein production in this strain (Figure 2-6A). Notably in B312, β -galactosidase activity controlled by the *flaB* promoter was 40-fold higher than activity from the *ospC* promoter (Figure 2-7A). This significant difference in β -galactosidase activity driven by the *flaB* and *ospC* promoters in B312 was somewhat surprising because similar levels of FlaB and OspC proteins were detected by immunoblot or Coomassie blue staining (Figure 2-6B and C, respectively and ref (25)). Thus, I investigated the transcript levels of *ospC* and *lacZBb* in B312, normalizing to *flaB* transcript levels. Endogenous *ospC* transcript levels in B312 lacking a shuttle vector and B312 transformants was roughly half of *flaB* transcript levels (56 *ospC* transcripts for every 100 *flaB* transcripts) (Figure 2-7B, solid blue bar). Similarly, *lacZBb* transcript levels in B312 were dramatically lower when controlled by the *ospC* promoter compared to the *flaB* promoter (Figure 2-7B, hatched blue bars). As expected, no *ospC* transcript or *lacZBb* transcript controlled by the *ospC* promoter was detected in A34. Notably, the *lacZBb* transcript levels driven by either the *flaB* or *ospC* promoters were 5-10 fold higher than the endogenous gene transcripts, presumably reflecting the difference in copy number between the shuttle vector (*lacZBb*) and genome (*flaB* or *ospC*) (37). This increase in gene expression from the shuttle vector may be beneficial when analyzing *lacZ* expression from weak *B. burgdorferi* promoters.

I also assessed β -galactosidase activity controlled from the *ospC* promoter in *B. burgdorferi* colonies grown on BSK solid medium without phenol red. As expected, colonies of A34 harboring pBHospCp-*lacZBb* remained white after

overnight treatment with X-gal (Figure 2-7C), indicating these clones do not express *ospC* or *lacZBb* under control of the *ospC* promoter and thus did not synthesize the β -galactosidase protein. Even when OspC production was verified by immunoblot and Coomassie blue staining (see Figure 2-6), not all of the B312 colonies harboring pBHospCp-lacZBb turned blue after X-gal treatment, suggesting that *ospC* expression varied at the individual colony level (Figure 2-7D). This variation in expression of β -galactosidase appeared to be due to stochastic differences in expression of the *ospC* promoter in this genetic background rather than an intrinsic property of the B312 strain, as B312 colonies harboring the pBHflaBp-lacZBb plasmid did not vary in intensity (Figure 2-7E). Additionally, plasmid loss was not responsible for the variation seen with pBHospCp-lacZBb, since at least 96% of colonies grown without antibiotic selection still contained the shuttle vector (Figure 2-8). Together, these results demonstrate that β -galactosidase activity and *lacZBb* gene expression accurately reflect promoter strength and regulation of gene expression in *B. burgdorferi* (Figure 2-7). Results from the plate assays also indicate variation in *ospC* expression in B312, with only some colonies transcribing *lacZBb* from the *ospC* promoter. This clonal variation in *ospC* regulation in B312 could not have been observed with immunoblots and Coomassie blue staining alone, which reflect the protein production by the bulk population in culture, and illustrates the utility of having *lacZ* as a reporter gene in *B. burgdorferi*.

Discussion

B. burgdorferi has a complicated genetic system encompassing at least 20 plasmids in addition to the linear chromosome. Furthermore, the infectious cycle of

Figure 2-8: pBHospCp-lacZBb stability in B312

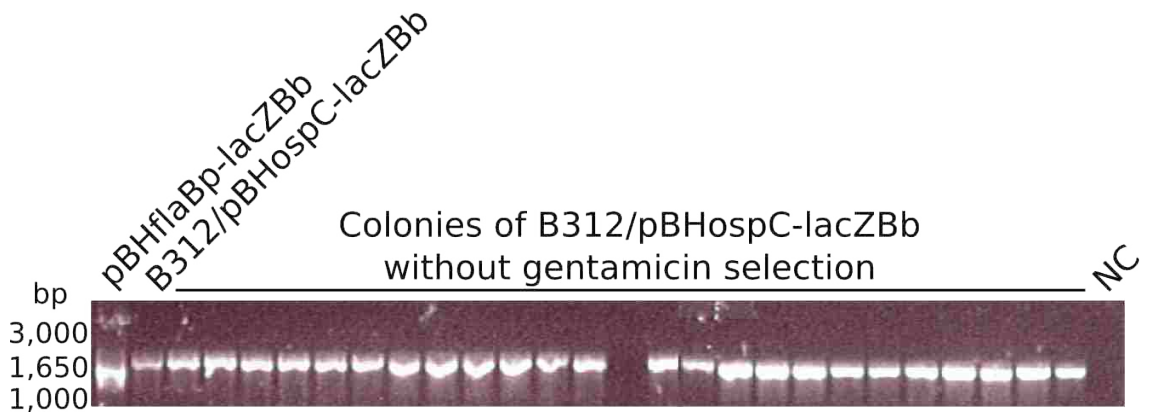


Figure 2-8 legend: PCR amplification of *lacZBb* from B312 colonies grown without selection. B312/pBHospCp-*lacZBb* was grown in solid medium without antibiotic selection. 26 colonies were screen by PCR for *lacZBb* using primers *lacZBb* Int-1 and *lacZBb* Mid Reverse (Table 2-2). The pBHflaBp-*lacZBb* plasmid (1st lane) was used as a positive control, as was a colony of B312/pBHospCp-*lacZBb* grown under gentamicin selection (2nd lane). Water served as a negative control (NC, last lane) in the PCR reaction. Numbers on the left refer to the size in base pairs (bp) based on the mobility of standards.

B. burgdorferi requires dramatic changes in gene expression as the spirochete moves from the vector to the host and vice versa. In order to understand gene regulation in *B. burgdorferi*, genetic tools, including reporter genes, are necessary. However, the existing reporter genes developed for use in *B. burgdorferi* have some limitations. CAT assays are often time consuming and require expensive radioactive substrates. Furthermore, CAT assays lack the sensitivity shown by other reporter genes (22), and CAT has only been used as a transient reporter gene in *B. burgdorferi* (18, 38). Firefly luciferase requires luciferin as a substrate, as well as ATP, magnesium, and oxygen. Additionally, a luminometer or scintillation counter is required for quantification, and the luciferase assay can lack sensitivity and reproducibility unless used in conjunction with inducible systems. Of the current reporter genes used in *B. burgdorferi*, GFP has the broadest utility. GFP emits fluorescence without the requirement for a substrate and can be used in both *in vitro* and *in vivo* formats. Still, a spectrofluorometer or flow cytometer is necessary for GFP quantification, making GFP a costly reporter gene. Additionally, GFP is a stable protein and often does not reflect transient or dynamic promoter activity (22).

My goal in these studies was to determine whether *lacZ*, one of the oldest and most widely used reporter genes, could be adapted to monitor transcriptional activity in *B. burgdorferi*. β -galactosidase activity, encoded by *lacZ*, can be measured using simple colorimetric substrates, and thus does not require expensive materials or equipment. Furthermore, β -galactosidase activity can be detected in liquid or solid media and some substrates allow *in vivo* detection as well (39). I used the constitutive *B. burgdorferi flaB* promoter to express either an *E. coli lacZ* gene

(*lacZ_{Ec}*) or a *B. burgdorferi* codon-optimized *lacZ* gene (*lacZ_{Bb}*). Results show that the β -galactosidase protein is produced in *B. burgdorferi* and activity is detected in both liquid (Figure 2-4) and solid media (Figure 2-5). As anticipated, β -galactosidase activity with the codon-optimized *lacZ_{Bb}* was considerably higher in *B. burgdorferi* than that of the original *E. coli lacZ* gene (Figure 2-4). The greater activity of *lacZ_{Bb}* likely reflects more efficient translation of the mRNA when the gene is optimized for the codon-preference of *B. burgdorferi*.

To determine whether *lacZ* expression and β -galactosidase activity appropriately reflect endogenous gene activity in *B. burgdorferi*, I introduced an *ospC* promoter-*lacZ_{Bb}* gene fusion into two *B. burgdorferi* clones that differ in their OspC protein production. When B312 colonies harboring *lacZ_{Bb}* driven by the *ospC* promoter were assayed with X-gal, β -galactosidase activity, and presumably *ospC* promoter activity, varied from colony to colony (Figure 2-7D), suggesting clonal regulation of *ospC* expression in B312. Furthermore, the β -galactosidase activity and *lacZ_{Bb}* transcript reflected endogenous *ospC* promoter activity in *B. burgdorferi* (Figure 2-7A and B). Although the FlaB and OspC protein levels were not markedly different from each other in B312 when assessed by Coomassie blue staining or immunoblot (Figure 2-6), I noticed a dramatic difference in β -galactosidase activity between the *flaB* and *ospC* promoters when they were used to express *lacZ_{Bb}* (Figure 2-7). In B312, β -galactosidase activity produced from pBHflaBp-*lacZ_{Bb}* was 40-fold higher than from pBHospCp-*lacZ_{Bb}*. Similarly, *lacZ_{Bb}* transcript was 6-fold higher when driven by the *flaB* promoter compared to the *ospC* promoter. Moreover, the endogenous *ospC* transcript level in B312 was approximately half of the endogenous *flaB* transcript level (Figure 2-7), and β -galactosidase protein was

only detected when the *flaB* promoter was used to drive expression of *lacZ* (Figure 2-6). Since *ospC* transcript was detected at a lower level than the *flaB* transcript and β -galactosidase activity was much lower when *lacZ**Bb* was controlled by the *ospC* promoter, the amount of β -galactosidase protein produced with the *ospC* promoter in B312 could be below the levels of detection for immunoblot. These results demonstrate different strengths of the *flaB* and *ospC* promoters and suggest post-transcriptional regulation occurs in *B. burgdorferi*; further studies are needed to investigate this possibility.

One advantage of using reporter gene fusions to assess the transcriptional activity of various promoters is that it eliminates differences in post-transcriptional regulation that may affect endogenous protein levels. The reporter gene transcript and encoded protein are the same irrespective of which promoter is used to express it. The reporter genes previously used in *B. burgdorferi* all require expensive substrates and/or specialized equipment for detection and quantification. In contrast, β -galactosidase activity can be detected with simple colorimetric substrates and quantified with a spectrometer. In fact, *B. burgdorferi* promoters have previously been fused to *lacZ*, but β -galactosidase activity was assessed in *E. coli*, not *B. burgdorferi* (26, 40). However, since many studies have detected significant differences in the expression profiles of *B. burgdorferi* genes in *E. coli* (18, 38, 41, 42), using *lacZ* as a reporter gene directly in *B. burgdorferi* will allow a more accurate analysis of native promoter activity. Additionally, there are many substrates for β -galactosidase, which allow *lacZ* to be used in multiple applications, including simple *in vitro* assays to assess promoter activity and rapid screening for

transformants on solid media. Thus, adding *lacZ* to the repertoire of genetic tools for *B. burgdorferi* should facilitate investigation of the regulatory mechanisms that allow *B. burgdorferi* to adapt to different environments.

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CHAPTER THREE

Regulation of the virulence determinant OspC by *bbd18* on linear plasmid lp17 of
Borrelia burgdorferi

Forward

This chapter is adapted from Sarkar A, Hayes BM, Dulebohn DP, Rosa PA. 2011 Journal of Bacteriology. 193(19): 5365-73. Copyright © 2011, American Society for Microbiology. All rights reserved. Dr. Amit Sarkar, a former fellow in the lab, piloted this study and I adapted the *lacZ* reporter (chapter 2) to aid in the identification of *bbd18*. As such, Figure 3-2 is based solely on work done by Dr. Sarkar, while the rest of the figures were a combined effort. Also note the use of ‘we’ instead of ‘I’ to show co-ownership of this project.

Abstract

Persistent infection of a mammalian host by *Borrelia burgdorferi*, the spirochete that causes Lyme disease, requires specific down-regulation of an immunogenic outer surface protein, OspC. Although OspC is an essential virulence factor needed by the spirochete to establish infection in the mammal, it represents a potent target for the host’s acquired immune response and constitutive expression of OspC results in spirochete clearance. In this study, we demonstrate that a factor encoded on a linear plasmid of *B. burgdorferi*, lp17, can negatively regulate *ospC* transcription from the endogenous gene on the circular plasmid cp26 and from an *ospC* promoter-*lacZ* fusion on a shuttle vector. Furthermore, we have identified *bbd18* as the gene on lp17 that is responsible for this effect. These data identify a

novel component of *ospC* regulation and provide the basis for determining the molecular mechanisms of *ospC* repression *in vivo*.

Introduction

Borrelia burgdorferi, the causal organism of Lyme disease (1-3), maintains its complex enzootic life cycle in two different environmental niches, *Ixodes* ticks and mammalian hosts (4). To achieve this, *B. burgdorferi* senses key changes in its surroundings and undergoes dramatic adaptive changes in gene expression. As part of this adaptive response, spirochetes in the midguts of infected ticks initiate synthesis of an abundant outer surface protein, OspC, when the ticks take in a blood meal (5-7). The incorporation of OspC on its outer membrane prepares *B. burgdorferi* for transmission to a mammalian host, where it establishes persistent infection. We have previously demonstrated that *B. burgdorferi* mutants lacking functional OspC cannot initiate mammalian infection following transmission by infected ticks (8-11). However, since OspC represents a potent neutralizing target, *ospC* expression must be downregulated after initiation of infection to avoid clearance of the spirochete by the host's acquired immune response (12-15).

As an infected nymphal tick attaches to a mammalian host and feeds, spirochetes residing in the tick midgut experience changes in temperature, pH and nutrients, which signal a global adaptive response in gene expression through a novel regulatory cascade involving the response regulator Rrp2 and the alternative sigma factors RpoN (σ^N , σ^{54}) and RpoS (σ^S , σ^{38}) (16-19). In addition to *ospC*, a number of other *B. burgdorferi* genes are induced during tick feeding through this RpoN/RpoS signaling pathway (17, 20-25). However, unlike *ospC*, many of these

genes continue to be expressed during mammalian infection (26). Hence, subsequent repression of *ospC* is a gene-specific mechanism required to avoid immune clearance.

Xu and colleagues have recently described a palindromic sequence immediately upstream of the *ospC* promoter that represents a potential operator site to which a repressor could bind (27, 28). Although *B. burgdorferi* mutants lacking this palindromic sequence can initiate mammalian infection, *ospC* expression is not downregulated in these mutants and thus they are subsequently recognized and cleared by neutralizing antibodies of the acquired immune response (27). However, the invoked repressor that binds to the operator site and downregulates *ospC* expression *in vivo* has not been identified.

In an earlier study, Sadziene and colleagues described several highly attenuated *B. burgdorferi* clones that constitutively synthesize OspC during *in vitro* growth, in contrast to the parental B31 strain from which these clones were derived (29). These *B. burgdorferi* clones had lost many or all linear plasmids during extended *in vitro* passage but retained the 26 kilobase (kb) circular plasmid (cp), cp26, which carries the *ospC* gene (29). Synthesis of OspC correlated with loss of a particular linear plasmid (lp), lp17, and *ospC* expression was highest in clones lacking both lp17 and another plasmid, lp54. These authors proposed that lp17 encodes a putative repressor that typically silences *ospC* in strain B31 during *in vitro* growth; they also suggested that further evidence for this repressor could be provided by restoration of lp17 to clones from which it had been lost (29). Although this was technically infeasible when proposed in 1993, the genetic system of *B. burgdorferi* has developed to a stage where displacement and restoration of

individual plasmids are now possible (30-32). In this report, we describe a series of experiments in which we investigate the ability of both full-length and truncated forms of lp17, as well as lp17 gene sequences introduced on a shuttle vector, to repress *ospC* gene expression.

Materials and Methods

Bacterial strains and culture conditions.

All *B. burgdorferi* strains were inoculated from frozen stocks into liquid Barbour-Stoenner-Kelly (BSKII) medium supplemented with 6% rabbit serum (PelFreez Biologicals, Rogers, AR) and grown at 35°C under 2.5% CO₂ (33). *B. burgdorferi* strains, *Escherichia coli* strains and plasmids used in this study are described in Table 3-1.

Transformation of *B. burgdorferi*.

B. burgdorferi was transformed by electroporation as previously described (34, 35). Briefly, 10–15 µg of plasmid DNA or genomic DNA was introduced by electroporation into competent *B. burgdorferi* cells, freshly prepared from an exponential phase culture. Following electroporation, the cells were resuspended in 5 ml of BSKII and allowed to recover for 18–24 h at 35°C. The spirochetes were then diluted to 20 ml of BSKII supplemented with kanamycin (200 mg/ml), distributed at 200 µl/well in 96 well flat bottom Costar plates (Corning, Lowell, MA) and incubated at 35°C with 2.5% CO₂. Alternatively, recovered cells were plated in solid BSKII medium with kanamycin (200mg/ml) and/or gentamicin (40µg/ml) and incubated at 35°C with 2.5% CO₂.

Screening of transformants.

Table 3-1: Strains and Plasmids used in Chapter Three

Strain or plasmid	Description	Reference
<i>B. burgdorferi</i> strains		
B31-A3	Infectious derivative of wild-type B31-MI, lacks cp9	(36)
ospCK1	ospC mutant; isogenic derivative of B31-A3; containing all B31 plasmids except cp9; kanamycin resistant	(11)
ospCK1+ospC	ospCK1 derivative that constitutively expresses <i>ospC</i> (ospCK1/pBSV2G-flaBp-ospC); containing all B31 plasmids except cp9; kanamycin- and gentamicin-resistant	(37)
B312	high passage, non-infectious clone containing lp54, cp26, cp32-1, cp32-3, cp32-4, cp32-7, cp32-8; synthesizes OspA/B and OspC <i>in vitro</i>	(29); this study
B31-A	non-infectious clone of the prototype <i>B. burgdorferi</i> sensu stricto isolate B31	(38)
B31-A, lp17::kan	B31-A derivative carrying pKK81 integration on lp17; kanamycin resistant	(39)
B312_lp17.8	B312 derivative harboring lp17::kan; kanamycin-resistant	This study
B312 +pGCB409	B312 derivative harboring pGCB409; kanamycin-resistant	(40); this study
B312+pGCB413	B312 derivative harboring pGCB413; kanamycin-resistant	(40); this study
B312+pGCB426	B312 derivative harboring pGCB426; kanamycin-resistant	(40); this study
B312+pGCB473	B312 derivative harboring pGCB473; kanamycin-resistant	(40); this study
B312+pBSV2*-7'	B312 derivative harboring <i>bbd15-bbd18</i> region of lp17 on a shuttle vector; kanamycin-resistant	this study
B312 + pBSV2*-NP-bbd18	B312 derivative harboring <i>bbd18</i> gene under the control of native promoter (NP) on a shuttle vector; kanamycin-resistant	this study
B312 + pBSV2*-flaBp-bbd18	B312 derivative harboring <i>bbd18</i> under the control of the constitutive flab promoter on a shuttle vector; kanamycin-resistant	this study
B312+ pBSV2*-bbd18	B312 derivative harboring <i>bbd18</i> without a promoter on a shuttle vector; kanamycin-resistant	this study
B312 + pBHospCp-lacZBb*	B312 derivative harboring <i>lacZBb*</i> under the control of the <i>ospC</i> promoter on a shuttle vector; gentamicin-resistant	(41); this study
B312 + pBHospCp-lacZBb* + lp17::kan	B312 derivative harboring <i>lacZBb*</i> under the control of the <i>ospC</i> promoter on a shuttle vector and lp17::kan; gentamicin- and kanamycin-resistant	(41); this study
<i>E. coli</i> strains		
Top10 + pOKflaBp-bbd18	Top10 with a constitutive <i>bbd18</i> gene on pOK12; kanamycin-resistant	(42); this study
Top10 + pBH-lacZBb*	Top10 with a promoter-less <i>lacZBb</i> gene in pBSV2G; gentamicin-resistant	(41); this study

Top10 + pBHospCp-lacZBb*	Top10 with <i>lacZBb</i> under the control of the <i>ospC</i> promoter in pBSV2G; gentamicin- resistant	(41); this study
Top10 + pOKflaBp-bbd18 + pBH-lacZBb*	Top10 with both the constitutive <i>bbd18</i> gene on pOK12 and the promoter-less <i>lacZBb</i> gene on pBSV2G; kanamycin- and gentamicin- resistant	(41, 42); this study
Top10 + pOKflaBp-bbd18 + pBHospCp-lacZBb*	Top10 with both the constitutive <i>bbd18</i> gene on pOK12 and <i>lacZBb</i> under the control of the <i>ospC</i> promoter in pBSV2G; kanamycin- and gentamicin- resistant	(41, 42); this study
Plasmids		
pBSV2*	pBSV2 derivative created by disrupting the EcoRI site at nt. 1852	(43)
pBSV2*-7'	pBSV2* with a 2721bp insert spanning <i>bbd15-18</i> (nt. 9360-12081) of lp17	this study
pBSV2*-NP-bbd18	pBSV2* with an insert encompassing <i>bbd18</i> and flanking sequences (nt. 10881-12056) of lp17 (native promoter)	this study
pBSV2*-flaBp-bbd18	pBSV2* with the constitutive <i>flaB</i> promoter driving expression of <i>bbd18</i> (nt. 11648-10881 of lp17).	this study
pBSV2*-bbd18	pBSV2* carrying a promoter-less <i>bbd18</i> gene (nt. 11648-10881 of lp17).	this study
pGCB409	lp17 deletion variant from <i>B. burgdorferi</i> strain GCB409 carrying <i>bbd1</i> through <i>bbd23</i>	(40)
pGCB413	lp17 deletion variant from <i>B. burgdorferi</i> strain GCB413 carrying <i>bbd7</i> through <i>bbd25</i>	(40)
pGCB426	lp17 deletion variant from <i>B. burgdorferi</i> strain GCB426 carrying <i>bbd1</i> through 5' of <i>bbd20</i>	(40)
pGCB473	lp17 deletion variant from <i>B. burgdorferi</i> strain GCB473 carrying <i>bbd1</i> through <i>bbd14</i>	(40)
pBH-lacZBb*	Promoter-less <i>Borrelia</i> codon-optimized <i>lacZBb</i> gene on the shuttle vector pBSV2G with BspHI restriction site added upstream of <i>lacZBb</i>	(41); this study
pBHospCp-lacZB*	pBH-lacZBb* with the <i>ospC</i> promoter driving expression of <i>lacZBb</i>	(41); this study
pOKflaBp-bbd18	<i>flaBp-bbd18</i> cassette from pBSV2*-flaBp-bbd18 cloned into pOK12 to be compatible with <i>lacZBb</i> shuttle vectors	(42); this study

After 7-8 days, 20 ml of bacterial cultures from positive wells (identified by phenol red indicator color change and confirmed by dark field microscopy) were inoculated into 5 ml of BSKII supplemented with kanamycin and incubated at 35°C. Typically, approximately 10 of 96 wells were positive for growth and hence transformants could be considered clonal (44). Total genomic DNA was isolated from outgrowth cultures with a Wizard genomic DNA purification kit (Promega, Madison, WI). Transformants were distinguished from spontaneous resistance mutants by polymerase chain reaction (PCR) with total genomic DNA, using appropriate primers (Table 3-2). Alternatively, colonies in solid media were screened directly by PCR to confirm the stable presence of introduced DNA.

B312 transformants carrying full length and truncated forms of lp17.

Genomic DNA isolated from B31-A, lp17::*kan* (39) and plasmid DNA isolated from four different *B. burgdorferi* clones (GCB409, GCB413, GCB426 and GCB473) harboring truncated forms of lp17 (40) were used to generate B312 transformants carrying either full length or the respective lp17 deletion variant (Table 3-1). B312 clones carrying the full-length lp17 were confirmed by PCR using primers specific for kanamycin and several lp17 genes (Table 3-2). The deletions in lp17 from the donor GCB strains were confirmed by PCR and Southern blot analysis (Dr. Amit Sarkar, personal communication). The deleted forms of lp17 were designated by the respective GCB strain from which they were derived (e.g., pGCB409 is the lp17 variant carried by GCB409). B312 transformants were initially screened by PCR for the kanamycin-resistance cassette present on lp17 and then checked by PCR for additional lp17 sequences to confirm introduction of the desired lp17 deletion variant from GCB clones. The complete plasmid content of each B312 transformant

Table 3-2: Primers and probes used in Chapter Three

	Name	Sequence (5'-3') ^a	Reference
1	flaB qPCR forward	TCCTTCCTGTTGAACACCCTCT	(45)
2	flaB qPCR reverse	TCTTTTCTCTGGTGAGGGAGCT	(45)
3	flaB qPCR probe	AAACTGCTCAGGCTGCACCGGTTC	(45)
4	ospC qPCR forward	ACGGATTCTAATGCGGTTTACTT	(46)
5	ospC qPCR reverse	CAATAGCTTTAGCAGCAATTCATCT	(46)
6	ospC qPCR probe	CTGTGAAAGAGGTTGAAGCGTTGCTGTCAT	(46)
7	<i>guaA</i> forward	TGACTCATGATAATTTGAAATT	This study
8	<i>ospC</i> reverse	CACCTTCTTTACCAAGATCTGT	This study
9	bbd11 forward	GTGTATACTGACCCAAGGTCAA	(23)
10	bbd11 reverse	AGAAATGGGGTTACATTTGAAGCTTGG	(23)
11	bbd14 forward	AACGCTCAAGGCTAAAATCA	(23)
12	bbd14 reverse	CTGTCAGTTTTTCCATTCATCATCC	(23)
13	bbd21 forward	GCATTTTCATATTCAAAAAGGTGGTGTGGG	(23)
14	bbd21 reverse	TAGGCATAAAGCATCTCCCA	(23)
15	kan 5' NdeI forward	<u>CATATGAGCCATATTCAACGGGAAACG</u>	(38)
16	kan reverse	TTAATTAATGAGCTAGCGCCGT	This study
17	bbd14 end forward	GCTCTAGAAATTAATAATCAAAATTAATTGb	This study
18	bbd19 start reverse	GCTCTAGAGATAAAAATAAAATTCATTTATCb	This study
19	<i>lacZBb</i> Int-1 forward	TGGTGTAAATGGAAGATGGGTTGG	This study
20	<i>lacZBb</i> Mid reverse	GATAGACCATTTAGGAACAGCAGG	This study
21	ospC promoter-BamHI forward	CCCGCGGATCCAATTA AAACTTTTTTTTATTAAAGTA	(41)
22	ospC promoter-BspHI reverse	CCCCGTCATGATTTGTGCCTCCTTTTTATTAT	This study
23	bbd18_NP forward	CCGGAATTCAGAAATTTACTTACAATATTTAACCTTC	This study
24	<i>bbd18</i> forward	CCGGAATTCATGCAAAAAGAAATAACAATAAAC	This study
25	bbd18 +reverse	CCGGAATTCGTAAGGTAGAAAACGATCTACCAAT	This study

^a qPCR probes were labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) at the 5' and 3' ends, respectively. Restriction sites are underlined

was also determined by PCR (36) to confirm that no plasmids other than lp17 had been introduced.

Shuttle vector constructs harboring lp17 sequences and B312 transformants.

A 2721 bp fragment of lp17 extending from the 3' end of *bbd14* (nt. 9360) to the 5' end of *bbd19* (nt. 12081) was amplified from B31-A3 genomic DNA (primers 17 and 18, Table 3-2) and cloned into the pCR2.1 TOPO vector (Invitrogen). Clones harboring the desired lp17 fragment were identified by PCR amplification and restriction digest, and confirmed by sequencing. The ~2.7kb insert fragment was excised with *XbaI* (New England Biolabs, Beverly, MA) and ligated into *XbaI*-digested pBSV2* vector (43), yielding pBSV2*-7' (Table 3-1).

A 767 bp fragment of lp17 (nt. 11648-10881) comprising the BBD18 coding sequence and downstream sequences (no promoter) was amplified from B31-A3 genomic DNA (primers 24 and 25, Table 3-2) and cloned into pCR2.1 TOPO vector (Invitrogen). Clones harboring the desired fragment were identified by restriction digest and confirmed by sequencing. The insert fragment was excised with *EcoRI* (New England Biolabs, Beverly, MA) and ligated into *EcoRI*-digested pBSV2* (43), yielding pBSV2*-*bbd18* (Table 3-1). The same *bbd18* fragment was also cloned on the shuttle vector under the control of the constitutive *flaB* promoter, yielding pBSV2*-*flaBp-bbd18*. Finally, a 1176 bp fragment of lp17 encompassing the *bbd18* gene with 5' and 3' flanking sequences (nt. 12056-10881) was amplified from B31-A3 genomic DNA (primers 23 and 25, Table 3-2) and cloned into pBSV2* in a similar fashion, yielding pBSV2*-NP-*bbd18* (native promoter) (Table 3-1).

Plasmid DNA isolated from *E. coli* harboring pBSV2*, pBSV2*-7', pBSV2*-*bbd18* (promoter-less), pBSV2*-NP-*bbd18* (native promoter) or pBSV2*-*flaBp*-

bbd18 (constitutive *flaB* promoter) (Table 3-1) was used to transform B312. Transformants were selected in liquid BSK II medium in the presence of kanamycin (200µg/ml) and screened by PCR with specific primers for the kanamycin cassette (38) and *bbd18* (Table 3-2). Shuttle vectors from *B. burgdorferi* transformants were rescued in *E. coli* and characterized by restriction digest to confirm insert.

lacZBb reporter constructs and expression in B312.

The *ospC* promoter was amplified from B31-A3 genomic DNA (primers 21 and 22, Table 3-2), cloned into pCR2.1 (Invitrogen), and sequenced to confirm the insert. The *ospC* promoter fragment was excised with *BamHI* and *BspHI* and ligated into appropriately digested pBH_ *lacZBb** (a derivative of pBH_ *lacZBb* with a *BspHI* restriction site immediately upstream of *lacZBb*) (41), creating pBHospCp- *lacZBb** (Table 3-1).

Plasmid DNA from *E. coli* harboring pBHospCp- *lacZBb** (Table 3-1) was used to transform B312 and B312_lp17.8 (Table 3-1). Transformants were selected in solid media containing gentamicin (40µg/ml) and individual colonies screened by PCR for *lacZBb* and retention of the kanamycin cassette in B312_lp17.8 (Table 3-2).

B312 derivatives harboring pBHospCp- *lacZBb**, with or without lp17 marked with a kanamycin cassette (Table 3-1), were grown in BSKII solid medium lacking phenol red, supplemented with the appropriate antibiotic(s). After colony formation, approximately 0.5ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 20mg/ml in dimethylformamide) was added to detect β-galactosidase activity, as described previously (Chapter 1 and ref (41)). Colonies were screened for maintenance of the kanamycin cassette and *lacZBb* with the appropriate primers (Table 3-2).

BBD18 construct for co-expression in *E. coli*.

The *flaB* promoter-*bbd18* insert fragment was cut out of pBSV2**flaBp-bbd18* (Table 3-1) with NotI and ligated into appropriately digested pOK12 (42), creating pOK*flaBp-bbd18* (Table 3-1). *E. coli* (Top 10) was transformed with pOK*flaBp-bbd18*, pBH_ *lacZBb**, or pBHospCp-*lacZBb**, or both pOK*flaBp-bbd18* and one of the *lacZBb** plasmids. Resulting colonies were screened for the presence of *flaBp::bbd18* and/or *lacZBb* as appropriate and streaked on LB agar plates containing the appropriate antibiotic(s) and X-gal.

Quantitative reverse transcriptase PCR (qRT-PCR).

Total RNA was extracted from exponential phase *B. burgdorferi* cultures using the Nucleospin RNA II kit (Fisher Scientific, Pittsburgh, PA) according to the manufacturer's specifications and treated with RNase-free DNase I. Synthesis of cDNA was carried out using random hexamer primers and a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Branchburg, NJ). These reactions were also carried out in the absence of reverse transcriptase to serve as a control for residual DNA contamination. Newly synthesized cDNA was treated with RNase H (Ambion, Applied Biosystems) for 1 h at 37°C to remove RNA-DNA hybrids and samples were then cleaned and concentrated using a MinElute PCR purification kit (Qiagen, Valencia, CA). Concentrated cDNA samples were quantified by absorbance at 260 nm and diluted to 50 ng/μl in DNase- and RNase-free water (Applied Biosystems). Quantitative PCR was performed with 100ng cDNA using a TaqMan Universal PCR Master Mix (Applied Biosystems) and primer-probe combinations (Table 3-2) for *B. burgdorferi flaB* (flagellin) (45) and *ospC* (46) genes, using an Applied Biosystems 7900HT instrument. The relative copy numbers of *flaB* and *ospC*

transcripts were determined using a standard curve for each gene target that was generated with purified genomic DNA from 10^5 , 10^4 , 10^3 , 10^2 and 10^1 spirochetes. Samples were analyzed in triplicate and gene expression reported as the number of *ospC* per *flaB* mRNA copies. The amplification of samples without reverse transcriptase was similar to that for the no-template control. Data sets were compared using the GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego CA).

SDS-PAGE and immunoblotting.

B. burgdorferi protein lysate preparation and their separation by 12.5% SDS-PAGE were as previously described (47). Gels were run in duplicate and either stained with Coomassie Brilliant Blue (Sigma, St. Louis, MO) or blotted to nitrocellulose membranes (BioRad, Hercules, CA). Membranes were blocked overnight with 5% nonfat milk (BD Diagnostics, Franklin Lakes, NJ) in TBS-Tween 20 (20mM TRIS pH 7.5, 150mM NaCl, 0.05% Tween 20), and probed with rabbit anti-OspC polyclonal antiserum (1:1000 dilution) (10), anti-FlaB mouse monoclonal antibody H9724 (1:25 dilution) (48) and rabbit anti-BBD18 antiserum (1:500 dilution) (described below). Next, membranes were incubated with peroxidase-conjugated anti-rabbit or anti-mouse serum (Sigma). Finally, peroxidase activity was detected using Super Signal reagents (Thermo Scientific, Rockford, IL) and X-ray film (LabScientific Inc, Livingston, New Jersey).

BBD18 peptide antiserum.

A synthetic 15 amino acid peptide (CRHFDEQNKTNFNES), matching residues 120-133 of the annotated BBD18 protein and the addition of a cysteine residue at the N-terminus for conjugation, was used to generate BBD18-specific antisera in

rabbits (Genscript, Piscataway, NJ). Affinity-purified antibodies (Genscript) were used in immunoblot analyses (1:500 dilution) to detect BBD18.

Sequencing the *ospC* promoter region.

An approximately 1 kb fragment extending through the *guaA-ospC* intergenic region was amplified from both B31-A3 and B312 total genomic DNA (primers 7 and 8, Table 3-2) and cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) in *E. coli* Top 10 (Invitrogen). Inserts were sequenced using the ABI Big Dye Terminator Cycle sequencing Ready Reaction Kit with an ABI 3700 DNA sequencer (Applied Biosystems) and the sequences of the *guaA-ospC* intergenic region from B31-A3 and B312 were compared using DNA-Star software (DNASTAR, Inc. Madison, WI).

Results

Characterization of strain B312.

Specific down-regulation of immunogenic OspC during the early phase of mammalian infection is a key strategy adopted by *B. burgdorferi* for persistence in the mammalian host (11, 12, 15, 37). Sadziene *et al.* previously observed that the highly attenuated *B. burgdorferi* clone B312 expresses an otherwise cryptic *ospC* gene when lp17 is lost (29), prompting the hypothesis that an lp17-encoded gene product might be responsible for the specific and timely repression of *ospC* expression *in vivo*.

In order to address the influence of lp17 on *ospC* expression, we first determined the plasmid content of strain B312 by PCR, using specific primer pairs for each of the 21 plasmids of strain B31 (36, 49, 50). This analysis confirmed that

B312 lacks all B31 plasmids except cp26, lp54 and several cp32s (Dr. Sarkar, personal communication and ref (29)). We also analyzed the protein content of B312 and confirmed that OspC was synthesized (Figure 3-1A, lane 2), in contrast to wild-type (wt) B31, which does not synthesize OspC under typical *in vitro* growth conditions (Figure 3-1A, lane 1) (29, 51). Finally, we analyzed the sequence of the *ospC* promoter and upstream flanking region (187 bp) of clone B312 and found that it was identical to the wt B31 sequence (Dr. Sarkar, personal communication and ref (50, 52)). Thus, although clone B312 constitutively synthesizes OspC during *in vitro* growth, there are no changes in the 5' flanking region of the *ospC* gene that might account for this phenotypic switch.

Restoration of lp17 into B312

To investigate the hypothesis that lp17 negatively regulates *ospC* expression, the entire lp17 plasmid was introduced into B312 by transformation with genomic DNA from a *B. burgdorferi* strain in which a selectable marker, the kanamycin resistance cassette (38), had been inserted near the left telomere of lp17 (lp17::kan) (39). The complete plasmid content of the resulting transformant, B312_lp17.8, was determined by PCR (36) to confirm that no plasmids other than lp17 had been introduced (Dr. Sarkar, personal communication). As predicted, B312_lp17.8 no longer synthesizes OspC (Figure 3-1A, lane 3), consistent with a role for one or more lp17 genes in negative regulation of *ospC*.

The influence of lp17 on *ospC* expression was analyzed by quantitative reverse transcription (qRT) PCR of RNA from B312 clones with and without lp17; *ospC* transcript was normalized to *flaB* mRNA copies for each strain. This analysis demonstrated approximately 10-fold less *ospC* transcript in the B312 derivative

Figure 3-1: Negative regulation of *ospC* by lp17 in B312.

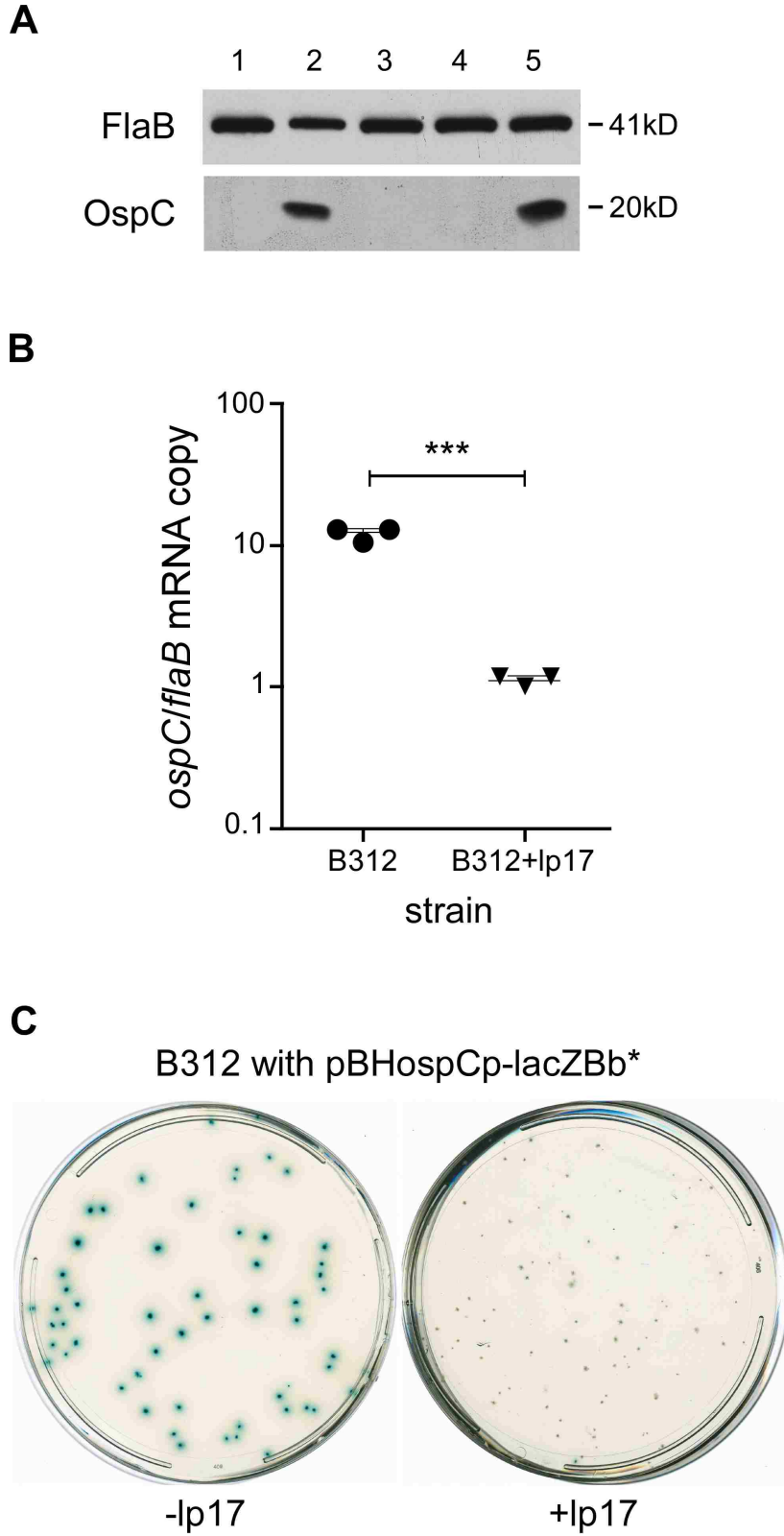


Figure 3-1 legend: **A.** Total *B. burgdorferi* protein lysates analyzed by immunoblot with OspC and FlaB antisera, as indicated: wild type clone B31-A3 (lane 1); B312 (lane 2); B312_lp17.8 (lane 3); mutant *ospCK1* (lane 4); *ospCK1+ospC* (lane 5). **B.** *ospC* transcript level in total RNA from B312 and B312_lp17.8 (+lp17) as measured by quantitative RT-PCR and normalized to the chromosomal *flaB* gene transcript. (***) indicates $P < 0.002$ based on Student's two-tailed t-test. **C.** *ospC* promoter activity in *B. burgdorferi* B312 (-lp17) and B312_lp17.8 (+lp17) monitored by a *lacZ* reporter gene. Beta-galactosidase activity in *B. burgdorferi* transformed with a shuttle vector carrying a *lacZ* reporter gene fused to the *ospC* promoter (pBHospCp-lacZBb*) was detected by the addition of X-Gal to solid BSKII medium without phenol red, resulting in blue B312 colonies (left), whereas B312_lp17.8 colonies remained white (right).

harboring lp17 relative to the original B312 clone lacking lp17 (Figure 3-1B). These data suggest that lp17 negatively regulates OspC production at the transcriptional level.

***ospC* transcription in B312 as monitored with a *lacZ* reporter gene**

We recently developed a *lacZ* reporter system for *B. burgdorferi* and used it to analyze *ospC* gene expression in various B31 clones, including B312 (Chapter 1 and (41)). To confirm that introduction of lp17 uniformly inhibited *ospC* expression in the B312 background, we monitored *ospC* promoter activity with a *lacZ* reporter construct, pBHospCp-*lacZ*Bb*, using a blue/white colony screen to assay β -galactosidase activity in a large numbers of transformants. B312 colonies harboring pBHospCp-*lacZ*Bb* turned blue after treatment with X-gal (Figure 3-1C), indicating expression from the *ospC* promoter and production of an active β -galactosidase enzyme, as reported previously (Chapter 1 and ref (41)). However, introduction of pBHospCp-*lacZ*Bb into B312_17.8 resulted in most colonies remaining white after incubation with X-gal (Figure 3-1C). We confirmed the presence of pBHospCp-*lacZ*Bb* in these colonies by PCR, suggesting that a factor encoded by lp17 repressed *lacZ*Bb expression from the *ospC* promoter in B312_17.8. Although we did detect a few blue colonies in B312_17.8 harboring pBHospCp-*lacZ*Bb (Figure 3-1C), this was a rare occurrence (~1 in 250). These blue colonies retained lp17, as confirmed by PCR, and had the same *ospC* promoter sequence as the *lacZ* reporter construct present in white colonies, potentially indicating a mutation in the putative *ospC* repressor or variation in some other component of *ospC* regulation (see Discussion).

Effect of truncated lp17 upon OspC production by B312 *in vitro*.

These initial observations support the hypothesis that sequences carried by

Figure 3-2: Introduction of truncated and cloned segments of lp17 into B312 and the resulting OspC phenotype.

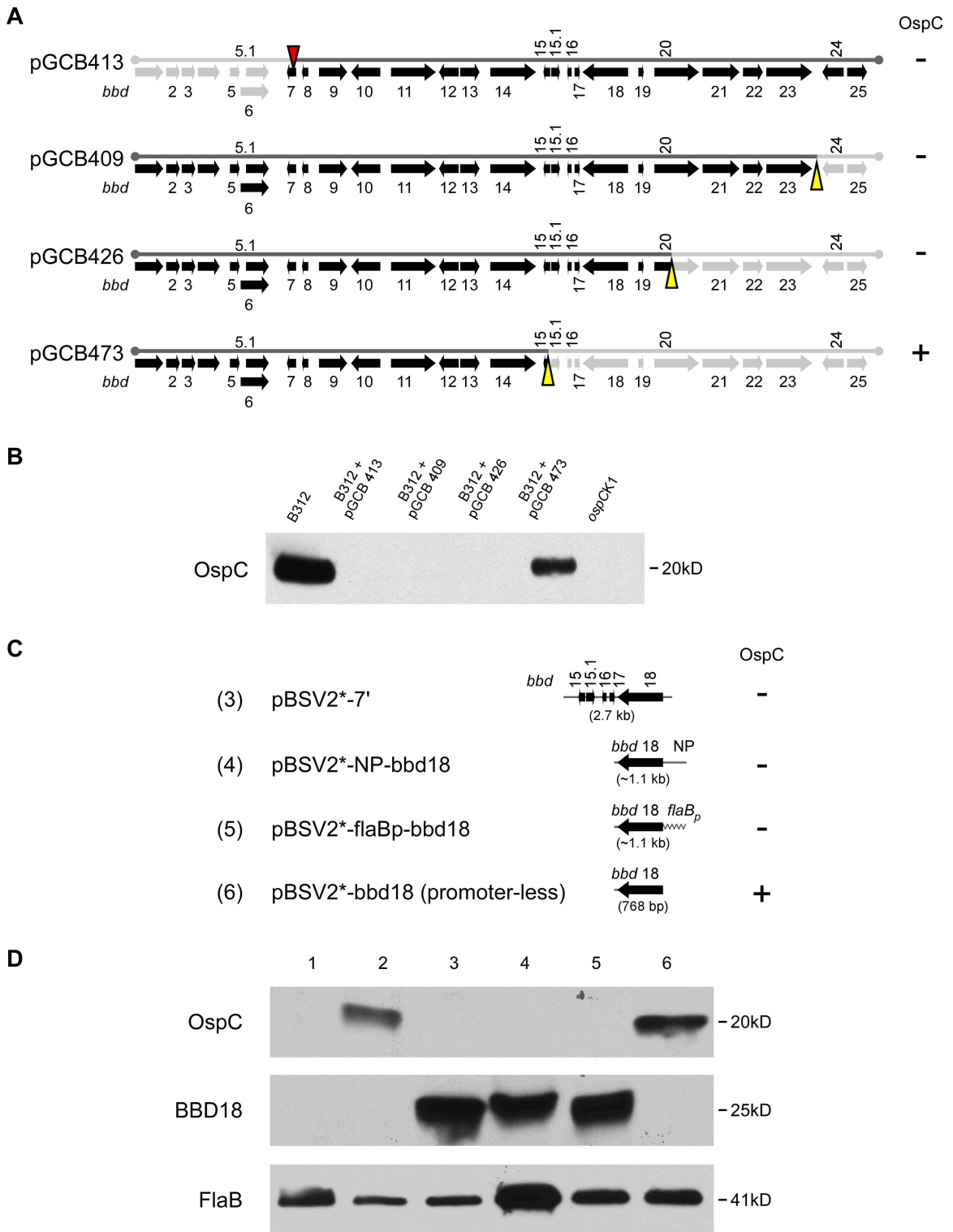


Figure 3-2 legend: **A.** Schematic diagram of truncated forms of lp17 created by insertion of replicated telomeres (40). lp17 sequences are deleted to the left of the insertion point for pGCB413 (red arrowhead above diagram) and to the right of the insertion points for pGCB409, pGCB426 and pGCB473 (yellow arrowheads beneath diagram). Light shading indicates lp17 sequences that are not present in the respective lp17 truncated variants. Gene designations are as previously annotated (49, 50). The presence (+) or absence (-) of OspC in protein lysates of B312 transformants carrying different truncated forms of lp17 is indicated to the right of each plasmid diagram. **B.** OspC immunoblots of B312 and transformants carrying truncated forms of lp17, and the *ospCK1* mutant. Whole cell lysates of *B. burgdorferi* strains, as identified at the top of each lane, were separated by SDS-PAGE and analyzed by immunoblot with anti-OspC antiserum. **C.** Schematic diagram of lp17 sequences introduced on the shuttle vector pBSV2*. The presence (+) or absence (-) of OspC in protein lysates of B312 transformants carrying these shuttle vectors is indicated to the right of each plasmid diagram. The number in parentheses to the left of each diagram corresponds with the lane designation in the immunoblot panels shown below. NP, native promoter; *flaBp*, constitutive promoter. **D.** OspC, BBD18 and FlaB immunoblots of *B. burgdorferi*. Whole cell lysates of *B. burgdorferi* wild type clone B31-A3 (lane 1), high passage clone B312 (lane 2), B312+pBSV2*-7' (lane 3), B312+pBSV2*-NP-bbd18 (lane 4), B312+pBSV2*flaB-bbd18 (lane 5) and B312+pBSV2*bbd18 (lane 6) were analyzed by immunoblot with antisera recognizing OspC, BBD18 and FlaB, as indicated. The estimated sizes of the proteins recognized in each immunoblot, relative to the mobilities of standards, are indicated to the right of each panel.

lp17 repress *ospC* transcription. In order to narrow down the putative lp17 gene(s) responsible for this effect, we utilized several lp17 deletion variants previously generated by Beaurepaire and Chaconas (Figure 3-2A) (40). These terminally truncated forms of lp17 were constructed by insertion of a synthetic replicated telomere at various points along the plasmid and subsequent cleavage by the endogenous telomere resolvase, with recovery of the lp17 fragment that retains essential replication functions and the selectable marker (40). We anticipated that transformation of B312 with a segment of lp17 harboring the putative *ospC* repressor gene would result in reduced OspC production, similar to what was found with the full-length plasmid. Conversely, introduction of an lp17 deletion variant that lacks the *ospC* repressor would not diminish *ospC* expression in B312. Thus, comparing the OspC phenotype of B312 transformants carrying different segments of lp17 would highlight the region of lp17 sufficient for repression of *ospC* and thereby facilitate subsequent identification of the sequences responsible for this effect.

We confirmed the structures of the lp17 variants in four clones, GCB409, GCB413, GCB426 and GCB473, whose deletions extend in from either the right or left telomeres of the plasmid (Figure 3-2A) (40). Results obtained by PCR amplification with primers targeting different regions of lp17, and Southern blot hybridization with probes for the left and right halves of lp17, were consistent with the predicted sizes and structures of the deleted forms of these plasmids (Dr. Sarkar personal communication and see Figure 4-4).

Transformation of B312 with these truncated forms of lp17 had varying effects on OspC synthesis depending upon which lp17 sequences were introduced

(Figure 3-2B). The complete plasmid content of each B312 transformant was also determined by PCR (36) to confirm that no plasmids other than lp17 had been introduced. Taken together, these data are consistent with negative regulation of *ospC* by an internal segment of lp17. Particularly informative were truncations extending in from the right telomere of lp17, which resulted in the complete absence of OspC in B312 harboring *bbd1-23* (pGCB409) or *bbd1-19* (pGCB426), yet abundant OspC production in B312 containing *bbd1-14* (pGCB473) (Figure 3-2B). These results suggest that the *bbd15-19* segment of lp17 can repress *ospC* expression.

Effect of BBD18 on *ospC* expression

To test this hypothesis directly, the implicated ~2.7kb fragment of lp17 was cloned into the shuttle vector pBSV2* and introduced into B312, in the absence of other lp17 sequences. As predicted, B312 transformants harboring the *bbd15-19* segment of lp17 (pBSV2*-7') no longer synthesize OspC (Figure 3-2C and D). These data indicate that gene(s) or sequences in this region of lp17 either directly or indirectly lead to repression of *ospC*. The lp17 sequences introduced on the shuttle vector were further limited to those encompassing only *bbd18*, the largest open reading frame in this region, with or without a promoter (Figure 3-2C). As shown by immunoblot analysis in Figure 3-2D, expression of *bbd18*, from either its native promoter or the constitutive *flaB* promoter, inversely correlated with *ospC* expression in clone B312. Introduction of the promoterless *bbd18* gene did not diminish *ospC* expression. These data demonstrate that expression of *bbd18* in clone B312 results in repression of *ospC*.

We have demonstrated that the *ospC* promoter-*lacZBb* reporter accurately reflects expression of the native *ospC* gene in B312 (Chapter 2, Figures 2-6, 2-7, and 3-1C and ref (41)). To investigate whether repression of *ospC* by BBD18 is direct or mediated through another *B. burgdorferi* factor, we utilized the *ospC* promoter-*lacZBb* reporter in *E. coli* and introduced constitutively expressed *bdd18* on a compatible plasmid. Conducting this experiment in a heterologous host in the absence of other *Borrelia* factors permits a clearer assessment of the mechanism of *ospC* repression by BBD18. Surprisingly, synthesis of BBD18 did not diminish *ospC* promoter activity in *E. coli*, as monitored by colony color (Figure 3-3A). In a quantitative assay for β -galactosidase activity in liquid cultures (Figure 3-3C), the expression of BBD18 led to lower overall activity units per mg protein. However, this was not specific to the *ospC* promoter, as *lacZ* expressed from the *flaB* promoter was also affected. This is likely just an artifact of how we report β -galactosidase activity since we normalize to total mg protein in the lysate. Regardless, *lacZBb* expression and corresponding β -galactosidase activity from the *ospC* promoter was significantly above activity from the promoter-less *lacZBb* (pBH-*lacZBb*) even when BBD18 was produced (Figure 3-3C), indicating BBD18 is unable to repress *lacZBb* expression from the *ospC* promoter in *E. coli*. While preliminary, these data suggest an indirect mechanism of repression of *ospC* by BBD18 in *B. burgdorferi*.

Discussion

In this study, we have confirmed the hypothesis that the presence of lp17 negatively influences expression of *ospC* in a highly attenuated *B. burgdorferi* clone, B312. By restoring all or part of the lp17 plasmid, we have shown that a ~2.7 kb

Figure 3-3: Assessing BBD18-mediated repression of the *ospC* promoter in *E. coli*.

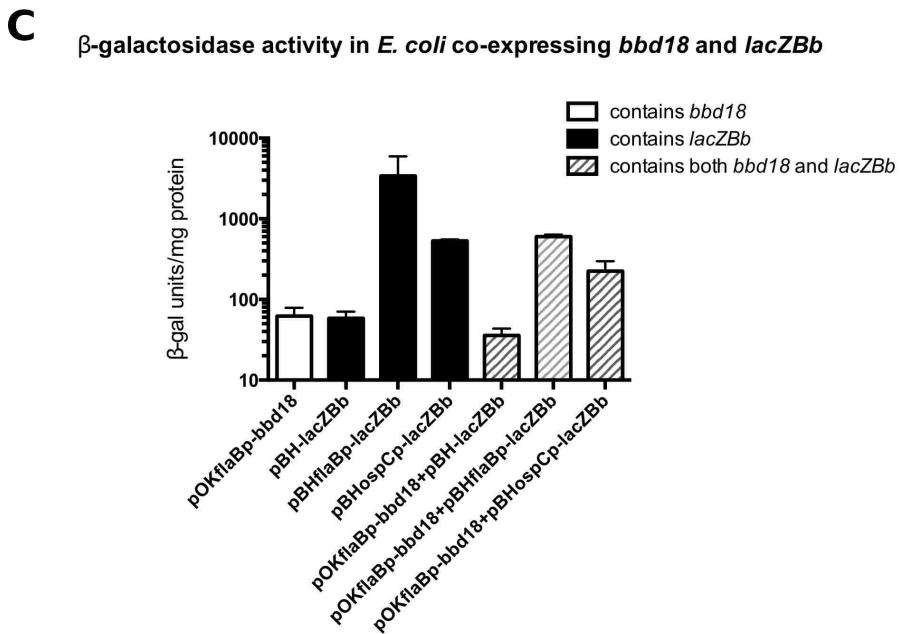
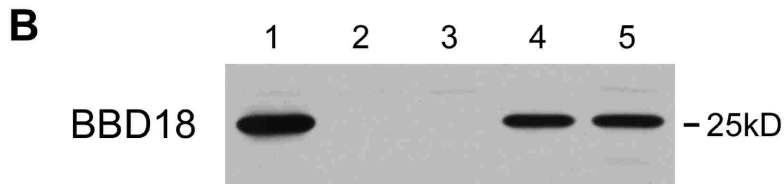
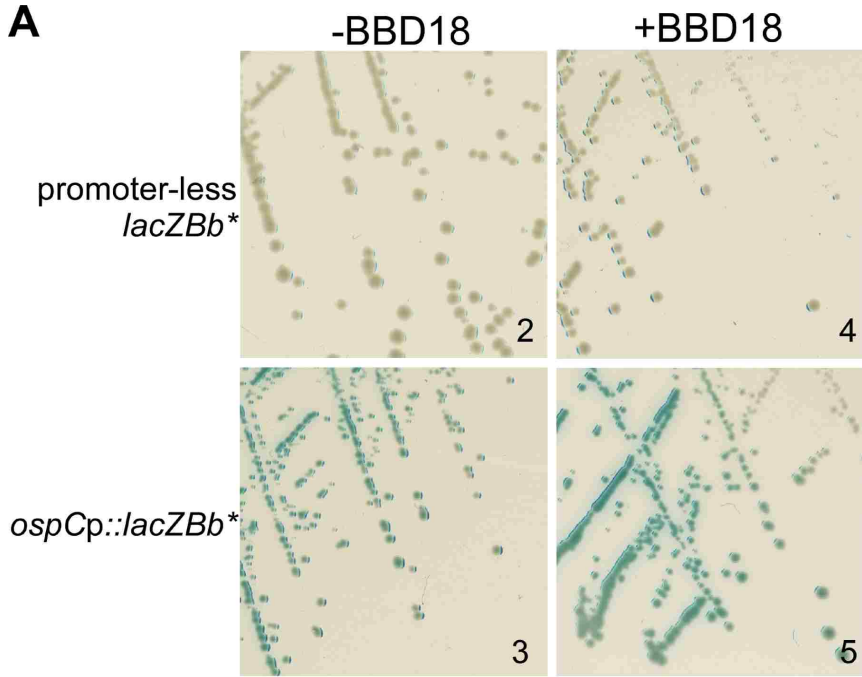


Figure 3-3 legend: **A.** *ospC* promoter-*lacZBb** expression in *E. coli* as monitored by colony color. Reporter constructs carrying *lacZBb**, with or without the *ospC* promoter (as identified at the left side of the figure), were introduced into *E. coli* that harbored or lacked a second plasmid with a constitutively expressed *bbd18* gene (as designated at the top of the figure) and streaked on plates containing X-gal. Expression of *lacZBb** from the *ospC* promoter in the presence or absence of BBD18 resulted in blue bacterial colonies (bottom panels), whereas bacteria carrying the promoter-less *lacZBb** formed white colonies in both conditions (top panels). The number of each panel corresponds with the lane designation in the following immunoblot. **B.** BBD18 immunoblot of *E. coli* harboring plasmids with the following genes: constitutively expressed *flaBp::bbd18* (lane 1); promoter-less *lacZBb** (lane 2); *ospC* promoter-*lacZBb** (lane 3); promoter-less *lacZBb** plus constitutively expressed *flaBp::bbd18* (lane 4); *ospC* promoter-*lacZBb** plus constitutively expressed *flaBp::bbd18* (lane 5). The estimated size of BBD18 relative to the mobilities of standards is indicated to the right of the immunoblot. **C.** Modified Miller ONPG assays (53) were performed on *E. coli* harboring plasmids encoding *bbd18* expressed from the *flaB* promoter and/or *lacZBb* without a promoter or expressed from the *flaB* or *ospC* promoters, as indicated. β -galactosidase activity units (Miller units) are reported per mg protein and levels represent mean \pm SD (n=3). Background activity of bacteria without a plasmid was subtracted from reported values.

region of lp17 is sufficient to repress *ospC* expression in clone B312, where all other linear plasmids but lp54 are missing. Furthermore, by introducing a subset of lp17 sequences from this region on a shuttle vector, we have demonstrated that expression of the *bbd18* gene alone is sufficient to mediate *ospC* repression in clone B312 of *B. burgdorferi*. The protein or RNA product(s) encoded by *bbd18* exerts a negative effect on *ospC* expression, and could act directly, by binding to the *ospC* promoter and blocking transcription, or indirectly, by altering the expression of other genes that regulate *ospC* expression. If working indirectly, BBD18 could either repress an activator or induce a repressor of *ospC* expression. All of these possibilities would result in an OspC-negative phenotype of B312 when BBD18 is present, whereas OspC synthesis would continue in its absence.

Initial results obtained with an *ospC* promoter- *lacZBb* reporter in a heterologous host support an indirect role for BBD18 in regulating *ospC* expression (Figure 3-3). *E. coli* carrying both the *ospC* promoter driving expression of *lacZBb* and a constitutively expressed *bbd18* gene form blue colonies, indicating that the *lacZBb* gene continues to be expressed from the *ospC* promoter even in the presence of BBD18 protein. A caveat to this experiment is the possibility that a *B. burgdorferi* co-factor or modification not present in *E. coli* could be required for BBD18 to bind the *ospC* promoter and block transcription. Alternatively, the amount of BBD18 protein made by *E. coli* (Figure 3-3B) may be insufficient or unavailable for efficient repression of the *ospC* promoter on a multi-copy plasmid. As a working model that accommodates the available data, however, we propose that BBD18 indirectly regulates *ospC* through induction of a repressor that binds to the *ospC* promoter and blocks transcription (Figure 3-4).

Figure 3-4: Proposed model of *ospC* regulation in wild type and B312 strains of *B. burgdorferi*

-mammalian signals

(temp, pH, cell density, ?)

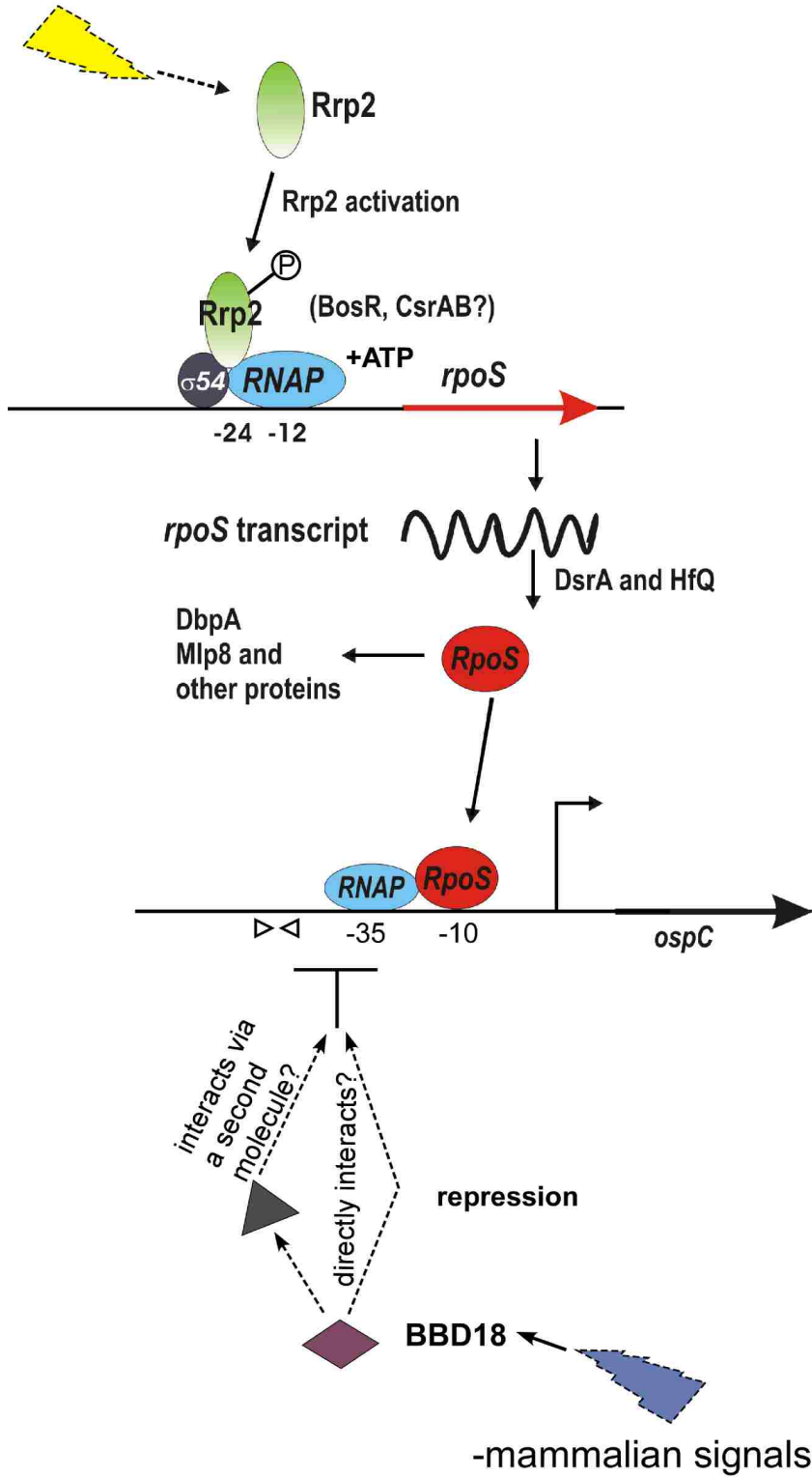


Figure 3-4 legend: Activation of the response regulator Rrp2 in wild type *B. burgdorferi* by environmental signals present during tick feeding leads to *rpoS* transcription through the alternative sigma factor RpoN (σ^{54}), which interacts with RNA polymerase (RNAP) (18, 19). RpoS in turn positively regulates *ospC* transcription, resulting in OspC on the spirochete's outer membrane during a critical initial stage of mammalian infection (8, 18). Persistent infection of mammals by wild type *B. burgdorferi* requires specific repression of *ospC* to evade host acquired immunity (15). Clone B312 constitutively synthesizes OspC in the absence of stimuli typically required for induction in wild type *B. burgdorferi*. Likewise, *bdd18* is constitutively expressed when lp17 is restored to B312, resulting in *ospC* repression. Inverted arrowheads ($\triangleright \triangleleft$) indicate putative operator site upstream of *ospC*. Parts of this figure are closely modeled after a figure by Burtnick *et al.* (54).

During the *in vivo* infectious cycle, synthesis of OspC is induced during tick feeding and present at the initiation of mammalian infection (6), but subsequently downregulated to avoid immune clearance, presumably through a repressor (11-13, 15, 26, 27, 55-57). During *in vitro* growth, most *B. burgdorferi sensu strictu* strains do not synthesize OspC unless culture conditions are altered to simulate tick feeding and the early stage of mammalian infection (6), resulting in induction of the Rrp2/RpoN/RpoS regulatory cascade (18, 19, 58). However, clone B312, which lacks many plasmids, constitutively synthesizes OspC without manipulation of culture conditions, yet has an *ospC* promoter identical to that of wt B31. Two unique features of B312 can be invoked to place the role of BBD18 within the context of our current understanding of *ospC* regulation in wt *B. burgdorferi*. First, RpoS should be induced in B312. Second, the putative *ospC* repressor should be made or activated in B312 when lp17 (*bbd18*) is present. Typically, transient induction of RpoS by shifting the temperature, pH or growth phase of cultures is needed to induce *ospC* expression in infectious B31 clones carrying lp17 (Figure 3-4), indicating that in a wt background, RpoS is made only under certain culture conditions and that the repressor is not made (28, 58). However, a constitutively activated RpoN-RpoS pathway also has been observed in some *ospA/B* mutants (59). We suggest that regulation of *ospC* in wt *B. burgdorferi* reflects a multi-layered network of both conditionally regulated and stochastic elements, as illustrated by the mixed phenotypic response of individual spirochetes in an isogenic *B. burgdorferi* population exposed to the same environmental stimuli (7). Plasmids lost by B312 presumably encode factors that contribute to other levels of *ospC* and *bbd18* regulation, both global and gene-specific. The *rpoS*-induced, *ospC*-repressed state of

B312 carrying lp17 fortuitously mimics the phenotypic state of wild-type *B. burgdorferi* during persistent mammalian infection, which presents a unique opportunity to investigate *ospC* repression *in vitro*.

The *bbd18* locus on lp17 implicated in *ospC* regulation encodes a single open reading frame in the annotated B31 sequence (49, 50). Database searches indicate that *bbd18* is well conserved among diverse *B. burgdorferi* sensu lato strains and that related sequences are present in at least some relapsing fever *Borrelia*, but do not identify any homologs of known or unknown function in other organisms. The predicted BBD18 protein has a fairly high percentage of charged (29%) and aromatic (11%) amino acid residues, comprising a basic protein with a molecular mass of 25,729 daltons and an isoelectric point (pI) of 9.39. Structural predictions suggest a predominantly alpha-helical protein with a few beta-sheets, connected by a number of coiled regions. Although the general characteristics and predicted structure of BBD18 are compatible with DNA interaction, analyses of the BBD18 sequence using Pfam and InterPro databases (60, 61) do not reveal homology with known DNA-binding proteins, DNA-binding domains or transcriptional regulators. Ongoing studies will determine if the entire BBD18 open reading frame is needed for *ospC* repression in *B. burgdorferi* and whether BBD18 can bind DNA in a sequence-specific manner.

In this study, we utilized a recently developed *lacZ* reporter system for *B. burgdorferi* (Chapter 1 and (41)) to demonstrate that *bbd18* regulates *ospC* at the transcriptional level, perhaps indirectly. We are currently using this tool in conjunction with other approaches to delineate which promoter sequences are responsible for BBD18-mediated repression of *ospC* in B312. Subsequent

experiments will determine whether deletion of *bbd18* in wt B31 clones results in continued *ospC* expression *in vivo* and abrogation of persistent *B. burgdorferi* infection, as predicted. Ultimately, this combined *in vitro* and *in vivo* approach should elucidate the molecular mechanisms by which the essential virulence factor OspC is regulated in the mammalian host.

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CHAPTER FOUR

Characterizing the role of BBD18 in the infectious cycle of *Borrelia burgdorferi*

Forward

The majority of the work presented in this chapter was conducted by me. However, this project, which was started by a former fellow in the lab Dr. Amit Sarkar, has become a collaborative effort with Dr. Daniel Dulebohn, a current fellow in the lab, and Figure 4-9 portrays experiments done exclusively by him. The structural prediction alluded to in this chapter was done by Xavier Ambroggio, a Computational Structural Biologist from the Office of Cyberinfrastructure and Computational Biology, NIAID, NIH. I am also extremely grateful to Tom Schwan for assistance with tick dissections and immunofluorescence and Aaron Bestor for training and assistance in conducting mouse and tick experiments.

Abstract

In order to cause infection in mammals, *B. burgdorferi* spirochetes within a feeding tick sense environmental changes and subsequently alter their gene expression and protein profiles. The induction of the Rrp2-RpoN-RpoS cascade is central to this adaptive response and is required for the upregulation of genes important for mammalian infection, including outer surface protein (Osp) C. OspC is required for early infection but is also a target of the host's adaptive immune system, and thus OspC is repressed soon after *B. burgdorferi* establishes infection. Previously, we identified BBD18 as a repressor of OspC in a non-infectious clone of *B. burgdorferi*. Here, we have investigated the role of *bbd18* during the infectious

cycle by constitutively expressing or inactivating *bbd18* in an infectious clone of *B. burgdorferi*. We show that spirochetes harboring a constitutively expressed *bbd18* are non-infectious in mice by needle inoculation and tick bite, presumably because they do not induce OspC. In contrast, *B. burgdorferi* lacking *bbd18* (and other genes on the right end of lp17) can establish infection and persist in the mouse model but show a defect in tissue colonization. Using *in vitro* approaches, we show that the putative *ospC* operator is not required for *ospC* repression by BBD18 and that production of BBD18 affects expression of other RpoS-dependent genes in addition to OspC. Site-directed mutagenesis within a proposed nucleic acid-binding domain in BBD18 abrogates its ability to repress OspC and prevent infection in mice when constitutively expressed. Together these data demonstrate that irregular regulation of BBD18 alters the infectivity of *B. burgdorferi* and suggest that regulation of RpoS-dependent genes by BBD18 might occur through putative BBD18-nucleic acid interactions. We propose that BBD18 is a novel plasmid-encoded player in the control of the RpoS regulon, which is critical for *B. burgdorferi* to establish infection in mammals.

Introduction

The causative agent of Lyme disease, *Borrelia burgdorferi* (1-3), is maintained in an enzoonotic cycle involving *Ixodes* ticks and mammalian hosts (4). Persistence in this cycle requires *B. burgdorferi* to sense environmental changes and alter its gene expression and protein profiles. A key example of this adaptation occurs when infected ticks take in a blood meal and spirochetes in the tick midgut upregulate Outer surface protein (Osp) C, which prepares *B. burgdorferi* for

transmission to the mammalian host (5-7). Our laboratory previously showed that *B. burgdorferi* mutants lacking a functional OspC are non-infectious by tick bite (8-11). However, OspC is also a target for neutralizing antibodies and *ospC* expression is downregulated after *B. burgdorferi* establishes infection in order to avoid clearance by the host's adaptive immune response (12-15).

Spirochetes within a feeding tick experience a change in temperature, pH, and other host factors, which leads to induction of the RpoN-RpoS regulatory pathway (16-19). This cascade is responsible for the upregulation of genes associated with mammalian infection, including *ospC* (20-25). However, while *ospC* is repressed to avoid immune pressure, other RpoS-induced genes continue to be expressed throughout infection (26, 27). These observations lead to the hypothesis that a specific mechanism is required to repress *ospC* (12).

Previous work by Xu *et al.* showed that an inverted repeat sequence or putative operator, upstream of the *ospC* promoter, was necessary for *in vivo* repression of *ospC*. *B. burgdorferi* mutants lacking this palindromic sequence successfully established infection in mice but subsequently were cleared by anti-OspC neutralizing antibodies, presumably because the spirochetes could not downregulate *ospC* expression (28, 29). More recently, our lab described a novel gene on lp17, *bbd18*, that when expressed led to the downregulation of *ospC* at the transcriptional level (30). Because our previous work with BBD18 was conducted in the non-infectious *B. burgdorferi* clone B312, we wanted to confirm that BBD18 can repress OspC in a wild-type (wt) clone and investigate the role of BBD18 in the infectious cycle. More specifically, we were interested in whether BBD18 is the

invoked *in vivo* repressor that binds to the palindromic sequence described by Xu *et al.* (28).

During the course of these studies, Casselli *et al.* demonstrated that *B. burgdorferi* clones carrying a truncated version of lp17 lacking a 6.7kB segment that encompasses *bbd18* were able to establish infection in mice, but had delayed tissue colonization. They also reported that these clones, which lack *bbd18*, had increased OspC production and that restoration of *bbd18* reversed this *in vitro* phenotype (31). These results are not wholly inconsistent with our hypothesis that BBD18 is responsible for repression of *ospC* during the infectious cycle. Here, we have taken a different approach and determined the effect of a constitutively expressed *bbd18* on mammalian infectivity and tick transmission. We have also used the *lacZ* expression system in *B. burgdorferi* (32), as well as other direct and indirect techniques, to further investigate the mechanism of *ospC* repression by BBD18.

Material and Methods

All chemicals and materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Bacterial Strains and Growth Conditions.

B. burgdorferi strains (Table 4-1) were inoculated from frozen stocks into Barbour-Stoener-Kelly (BSK II) medium (33) with or without phenol red (32). *B. burgdorferi* cultures were generally grown at 35°C and plates were incubated under 2.5% CO₂. Spirochetes were enumerated using dark-field microscopy and a Petroff-Hausser counting chamber and prepared for electroporation as described previously (34). Competent *B. burgdorferi* was transformed with ~10µg plasmid

Table 4-1 Strains and Plasmids Used in Chapter Four

strain or plasmid	Description	Reference
<i>B. burgdorferi</i> strains		
B31-S9 (S9)	infectious, <i>bbe02</i> mutant; derivative of B31-A3-68; streptomycin resistant	(35)
S9/pBSV2*- <i>flaBp</i> - <i>bbd18</i> -A	B31-S9 with <i>bbd18</i> under control of <i>flaB</i> promoter on shuttle vector, lacks cp32-1 and lp28-4; kanamycin and streptomycin resistant	this study
S9/chase-pBSV2G	derivative of B31-S9/pBSV2*- <i>flaBp</i> - <i>bbd18</i> -A in which pBSV2G was used to displace pBSV2*- <i>flaBp</i> - <i>bbd18</i> ; gentamicin and streptomycin resistant	this study
S9/pBSV2*- <i>flaBp</i> - <i>bbd18</i> -B	independent clone of B31-S9 with <i>bbd18</i> under control of <i>flaB</i> promoter on shuttle vector, retains full plasmid content of B31-S9; kanamycin and streptomycin resistant	this study
B31-A/lp17::kan	B31-A derivative carrying pKK81 integration on lp17; kanamycin resistant	(36)
S9Δ <i>bbd18</i>	S9 derivative in which the <i>bbd18</i> ORF is disrupted with <i>flgBp</i> - <i>aacC1</i> through allelic exchange with pOK12- <i>bbd18</i> -KO; streptomycin and gentamicin resistant	this study
S9Δ <i>bbd18</i> /lp17::kan	S9Δ <i>bbd18</i> complemented with lp17::kan; streptomycin and kanamycin resistant	this study
S9Δ <i>bbd18</i> /pBSV2*NP- <i>bbd18</i>	S9Δ <i>bbd18</i> complemented with <i>bbd18</i> expressed from its native promoter on a shuttle vector; streptomycin, gentamicin, and kanamycin resistant	this study
S9/pGCB426	S9 derivative harboring the lp17 truncation, pGCB426 (<i>bbd1</i> -20); kanamycin resistant	this study
S9/pGCB473	S9 derivative harboring the lp17 truncation, pGCB473 (<i>bbd1</i> -14); kanamycin resistant	this study
B31-A3 (A3)	Infectious clonal derivative of wild-type B31-MI, lacks cp9	(37)
A3Δ <i>rpoS</i>	<i>rpoS</i> mutant, derivative of B31-A3; kanamycin resistant	(37)
B312	high-passage non-infectious B31 clone, synthesizes OspC <i>in vitro</i> without manipulation	(30, 38)
B312/pBHospCp- <i>lacZBb</i> *	B312 derivative harboring <i>lacZBb</i> * on shuttle vector under control of <i>ospC</i> promoter; gentamicin resistant	(30)

B312/pBHP7F-lacZBb*	B312 derivative harboring <i>lacZBb*</i> on shuttle vector under control of a minimal <i>ospC</i> promoter lacking the palindromic operator sequence; gentamicin resistant	this study
B312/pBHospCp-flaBpRBS-lacZBb*	B312 derivative harboring <i>lacZBb*</i> under the control of an <i>ospC-flaB</i> fusion promoter (-35 up to +1 of <i>ospC</i> promoter fused with +1 to ATG of <i>flaB</i> promoter) on shuttle vector; gentamicin resistant	this study
B312/pBSV28-1-flaBp-bbd18	B312 derivative harboring <i>bbd18</i> under control of <i>flaB</i> promoter on shuttle vector; kanamycin resistant	this study
B312/pBH-ospCp-lacZBb*/pBSV28-1-flaBp-bbd18	B312 derivative with <i>lacZBb*</i> under control of <i>ospC</i> promoter and <i>bbd18</i> under control of <i>flaB</i> promoter on compatible shuttle vectors; kanamycin and gentamicin resistant	this study
B312/pBH-P7F-lacZBb*/pBSV28-1-flaBp-bbd18	B312 derivative with <i>lacZBb*</i> under control of minimal <i>ospC</i> promoter that lacks the palindromic operator sequence and <i>bbd18</i> under control of <i>flaB</i> promoter on compatible shuttle vectors; kanamycin and gentamicin resistant	this study
B312/pBHospCp-flaBpRBS-lacZBb*/pBSV28-1-flaBp-bbd18	B312 derivative harboring <i>lacZBb*</i> under the control of an <i>ospC-flaB</i> fusion promoter (-35 up to +1 of <i>ospC</i> promoter fused with +1 to ATG of <i>flaB</i> promoter) and <i>bbd18</i> under control of <i>flaB</i> promoter on compatible shuttle vectors; kanamycin and gentamicin resistant	this study
B312/pBSV2G-flaBp-recA	B312 derivative with <i>recA</i> under the control of the <i>flaB</i> promoter on a shuttle vector; gentamicin resistant	this study
B312 Δ <i>rpoS</i>	<i>rpoS</i> mutant in B312/pBSV2G-flaBp-recA; kanamycin and gentamicin resistant	(37); this study
B312/pBSV2*flaBp-bbd18	B312 derivative harboring wt <i>bbd18</i> under the control of the constitutive <i>flaB</i> promoter on a shuttle vector; kanamycin-resistant	(30)
B312/pBSV2*flaBp-bbd18 Δ 1	B312 derivative harboring a <i>bbd18</i> variant (K58A, S67A, C74A, K79A, D94A, and D96A) under the control of the constitutive <i>flaB</i> promoter on a shuttle vector; kanamycin-resistant	this study

B312/pBSV2*flaBp-bbd18Δ2	B312 derivative harboring a <i>bbd18</i> variant (N56A, I57A, K58A, S66A, S67A, N69A, I70A, and M72A) under the control of the constitutive <i>flaB</i> promoter on a shuttle vector; kanamycin-resistant	this study
B312/pBSV2*flaBp-bbd18Δ3	B312 derivative harboring a <i>bbd18</i> variant (K58E, K79E, D94R, and D96R) under the control of the constitutive <i>flaB</i> promoter on a shuttle vector; kanamycin-resistant	this study
B312/pBSV2*flaBp-bbd18Δ4	B312 derivative harboring a <i>bbd18</i> variant (S67A and V85A) under the control of the constitutive <i>flaB</i> promoter on a shuttle vector; kanamycin-resistant	this study
S9/pBSV2*-flaBp-bbd18Δ3	S9 derivative harboring a <i>bbd18</i> variant (K58E, K79E, D94R, and D96R) under the control of the constitutive <i>flaB</i> promoter on a shuttle vector; streptomycin and kanamycin-resistant	this study
S9/pBSV2*-flaBp-bbd18Δ4	S9 derivative harboring a <i>bbd18</i> variant (S67A and V85A) under the control of the constitutive <i>flaB</i> promoter on a shuttle vector; streptomycin and kanamycin-resistant	this study
Plasmids		
pBSV2*	derivative of the cp9-based shuttle vector, pBSV2, single EcoRI recognition site, kanamycin-resistance cassette	(39)
pBSV2G	derivative of the cp9-based shuttle vector, pBSV2, gentamicin-resistance cassette, incompatible with pBSV2*	(40)
pBSV2*flaBp-bbd18	pBSV2* with <i>bbd18</i> under control of constitutive <i>flaB</i> promoter	(30)
pBHospCp-lacZBb*	pBH-lacZBb* with <i>ospC</i> promoter driving expression of <i>lacZBb</i>	(30)
pBHP7F-lacZBb*	pBH-lacZBb* with minimal <i>ospC</i> promoter that lacks the palindromic operator sequence driving expression of <i>lacZBb</i>	this study
pBHospCp-flaBpRBS-lacZBb*	pBH-lacZBb* with an <i>ospC-flaB</i> fusion promoter (-35 up to +1 of <i>ospC</i> transcript fused with +1 to ATG of <i>flaB</i>) driving expression of <i>lacZBb</i> *	this study
pBSV28-1-flaBp-bbd18	pBSV28-1 with <i>bbd18</i> under control of the constitutive <i>flaB</i> promoter	(41); this study
pBSV2G-flaBp-recA	pBSV2G with <i>recA</i> under the control of the constitutive <i>flaB</i> promoter	Frank Gherardini unpublished; this study

pBSV2*NP-bbd18	pBSV2* with an insert encompassing <i>bbd18</i> and flanking sequences (nt. 10881-12056) of lp17 (native promoter)	(30)
pBSV2*flaBp-bbd18Δ1	pBSV2* with a synthetic <i>bbd18</i> gene containing amino acid changes K58A, S67A, C74A, K79A, D94A, and D96A; expressed from the constitutive <i>flaB</i> promoter	this study
pBSV2*flaBp-bbd18Δ2	pBSV2* with a synthetic <i>bbd18</i> gene containing amino acid changes N56A, I57A, K58A, S66A, S67A, N69A, I70A, and M72A; expressed from the constitutive <i>flaB</i> promoter	this study
pBSV2*flaBp-bbd18Δ3	pBSV2* with a synthetic <i>bbd18</i> gene containing amino acid changes K58E, K79E, D94R, and D96R; expressed from the constitutive <i>flaB</i> promoter	this study
pBSV2*flaBp-bbd18Δ4	pBSV2* with a synthetic <i>bbd18</i> gene containing amino acid changes S67A and V85A; expressed from the constitutive <i>flaB</i> promoter	this study
pOK12-bbd18-KO	suicide vector for allelic exchange with the <i>bbd18</i> ORF; <i>bbd18</i> and flanking region in pOK12; <i>flgBp-aacC1</i> inserted into the <i>bbd18</i> ORF	(42); this study
pGCB426	lp17 deletion variant from <i>B. burgdorferi</i> strain GCB426 carrying <i>bbd1</i> through 5' end of <i>bbd20</i>	(30, 43)
pGCB473	lp17 deletion variant from <i>B. burgdorferi</i> strain GCB473 carrying <i>bbd1</i> through <i>bbd14</i>	(30, 43)

DNA (see below and Table 4-1) purified from *E. coli*. Transformations of B312 and B31-S9 (subsequently referred to as S9) were selected in solid BSK II medium with the appropriate antibiotics: gentamicin (40µg/mL), kanamycin (200µg/mL), and/or streptomycin (50 µg/ml). Colonies arising in selective media were screened by PCR for *lacZBb*, *bbd18*, the gentamicin marker or the kanamycin marker with appropriate primers (Table 4-2) to confirm the stable introduction of plasmid DNA.

Routine cloning was performed using chemically competent Top10 *Escherichia coli* (Invitrogen, Carlsbad, CA). *E. coli* was plated on LB agar (10g/L Tryptone, 5g/L NaCl, 5g/L yeast extract, and 15g/L agar) with the appropriate antibiotic (kanamycin at 30µg mL⁻¹ or gentamicin at 5 µg mL⁻¹). Liquid cultures were grown in LB broth supplemented with the appropriate antibiotics (kanamycin at 100µg mL⁻¹ or gentamicin at 10 µg mL⁻¹)

Plasmid construction.

A minimal *ospC* promoter, lacking the inverted repeat (putative operator) sequence, was amplified from B31-A3 (A3) genomic DNA (gDNA) using primers P7F (28) and *ospC* promoter-BspHI Reverse (Table 4-2), cloned into pCR2.1 (Invitrogen), and sequenced to confirm insert. This promoter was excised with the restriction enzymes BamHI and BspHI (New England Biolabs, NEB, Ipswich, MA) and ligated into appropriately digested pBH-lacZBb* (30), creating pBHP7F-lacZBb* (Table 4-1). An *ospC-flaB* fusion promoter was amplified from A3 gDNA using primers *ospC*-35region Forward and *ospCp-flaBp*-RBS Reverse, which encodes the 5' untranslated region of *flaB* in the primer sequence (Table 4-2). This fusion promoter was cloned into pCR2.1 (Invitrogen), sequenced, and recloned into pBH-lacZBb* as described above, creating pBHospCp-flaBpRBS-lacZBb* (Table 4-1).

Table 4-2: Primers used in Chapter Four

Name	Sequence (5'-3') ^a	Reference
ospCp Forward	CCCGCGGATCCAATTTAAACTTTTTTTATTAAAGTA	(30, 32)
P7F	CACTGGATCCTTATTTTTTCAAATAAAAAATTGA	(28)
ospC promoter-BspHI Reverse	CCCCGTCATGATTTGTGCCTCCTTTTTATTAT	(30)
ospC-35region Forward	CCCGCGGATCCTTGAAAAACAAAATTGTTGGACT	this study
ospCp-flaBp-RBS Reverse	CCCCCTCATGAATCATTCTCCATGATAAAATTTAAATTTCT GACTTTGGCAAATCCTTTTGCCTTTATGAATTATTAGTCCAA CAATTTT	this study
<i>lacZBb</i> Int-1	TGGTGTAATGGAAGATGGGTTGG	(30)
<i>lacZBb</i> Mid Reverse	GATAGACCATTTAGGAACAGCAGG	(30)
kan 5' NdeI Forward	CATATGAGCCATATTCAACGGGAAACG	(30)
kan Reverse	TTAATTAATGAGCTAGCGCCGT	(30)
gent Forward	TCTCGGCTTGAACGAATTGTTACGT	(35)
gent Reverse	GGCAGTCGCCCTAAAACAAAGTT	(35)
<i>bbd13</i> Forward	ACGGTGTAAGACTTTATTCTTTAAAAGAATTCC	(44)
<i>bbd13</i> reverse	AAAATATGACCCATCTTTTTTGAAGC	(44)
<i>bbd18</i> -NdeI-For	GGGAATTCCATATGCAAAAAGAAATAACAATAAACTATAAT G	this study
<i>bbd18</i> -XmaI-Rev	TCCCCCGGGCATTTTACTTAAAAGCGTTTAATTTT	this study
<i>bbd18</i> -promoter Forward	CGCGGATCCAGAATTTACTTACAATATTTAACC	this study
<i>bbd18</i> -terminator Reverse	TTCCGCGGCCGCTATGGCCGACGTCGACAATCTTAAAGCATT GTTAAAATTTTATATTC	this study

^a underlined bases denote restriction sites

The *flaBp-bbd18* cassette was excised from pBSV2**flaBp-bbd18* (30) with NotI-HF (NEB) and ligated into appropriately digested and de-phosphorylated pBSV28-1 (41) to create pBSV28-1-*flaBp-bbd18* (Table 4-1).

Suicide vector construction

To inactivate *bbd18*, an allelic exchange vector was constructed as follows. The region of lp17 containing *bbd18* and its flanking regions (lp17 nt 12053-10684) was amplified by PCR using primers *bbd18*-promoter Forward and *bbd18* terminator Reverse (Table 4-2) and cloned into pCR2.1. This region was subsequently recloned into pOK12 to avoid introducing the ampicillin-resistance gene into *B. burgdorferi*. The *flgB* promoter driving expression of the *aacC1* gene (conferring gentamicin resistance) was amplified from pBSV2G and cloned into pCR2.1. The *flgBp-aacC1* cassette was excised out of pCR2.1 using HindIII, ends blunted with T4 DNA polymerase (NEB) and ligated into the unique MfeI site that is 32bp downstream of the ATG-start site of the *bbd18* open reading frame in pOK12. One clone, named pOK12-*bbd18*-KO (Table 4-1), in which the *flgBp-aacC1* cassette was inserted in the opposite orientation of the native *bbd18* gene, was chosen to inactivate *bbd18* in *B. burgdorferi*.

β -galactosidase assays.

To assess β -galactosidase activity of colonies grown in solid media, ~0.3mL 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-gal) dissolved in dimethylformamide (DMF) (10mg/ml) was spread on *B. burgdorferi* plates after colony formation.

SDS-PAGE and immunoblotting

Lysates from high density or temperature-shifted cultures, to enhance

induction of the RpoN/RroS regulon, were analyzed for protein content. For temperature shifts, duplicate 50mL *B. burgdorferi* cultures were first grown at 35°C. When the concentration reached approximately 5×10^5 - 1×10^6 spirochetes/mL, cultures were shifted to 25°C (i.e. room-temperature). 7-10 days later, one of the duplicate cultures was returned to 35°C for 48 hours. Equal numbers of spirochetes were harvested from both culture conditions. All samples were washed twice in HEPES-NaCl (HN) buffer (50mM each, pH 7.6) and resuspended in Laemmli loading buffer to be separated on 12.5% SDS-PAGE gels. Gels were run in duplicate and either stained with Coomassie blue or electrophoretically transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in TBS-Tween 20 (20mM TRIS pH 7.5, 150mM NaCl, 0.01% Tween 20) with or without nonfat milk (BD Diagnostics, Franklin Lakes, NJ) and probed with mouse monoclonal anti-FlaB H9724 (1:200) (45), rabbit polyclonal anti-OspC antiserum (1:1000 dilution) (10), rabbit anti-BBD18 antiserum (30), chicken polyclonal anti-BBA66 antibody (1:6,000)(46), rabbit polyclonal anti-DbpA antibody 200-401-B98S (1:1000 dilution; Rockland Immunochemicals, Gilbertsville, PA), or UGA-17 rabbit polyclonal anti-RpoS antibody (1:500)(a kind gift from F. Gherardini). Horseradish peroxidase-conjugated secondary antibodies that recognize mouse, rabbit, or chicken immunoglobulins were diluted 1:10,000, 1:50,000, or 1:10,000, respectively, in TBST with non-fat milk. Immunoreactivity was visualized using the Supersignal West Pico Chemiluminescence substrate (Thermo Scientific, Rockford, IL) and X-ray film (Labsscientific Inc., Livingston NJ).

Mouse Infection

All mouse studies were done in accordance with guidelines of the National

Institutes of Health and protocols were approved by the Rocky Mountain Laboratories Animal Care and Use Committee. The Rocky Mountain Laboratories are accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Infection studies were conducted with 6- to 8-week-old female RML mice, an outbred strain of Swiss-Webster mice reared at the Rocky Mountain Laboratories breeding facility. Mice were retro-orbitally bled prior to injection and then needle-inoculated with a target dose of 10^4 *B. burgdorferi* spirochetes (8×10^3 spirochetes intraperitoneally and 2×10^3 spirochetes subcutaneously). After 3 weeks, mice were bled to assess seroconversion to *B. burgdorferi* proteins, and ear punch biopsies placed into BSK II medium to assess the presence of spirochetes in these tissues. At 4-8 weeks post-inoculation, mice were euthanized and ear, bladder, and joint tissues were cultured in BSK II medium for final assessment of infection.

Tick Transmission Studies

Ixodes scapularis larvae were artificially infected as previously described (47) Briefly, larval *I. scapularis* were partially dehydrated for 48 hours at a relative humidity of 85% before an aliquot of *B. burgdorferi* culture ($\sim 1 \times 10^8$ spirochetes/mL) was added. The ticks were incubated with *B. burgdorferi* for 45 min at 35°C, washed twice with PBS, and then allowed to recover for 48 hours before feeding on naïve mice to repletion (~ 100 /mouse). To assess colonization by *B. burgdorferi*, a subset of fed larvae were crushed at 7 days post-feeding and either plated in solid BSK medium or placed in liquid BSK medium. Remaining fed *I. scapularis* larvae were allowed to molt to nymphs and recover (approximately 12 weeks post-larval feeding) before feeding on naïve mice (2-4 nymphs/mouse). Two

or three *I. scapularis* nymphs were removed at 48 hours post-attachment and prepared for immunofluorescence (see below). Remaining attached nymphs (1-2/mouse) were allowed to feed to repletion and at 7 days post-feeding were crushed and plated in BSK solid medium to enumerate spirochetes. Mice that were fed on by infected *I. scapularis* were euthanized at 3 weeks post-feeding and processed as described above to assess *B. burgdorferi* transmission via tick bite and ensuing infection.

Fluorescence Microscopy

Manually detached *I. scapularis* nymphs were dissected in phosphate buffered saline (PBS) on glass microscope slides. Tick smears were allowed to air dry before being passed over a flame to heat-fix. Samples were then permeabilized in acetone for 30 minutes. Mouse monoclonal antibody B5 recognizing OspC of *B. burgdorferi* B31 (kindly provided by Dr. Robert Gilmore and originally produced by Dr. Lamine MBow, (48)) was used to determine if spirochetes were producing OspC, and a hyperimmune convalescent rabbit anti-*B. burgdorferi* antiserum (Drs. Robert Karstens and Tom Schwan, RML, NIAID, NIH) was used to detect all spirochetes regardless of their OspC phenotype. Primary antibodies were detected using rhodamine-conjugated goat anti-rabbit (*B. burgdorferi*) and Alexa-488 labeled goat anti-mouse (OspC) sera. All antibodies were diluted 1:100 in PBS with 0.75% Bovine Serum Albumin (BSA) and incubated at 35°C for 30 minutes. Vectashield mounting medium (Vector Laboratories, Burlingame, CA) was used to prevent the photobleaching of fluorochromes. Images were taken with a Nikon Eclipse 80i microscope and a Nikon Digital Sight Qi1Mc camera.

Southern Blotting

Genomic DNA was isolated from *B. burgdorferi* cultures using the Promega Wizard Genomic DNA purification kit (Promega, Madison, WI). Approximately 275ng of uncut gDNA was separated on a 0.5 % agarose gel by field-inversion electrophoresis with program 2 on the PPI-200 Pulse-Field Gel Controller (MJ Research, Watertown, MA) The gel was depurinated, denatured, neutralized, and blotted onto a Magnagraph nylon transfer membrane (GE, Boulder, CO). gDNA was cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene, Los Angeles, CA). Probes were generated by PCR amplification of *B. burgdorferi* B31-A3 gDNA with *bbd13*, *aacC1*, or the kanamycin-resistant gene primers (Table 4-2) and a DIG probe synthesis kit (Roche, Indianapolis, IN), according to the manufacturer's instructions. Hybridization of the labeled probe with the membrane-bound DNA and signal development were done as described before (Chapter 2, (30)).

Site-directed Mutagenesis of BBD18

Based on structural predictions (see below) and amino acid conservation within both Lyme disease spirochetes and relapsing fever spirochetes (unpublished sequences, Dr. Tom Schwan, RML, NIAID, NIH), we designed four *bbd18* variants, each with multiple amino acid substitutions. The changes are as follows: *bbd18-Δ1* has lysine 58 changed to alanine (K58A), S67A, C74A, K79A, D94A, and D96A; *bbd18-Δ2* has N56A, I57A, K58A, S66A, S67A, N69A, I70A, and M72A; *bbd18-Δ3* has K58E, K79E, D94R, and D96R; *bbd18-Δ4* has S67A, and V85A. These BBD18 variants had similar predicted secondary structures as wt BBD18 (PSIPRED Protein Structure Prediction Server; available at <http://bioinf.cs.ucl.ac.uk/psipred/>). Synthetic genes with the specific nucleotide changes were synthesized by GenScript Corporation (Piscataway, NJ) with EcoRI recognition sites at both ends. These

synthetic genes were excised from pUC57 with EcoRI-HF (NEB) and ligated into appropriately digested and de-phosphorylated pBSV2*flaBp (39) in a manner following the construction of pBSV2*flaBp-bbd18, which contains the wt *bbd18* gene under expression of the constitutive *flaB* promoter (30). Resulting clones were screened by PCR to confirm orientation and presence of the insert and then sequenced to confirm that no other mutations had arisen during cloning. These shuttle vectors were transformed into B312 and assessed for their ability to repress OspC by immunoblot. pBSV2*flaBp-bbd18 Δ 3 and pBSV2*flaBp-bbd18 Δ 4 were also transformed into S9.

Single (or double) amino acid *bbd18* variants were created using the Quikchange Lightning Single or Multi Site-directed mutagenesis kits (Agilent, Santa Clara, CA) and pBSV2*flaBp-bbd18, according to the manufacturer's instructions. Primers were designed with the primer design tool available on their website (<https://www.genomics.agilent.com>). We created the following single residue variants: K58E, K79E, D94R, D96R; and the following double residue variants: K58E/D94R, K79E/D96R, and D103R/K113E (control). Resulting clones were sequenced to confirm that only the specified mutations had been introduced. The shuttle vectors were subsequently transformed into B312 and assessed for the ability to repress OspC by immunoblotting as above.

Complementation of the *bbd18* mutant

To complement the *bbd18* mutant, lp17 harboring the *flgBp-aacC1* insertion in the *bbd18* open reading frame was displaced with an lp17 that was marked with a kanamycin-resistance cassette. This lp17::kan (Table 4-1) was created by the intergration of pKK81 into lp17 in B31-A (36) (30). pBSV2*NP-bbd18 (Table 4-

1)(30), which harbors a wt *bbd18* gene expressed from its native promoter, was also used to transform the *bbd18* mutant. Both complemented clones retained the same plasmid content as S9- Δ *bbd18* and the parental S9.

Results

***B. burgdorferi* constitutively expressing *bbd18* is non-infectious in the mouse model.**

The presence of OspC on the surface of *B. burgdorferi* not only prepares spirochetes for mammalian infection but is also a target for the mammalian immune system. Thus, OspC must be specifically downregulated during infection to prevent clearance of the spirochete (12). As described in chapter 3, we found that BBD18, a novel protein encoded on lp17, caused repression of *ospC* at the transcriptional level in the non-infectious high-passage *B. burgdorferi* strain B312 (30).

To address whether BBD18 is important during the infectious cycle, we wanted to constitutively express *bbd18* in an infectious *B. burgdorferi* clone and determine whether that interferes with infection or transmission. We hypothesized that if *bbd18* expressed by a constitutive promoter represses *ospC*, which is an essential virulence factor for infection in mice, a low-passage *B. burgdorferi* clone constitutively expressing *bbd18* would be unable to upregulate OspC and therefore would be non-infectious in the mouse model. To achieve this, we utilized low-passage S9, a virulent clone of *B. burgdorferi* that lacks two restriction modification systems that have been shown to hinder efficient shuttle vector transformations into *B. burgdorferi* (35, 49, 50). S9 was transformed with pBSV2**flaBp*-*bbd18* (30), a shuttle vector harboring *bbd18* expressed from the constitutive *flaB* promoter.

Independent transformations yielded in two transformants, S9/pBSV2*flaBp-bbd18-A and S9/pBSV2*flaBp-bbd18-B. When the complete plasmid profile was analyzed, we found that S9/pBSV2*flaBp-bbd18-B retained all parental plasmids, while S9/pBSV2*flaBp-bbd18-A had lost lp28-4 and cp32-1; the loss of either of these plasmids has not been associated with a loss of virulence (a representative image of the *B. burgdorferi* plasmid profile is available in Figure 4-4C). To our knowledge, however, infectivity of *B. burgdorferi* lacking both cp32-1 and lp28-4 has not been tested. To confirm that any observed differences from wt S9 were caused by constitutive expression of *bbd18*, we displaced pBSV2*flaBp-bbd18 from S9/pBSV2*flaBp-bbd18-A with the incompatible plasmid pBSV2G. Southern blot analysis with probes specific for the gentamicin- (pBSV2G) or kanamycin- (pBSV2*flaBp-bbd18) resistance genes was used to confirm the presence of pBSV2G and absence of pBSV2*flaBp-bbd18 (Figure 4-1). This clone, referred to as S9/chase-pBSV2G, retained the same plasmid profile as the parent strain (S9/pBSV2*flaBp-bbd18-A) yet lacked constitutive *bbd18*.

Groups of 5 mice were injected with S9, S9/pBSV2*flaBp-bbd18-A, S9/chase-pBSV2G, or S9/pBSV2*flaBp-bbd18-B. After 3 weeks, mice inoculated with S9 or S9/chase-pBSV2G were seropositive for *B. burgdorferi* proteins, but mice inoculated with S9/pBSV2*flaBp-bbd18-A or S9/pBSV2*flaBp-bbd18-B did not seroconvert (Table 4-3). In addition, spirochetes were isolated from tissues (ear, bladder, and ankle joint) of mice inoculated with S9 or S9/chase-pBSV2G but not from mice inoculated with S9/pBSV2*flaBp-bbd18-A or -B (Table 4-3). These results demonstrate that constitutively expressed *bbd18* renders *B. burgdorferi* non-infectious by needle inoculation. Restoration of infectivity in S9/chase-pBSV2G also

Figure 4-1: S9 with pBSV2*flaBp-bbd18 or pBSV2G

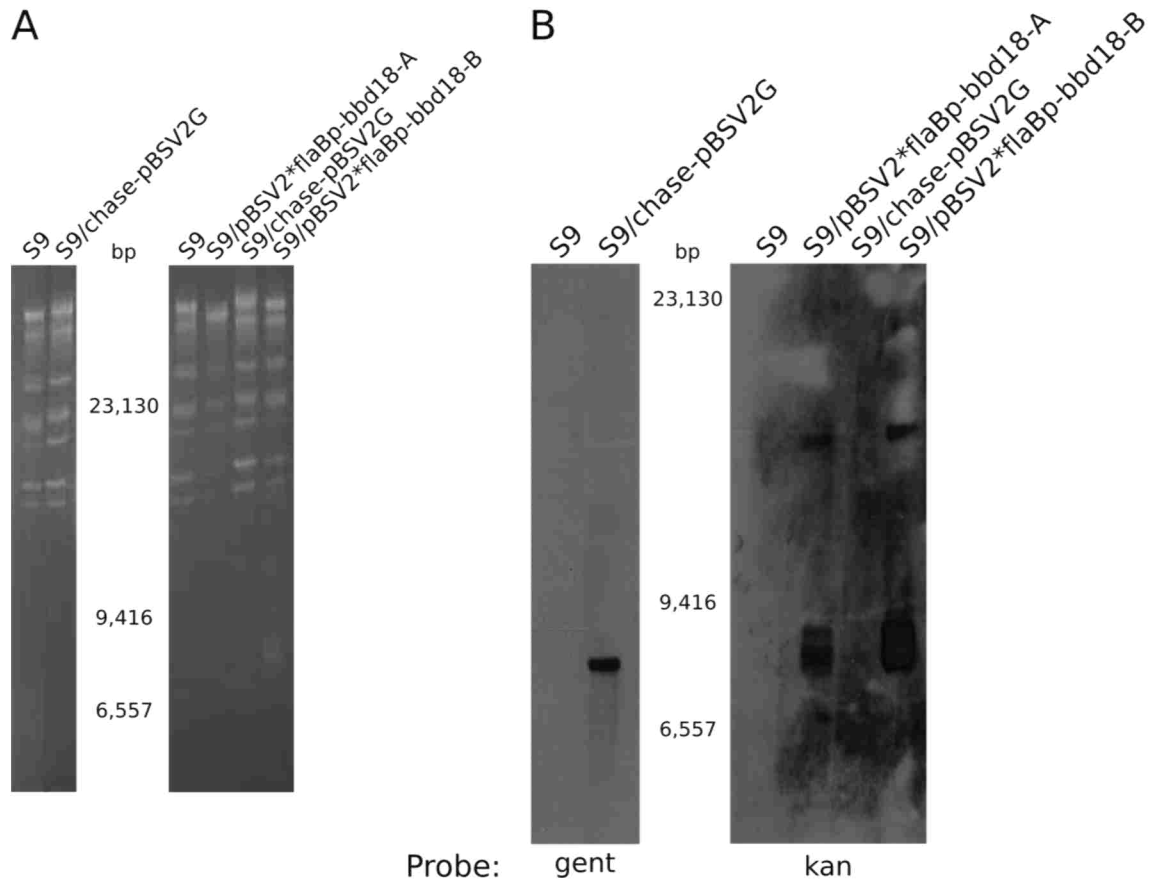


Figure 4-1 legend: **A.** gDNA from spirochetes harboring a constitutive *bbd18* separated by phase-inversion gel electrophoresis. Uncut gDNA, isolated from wt S9, S9/pBSV2*flaBp-bbd18-A, S9/chase-pBSV2G, or S9/pBSV2*flaBp-bbd18-B, as indicated, was analyzed by gel electrophoresis and visualized using Gel Red and ultra violet light. The size in base pairs (bp) based on the migration of standards is listed in between the panels. **B.** gDNA hybridization with probes recognizing the gentamicin- (gent, left) or kanamycin- (kan, right) resistance genes. Nylon membrane-bound gDNA transferred from gels shown in A was incubated with a DIG-labeled probe corresponding to gent or kan resistance cassettes, which are carried by pBSV2G or pBSV2*flaBp-bbd18, respectively. Probe hybridization was visualized using an anti-DIG antibody, chemiluminescent substrates, and X-ray film. The size in base pairs (bp) based on the migration of standards is listed between the two panels.

Table 4-3: Constitutive expression of BBD18 prevents *B. burgdorferi* infectivity in mice by needle inoculation

strain	No. of sero-positive mice /no. of injected mice ^a	No. of tissue reisolates/no. of injected mice (ear, bladder, joint) ^b
S9	5/5	5/5, 5/5, 5/5
S9/pBSV2*flaBp-bbd18-A	0/5	0/5, 0/5, 0/5
S9/chase-pBSV2G	4/5	4/5, 4/5, 4/5
S9/pBSV2*flaBp-bbd18-B	0/5	0/5, 0/5, 0/5

^a seroconversion to *B. burgdorferi* proteins was assessed at 3 weeks post-inoculation

^b mice were euthanized at 5 weeks post-inoculation

corroborated these findings and indicated that the inappropriate expression of *bbd18* was the sole cause of the loss of infectivity from S9/pBSV2*flaBp-bbd18-A. In addition, the fact that S9/chase-pBSV2G was infectious (Table 4-3) despite lacking both cp32-1 and lp28-4, verified that these plasmids, alone or in combination, are not critical for the infectivity of *B. burgdorferi* by needle inoculation.

Constitutively expressed *bbd18* prevents the upregulation of OspC by *B. burgdorferi* within feeding ticks.

B. burgdorferi overexpressing *bbd18* were non-infectious by needle inoculation, but in nature *B. burgdorferi* spirochetes are transmitted by *Ixodes* ticks (4). We wanted to investigate the potential impact on *B. burgdorferi* of overexpressing *bbd18* within ticks and during natural transmission to the mammalian host. We would typically do this by feeding larval *I. scapularis* ticks on infected mice and assess acquisition of *B. burgdorferi* before allowing the ticks to molt into nymphs. However, mice inoculated with S9/pBSV2*flaBp-bbd18-A or S9/pBSV2*flaBp-bbd18-B were not infected. Thus, we followed a well-established protocol (47) to artificially infect *I. scapularis* larvae with S9, S9/pBSV2*flaBp-bbd18-A, S9/chase-pBSV2G, or S9/pBSV2*flaBp-bbd18-B. The majority of ticks analyzed seven days after larval feeding contained viable spirochetes (Figure 4-2A), indicating that the artificial infection was successful. The remaining fed larval *I. scapularis* were allowed to molt and recover (~12 weeks after larval feeding) before groups fed on naïve mice (~5 ticks/mouse).

Infected nymphs either fed to repletion (1-4/mouse) to determine whether *B. burgdorferi* overexpressing *bbd18* were infectious in mice via tick transmission (Table 4-4) or were manually detached at 48 hours post-attachment to assess the

Figure 4-2: Constitutive expression of *bbd18* does not affect tick colonization or persistence

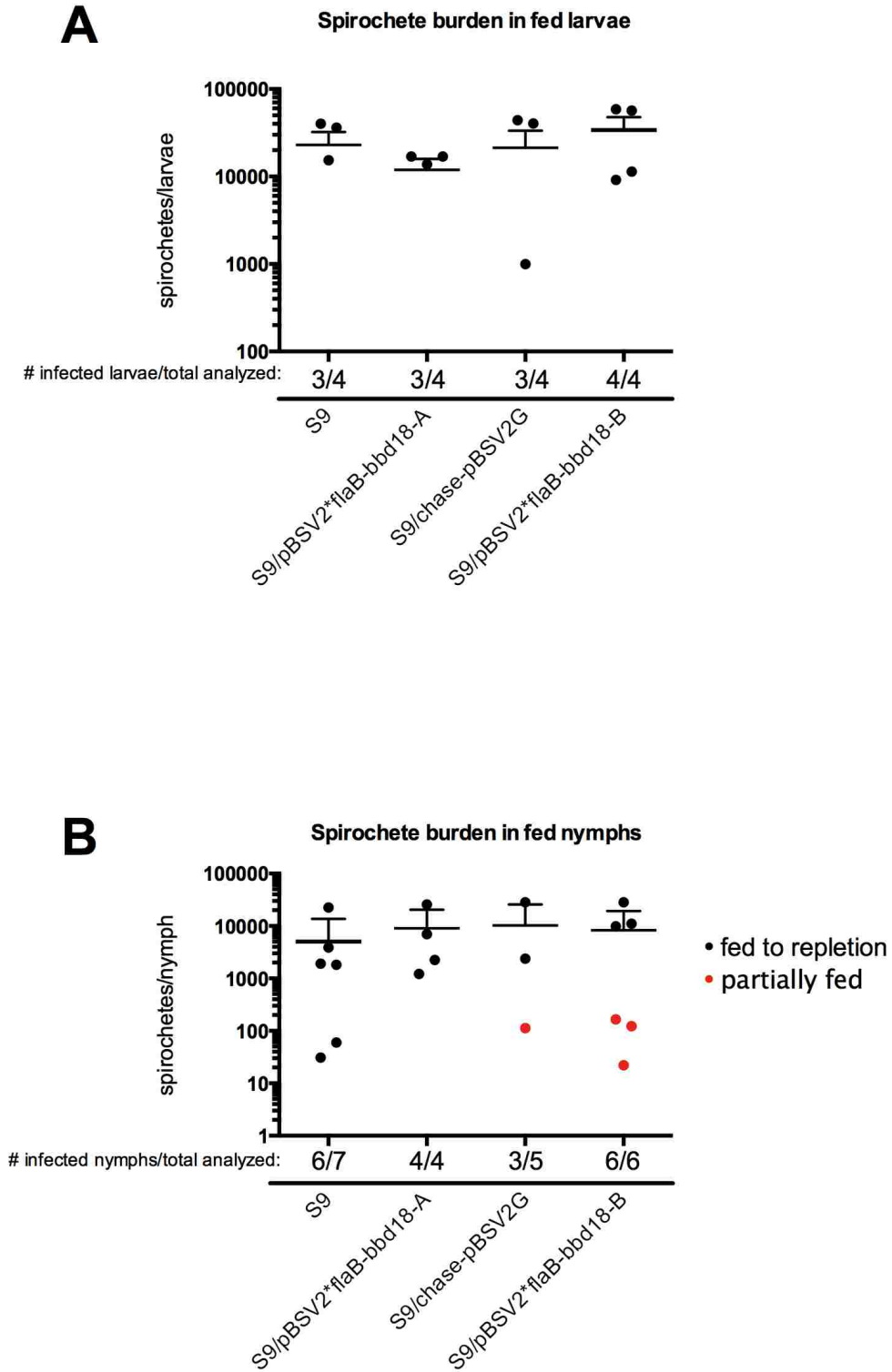


Figure 4-2 legend: **A.** Spirochete burden in fed artificially infected larval *I. scapularis*. Larvae were artificially infected (47) with S9, S9/pBSV2*flaBp-bbd18-A, S9/chase-pBSV2G, or S9/pBSV2*flaBp-bbd18-B, as indicated, and fed on naïve mice. Each point denotes the number of viable spirochetes per tick as determined by the CFU on solid media when fed larval ticks were individually crushed and plated seven days after drop off, and bars represent mean + SD. The fraction below the X-axis indicates the number of infected ticks over the total analyzed (four/strain). **B.** Spirochete burden in fed nymphs, which were artificially infected as larvae. Remaining fed artificially infected larvae (other than the subset analyzed for infection in A) were allowed to molt before being fed on naïve mice as nymphs. Each point denotes the number of viable spirochetes per nymph as determined by the CFU on solid media when fed ticks were individually crushed and plated seven days after drop off, and bars represent mean + SD. Black points indicate nymphs that fed to repletion, whereas red points indicate nymphs that were only partially fed. The fraction below the X-axis indicates the number of infected ticks over the total analyzed (4-7/strain).

Table 4-4: Transmission of *B. burgdorferi* by infected ticks

strain	No. of infected mice/mice fed on by infected larvae ^a	No. of seropositive mice /mice fed on by infected nymphs ^b	No. of mouse reisolates/mice fed on by infected nymphs (ear, bladder, joint) ^c
S9	1/1	2/2	2/2
S9/pBSV2*flaBp-bbd18-A	0/1	0/4 ^d	0/4 ^d
S9/chase-pBSV2G	1/1	1/1	0/1
S9/pBSV2*flaBp-bbd18-B	0/1	0/1	0/1

^a results from serology confirmed with reisolation from ear, bladder and joint tissues when mice were euthanized at 3 weeks post larvae drop-off

^b seroconversion to *B. burgdorferi* proteins assessed when mice were euthanized 3 weeks after nymph drop-off

^c mice were euthanized at 3 weeks after nymph drop-off

^d includes data from independent experiments by Dr. Amit Sarkar

upregulation of OspC on the surface of *B. burgdorferi* in feeding ticks (see below). S9 and S9/chase-pBSV2G were transmitted by infected nymphs to mice where they established infection, as measured by seroconversion and tissue reisolation (Table 4-4). In contrast, mice fed on by ticks infected with S9/pBSV2*flaBp-bbd18-A or S9/pBSV2*flaBp-bbd18-B neither seroconverted to *B. burgdorferi* proteins nor were spirochetes reisolated from tissues when mice were euthanized at 3 weeks (Table 4-4). Although *B. burgdorferi* containing pBSV2*flaBp-bbd18 were not able to establish infection in mice by needle inoculation (Table 4-3) or tick transmission (Table 4-4), the overexpression of *bbd18* did not affect the persistence of spirochetes within ticks, and spirochete numbers within nymphs were not significantly different between strains when assessed seven days after drop off (Figure 4-2B). Additionally, since S9/chase-pBSV2g persisted through the tick molt and was transmitted to naïve mice (Table 4-4 and Figure 4-2), cp32-1 and lp28-4 are not required for *B. burgdorferi* survival in its natural infectious cycle. We have shown that *B. burgdorferi* that constitutively express *bbd18* are non-infectious by needle-inoculation (Table 4-3) and tick bite (Table 4-4), but we wanted to investigate the mechanism by which this occurs. Since BBD18 represses OspC synthesis *in vitro*, we hypothesized that the observed loss of infectivity of S9/pBSV2*flaBp-bbd18-A and S9/pBSV2*flaBp-bbd18-B resulted because BBD18 repressed OspC within feeding ticks and during early infection where wt *B. burgdorferi* normally produce OspC. Thus, we determined the OspC phenotype of S9, S9/pBSV2*flaBp-bbd18-A, and S9/pBSV2*flaBp-bbd18-B within feeding nymphs that were forcibly removed at 48 hours post-attachment. We used this time point because Piesman and Schwan reported the highest proportion of OspC-positive

spirochetes at day 2 after attachment (6). We used a monoclonal mouse antibody recognizing OspC to analyze *B. burgdorferi* that had induced OspC (Figure 4-3, left panels) and a convalescent rabbit serum to visualize the entire *B. burgdorferi* population (Figure 4-3, right panels) within dissected ticks. We only detected OspC on spirochetes in ticks infected with the wt S9 strain, which did not constitutively express *bbd18*, as shown by green spirochetes in Figure 4-3 (top left). Although there were no OspC-positive spirochetes in ticks containing S9/pBSV2*-flaBp-bbd18-A or S9/pBSV2*-flaBp-bbd18-B, spirochetes were evident within these ticks when analyzed using the anti-*B. burgdorferi* rabbit serum (Figure 4-3, red spirochetes, right panels). These data demonstrate that constitutive *bbd18* prevents the upregulation of OspC by *B. burgdorferi*, even in the natural environment of a feeding tick, where OspC is normally induced. Presumably this lack of OspC in S9/pBSV2*-flaBp-bbd18-A and S9/pBSV2*-flaBp-bbd18-B is central to why these strains were non-infectious in mice (Tables 4-3 and 4-4), as OspC is critical for establishing infection in mammals (8-11).

***B. burgdorferi* lacking *bbd18* are infectious in mice**

Previous *in vitro* results using a high passage clone of *B. burgdorferi* showed that expression of *bbd18* represses the critical virulence factor *ospC*, and in the absence of BBD18, *ospC* is expressed (Chapter 3, (30)). Our current data demonstrate that overexpression of *bbd18* in an infectious clone represses the upregulation of *ospC* in feeding ticks and prevents infection in mice. Thus, we hypothesized that an infectious clone of *B. burgdorferi* that lacks *bbd18* would be infectious in mice since OspC would not be repressed. However, if BBD18 is the *in vivo* *ospC* repressor required to downregulate *ospC* in response to immune pressure,

Figure 4-3: *B. burgdorferi* constitutively expressing *bbd18* do not upregulate OspC within feeding ticks

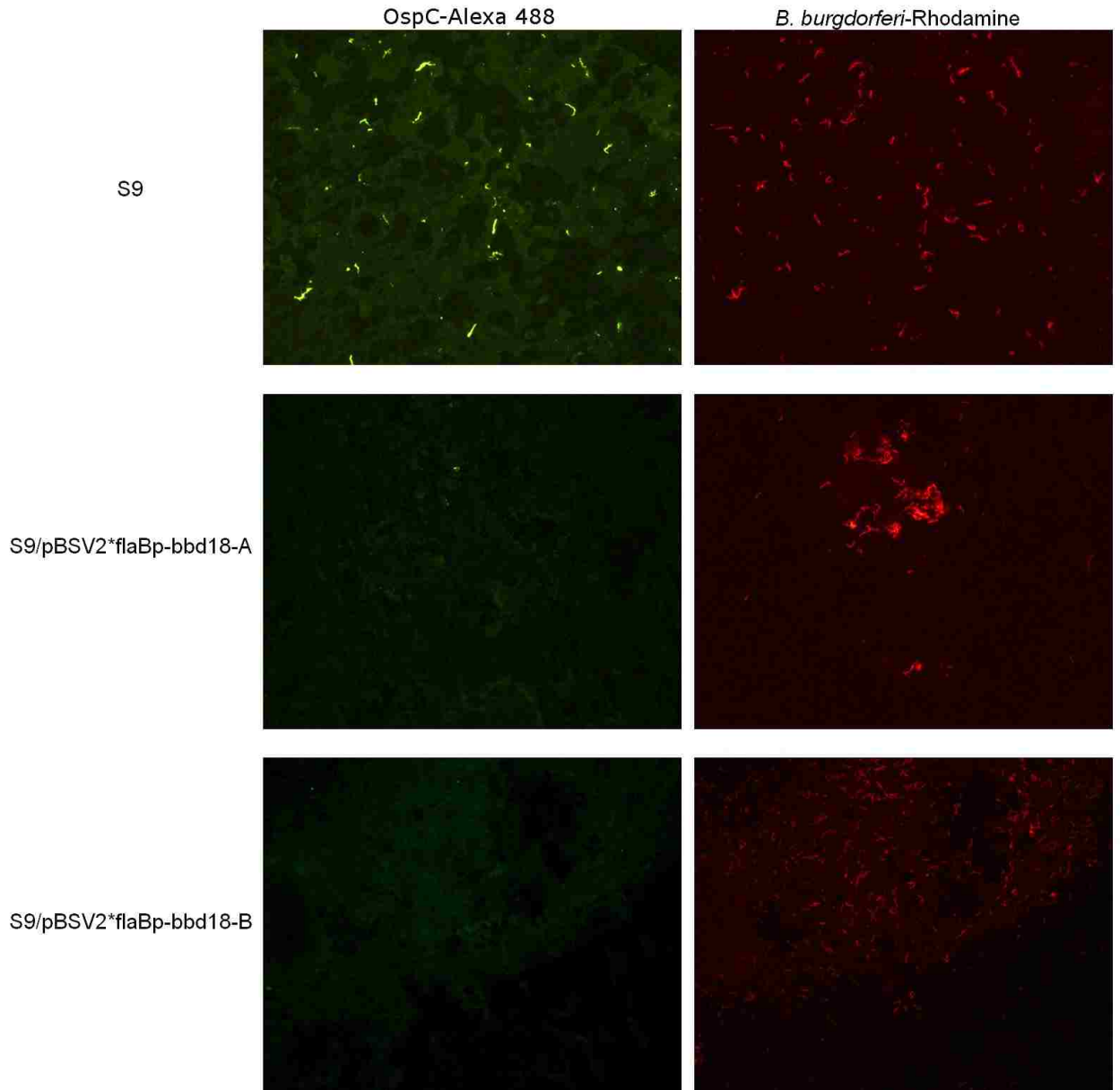


Figure 4-3 legend: Immunofluorescence microscopy of spirochetes within feeding *I. scapularis* nymphs. Nymphs infected with S9 (top), S9/pBSV2*flaBp-bbd18-A (middle), or S9/pBSV2*flaBp-bbd18-B (bottom) were manually detached after 2 days of attachment and assessed for the presence of OspC. Spirochetes within tick smears were co-stained with a mouse monoclonal antibody recognizing OspC (left panels) and polyclonal rabbit serum that detects all *B. burgdorferi* regardless of the OspC phenotype (right panels). Spirochetes were detected in each tick with the anti-*B. burgdorferi* rabbit serum (right panels, red spirochetes). However, OspC was only detected on the surface of wt S9 spirochetes (top left) and not on the spirochetes overexpressing *bbd18* (S9/pBSV2*flaBp-bbd18-A or S9/pBSV2*flaBp-bbd18-B, middle and bottom left, respectively). Images were taken with a Nikon Eclipse 80i microscope and a Nikon Digital Sight Qi1Mc camera.

then *B. burgdorferi* lacking *bbd18* should not persist, as the mammalian immune system would target spirochetes that continue to express *ospC*. A recent study investigating the role of genes encoded on lp17 used truncations of lp17 in an infectious *B. burgdorferi* clone (31). Casselli *et al.* injected mice with *B. burgdorferi* harboring a truncated version of lp17, in which the region encoding *bbd16-bbd25* (including *bbd18*) was deleted by the insertion of a synthetic telomere. This *B. burgdorferi* strain was able to establish infection and even persist up to 8 weeks but had a defect in colonizing the murine bladder (31). While attempting to inactivate *bbd18* (which proved to be not straightforward), we decided to repeat the experiments by Casselli *et al.*, and utilize lp17 truncations in the infectious clone S9 to further investigate the role of *bbd18* during the infectious cycle. We used gDNA from *B. burgdorferi* containing pGCB473 (30, 51), a truncated version of lp17 that only harbors *bbd1-bbd14* (lacking *bbd18*), to transform S9 and displace full-length lp17. We also used pGCB426 (30, 51), a truncated version of lp17 containing *bbd1-bbd20* (retaining *bbd18*) as a more appropriate control for S9/pGCB473. Southern blot analysis confirmed that full-length lp17 had been displaced from S9 using both pGCB473 and pGCB426 (Figure 4-4B), and the resulting strains S9/pGCB473 (Figure 4-4C) and S9/pGCB426 (data not shown) retained the full plasmid content as wt S9.

Groups of 5 mice were inoculated with S9, S9/pGCB473, or S9/pGCB426. After 3 weeks, all mice from groups infected with S9/pGCB426 or S9/pGCB473 had seroconverted to *B. burgdorferi* proteins (Table 4-5). These data demonstrate that spirochetes lacking the right end of lp17, and importantly *bbd18*, are able to establish infection in mice by needle inoculation. After euthanizing the mice at 8

Figure 4-4: Full-length lp17 displaced by pGCB426 and pGCB473 in S9

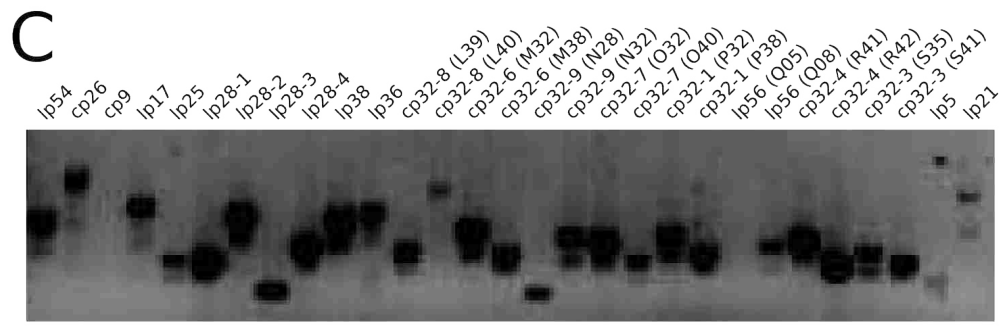
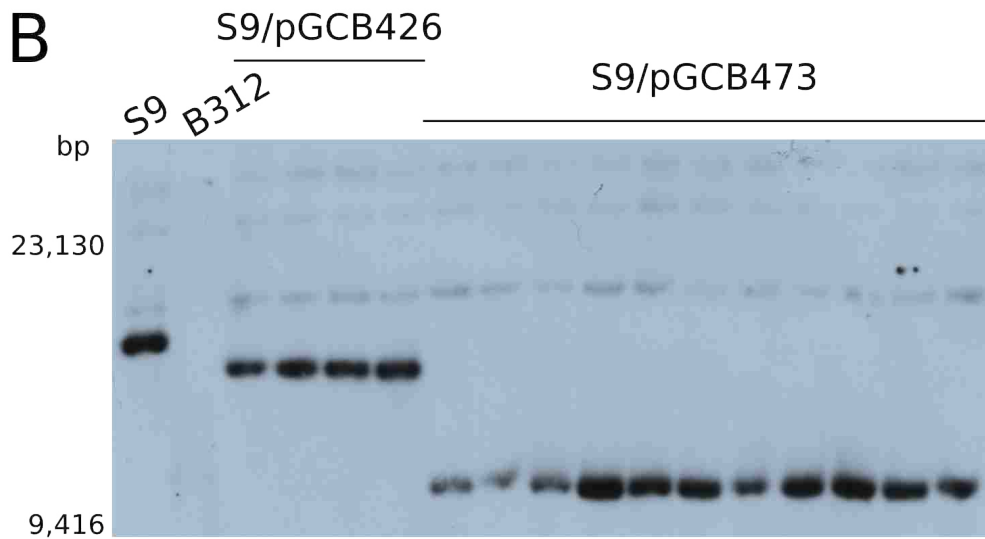
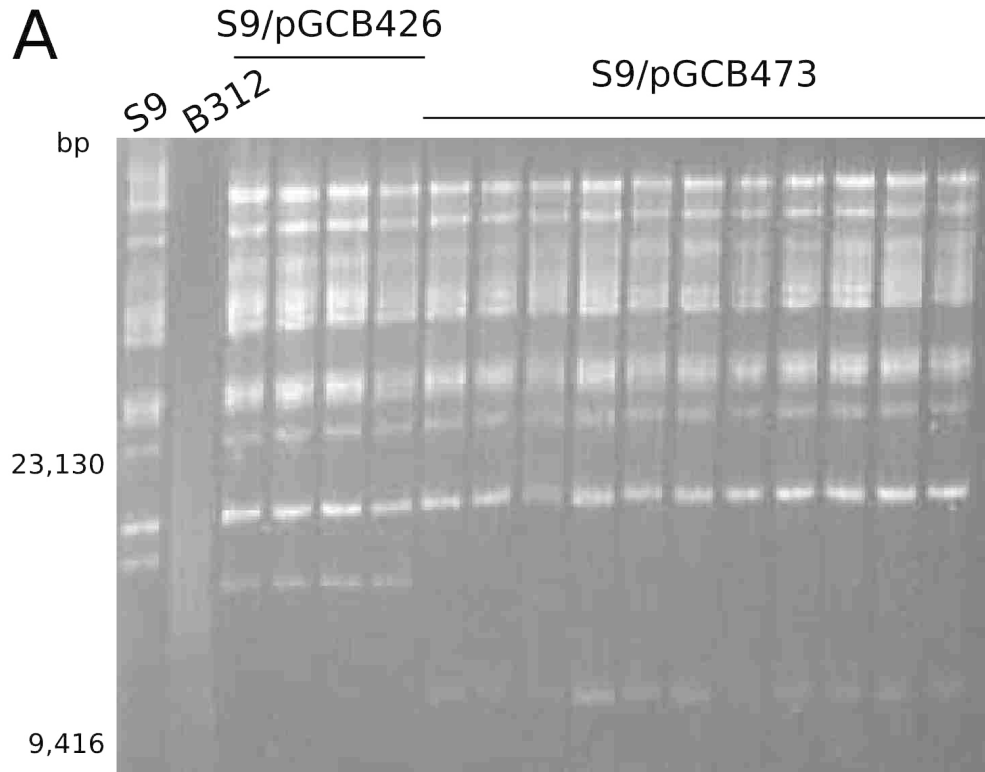


Figure 4-4 legend: **A.** Genomic DNA from S9 or S9 containing the lp17 truncations pGCB426 or pGCB473 separated by field-inversion gel electrophoresis. Uncut gDNA isolated from wt S9, B312, four transformants of S9/pGCB426, and 11 transformants of S9/pGCB473, as indicated, was analyzed by gel electrophoresis and visualized with Gel Red and ultraviolet light. The size in base pairs (bp) relative to the migration of standards is shown to the left. **B.** gDNA hybridization with an lp17 gene (*bbd13*) probe. Nylon-membrane-bound gDNA transferred from gels shown in A was incubated with a DIG-labeled probe corresponding to *bbd13*, which recognizes lp17 (full-length or smaller truncated forms). Hybridization of the probe was visualized using an anti-DIG antibody, chemiluminescent substrates, and X-ray film. **C.** Representative image of the plasmid profile of *B. burgdorferi*. One clone of S9/pGCB473 (A and B) had the same plasmid profile as parental S9, which lacks cp9 and lp56, when gDNA was analyzed using primers specific to individual *B. burgdorferi* plasmids (52) The plasmids corresponding to the specific amplicons are indicated above and gene numbers are specified when plasmids have more than one PCR target.

Table 4-5: *B. burgdorferi* harboring lp17-truncations are infectious in mice by needle inoculation

strain	No. of sero-positive mice/no. of injected mice ^a	No. of ear biopsy reisolates/no. of injected mice ^b	No. of mouse reisolates/no. of injected mice (ear, bladder, joint) ^c
S9	4/5	4/5	4/5, 4/5, 4/5
S9/pGCB426 (+ <i>bbd18</i>)	5/5	3/5	4/5, 0/5*, 1/5
S9/pGCB473 (- <i>bbd18</i>)	5/5	3/5	5/5, 2/5, 3/5

^a seroconversion to *B. burgdorferi* proteins was assessed at 3 weeks post-inoculation

^b ear biopsies were taken at 3 weeks post-inoculation

^c mice were euthanized at 8 weeks post-inoculation

* *P*-value <0.05 compared to S9 using the Fisher's exact test

weeks post-inoculation, spirochetes were reisolated from ear tissues of all S9/pGCB473-infected mice (lacks *bbd18*) and four out of five S9- and S9/pGCB426 infected mice (retains *bbd18*) (Table 4-5). However, we observed lower colonization of bladder and joint tissue for mice inoculated with *B. burgdorferi* harboring either of the lp17 truncations (Table 4-5). These results are in accord with the data from Casselli *et al.* in which they reported a complete loss in bladder colonization in mice infected with *B. burgdorferi* lacking *bbd16-25* at 8 weeks post-inoculation. Surprisingly, only the bladder colonization defect observed in mice infected with S9/pGCB426 was significantly different from colonization results with S9 (P-value = 0.0476 compared to S9 using Fisher's exact test) (Table 4-5), indicating that some factor encoded by *bbd20-25* (and not *bbd18*) contributes to the dissemination and/or colonization of peripheral tissue by *B. burgdorferi*.

Nonetheless, these data confirm that spirochetes lacking *bbd18* are infectious in the mouse model, but also suggest that *bbd18* is not the *in vivo* repressor necessary for the downregulation of *ospC* after *B. burgdorferi* establishes infection (12). To further investigate the role of *bbd18* during the infectious cycle, we wanted to investigate whether the lack of *bbd18* would have any effect on tick acquisition or transmission, which was not addressed by Casselli *et al.* (31). Therefore, we fed uninfected *I. scapularis* larvae on the mice infected with S9, S9/pGCB426 and S9/pGCB473. Although results from ear biopsies taken at 3 weeks post-inoculation confirmed that the mice on which ticks were fed contained viable spirochetes within skin tissues (Table 4-5), most larval ticks did not acquire spirochetes when they fed on infected mice at 4 weeks post-inoculation (Table 4-6), even from wt S9- infected mice, presumably reflecting some technical problem, but this experiment was not

Table 4-6: Larval ticks did not acquire *B. burgdorferi* from infected mice

mouse infection strain	no. infected larval ticks/no. analyzed
S9	3/10
S9/pGCB426 (+ <i>bbd18</i>)	0/10
S9/pGCB473 (- <i>bbd18</i>)	1/10

^a larval tick infection was determine by CFU on solid media or reisolation of spirochetes in liquid media

repeated because we had finally succeeded in inactivating the *bbd18* ORF in an infectious *B. burgdorferi* clone. Thus, we decided to use this “clean” *bbd18* mutant (S9 Δ *bbd18*, Table 4-1 and below) in subsequent studies to avoid any confounding results due to the deletion of the additional lp17-encoded genes missing from S9/pGCB426 and S9/pGCB473.

Interrupting the *bbd18* ORF in S9

We have shown that constitutive expression of *bbd18* abrogates *B. burgdorferi* infection in mice but wanted to investigate whether *bbd18* was necessary during the infectious cycle by interrupting the *bbd18* ORF in an otherwise infectious *B. burgdorferi* clone. We created an allelic exchange construct, pOK12-*bbd18*-KO, in which the *flgBp-aacC1* cassette, conferring gentamicin resistance, interrupts the *bbd18* ORF near the 5' end. After multiple attempts at transforming S9 with linearized pOK12-*bbd18*-KO, we identified a few colonies that amplified both a wt and mutant size *bbd18* fragment by PCR; the mutant band is about 1kb larger in size due to the insertion of the *flgBp-aacC1* cassette in the *bbd18* ORF (see Figure 4-5C). Since we had linearized the allelic exchange vector, we thought that the presence of both bands implied merodiploid lp17 plasmid content as opposed to the integration of the entire pOK12-*bbd18*-KO plasmid into lp17. After several serial passages under gentamicin selection and screening colonies by PCR, we identified a clone, referred to as S9 Δ *bbd18*, that only yielded a mutant-size *bbd18* band when screened by PCR (Figure 4-5C). Southern blotting confirmed that the *flgBp-aacC1* cassette had integrated on lp17 and a wt *bbd18* gene copy was not present (Figure 4-5). S9 Δ *bbd18* also retained the entire plasmid content as parental S9 (a representative image of the *B. burgdorferi* plasmid profile as determined by PCR is

Figure 4-5: Inactivation of *bbd18* in S9

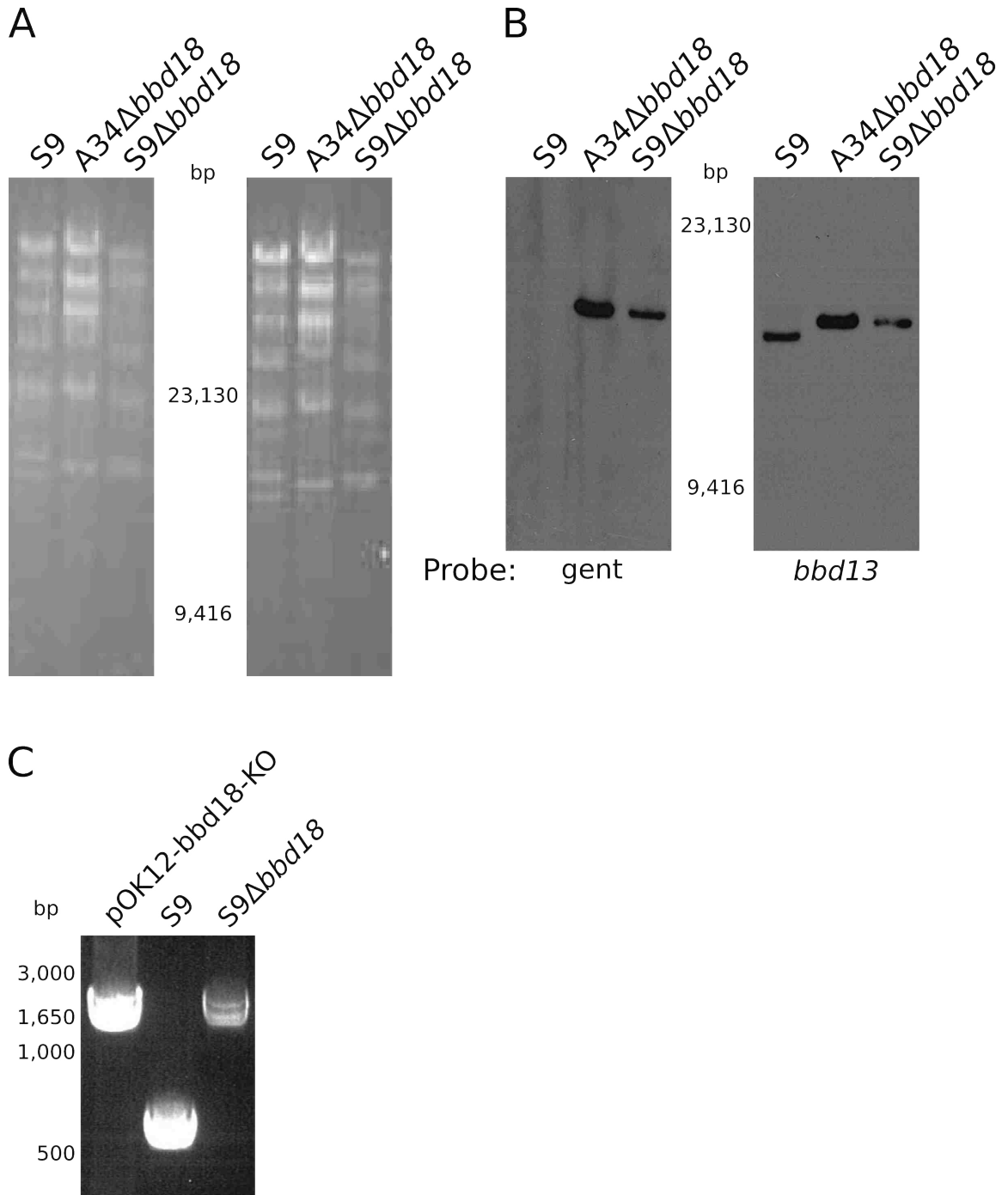


Figure 4-5 legend: **A.** Genomic DNA from S9, A34Δ*bbd18*, or S9Δ*bbd18* separated by field-inversion gel electrophoresis. Uncut gDNA, isolated from wt S9, A34Δ*bbd18*, or S9Δ*bbd18*, as indicated, was analyzed by gel electrophoresis and visualized using Gel Red and ultraviolet light. The size in base pairs (bp) relative to the migration of standards is listed between duplicate panels **B.** gDNA hybridization with probes recognizing *aacC1*, the gentamicin-resistance gene (*gent*, left blot) or *bbd13* (right blot). Nylon membrane-bound gDNA transferred from gels shown in A was incubated with a DIG-labeled probes corresponding to *gent* to localize the *flgBp-aacC1* insertion in the *B. burgdorferi* genome or *bbd13*, which is encoded on lp17. Probe hybridization was visualized using an anti-DIG antibody, chemiluminescent substrates, and X-ray film. The size in base pairs (bp) based on the migration of standards is listed in between the two panels. **C.** Agarose gel analysis of PCR amplification of the *bbd18* ORF. *bbd18* specific primers (Table 4-2) were used with the *bbd18* inactivation plasmid, pOK12-*bbd18*-KO, wt S9 gDNA, or a colony for the S9Δ*bbd18* clone, as indicated, to amplify the corresponding *bbd18* fragment. The inactivation of *bbd18* leads to a larger product because of the insertion of *flgBp-aacC1* into the *bbd18* ORF. The size in base pairs (bp) relative to the migration of standards is listed on the left.

displayed in Figure 4-4C).

In order to confirm that any observed differences from wt S9 were due to the inactivation of *bbd18*, we wanted to complement *S9Δbbd18* with a wt *bbd18* gene. However, we were concerned that expression of *bbd18* from a shuttle vector would not complement the phenotype of *S9Δbbd18*, since overexpression of *bbd18* alone in S9 also abrogated infection in mice (Table 4-3). To address this possible complication, we first utilized lp17::kan from the B31-A derivative carrying pKK81 integration on lp17 (30, 36) to displace lp17 harboring the *flgBp-aacC1* insertion in the *bbd18* open reading frame. Although this strategy would indicate that the phenotype of the *bbd18* mutant was due to a change in lp17, it would not necessarily specify *bbd18* as the culprit. Thus, we also used pBSV2*NP-*bbd18* (Table 4-1, (30)), which harbors a wt *bbd18* gene expressed from its native promoter, to transform *S9Δbbd18*. We hypothesized that by using the native *bbd18* promoter, we could limit the amount of *bbd18* expressed and control for the proper regulation of *bbd18*. If this latter strategy worked, we would be able to demonstrate that any phenotypic defect of *S9Δbbd18* was strictly due to the interruption of the native *bbd18* gene on lp17. Both complemented clones, *S9Δbbd18*/lp17::kan and *S9Δbbd18*/pBSV2*NP-*bbd18* (Table 4-1), retained the full plasmid content as *S9Δbbd18* and the parental S9 (see Figure 4-4C for an example of the S9 plasmid profile).

Groups of 5 mice were inoculated with S9, *S9Δbbd18*, *S9Δbbd18*/lp17::kan or *S9Δbbd18*/pBSV2*NP-*bbd18*. After 3 weeks, we assessed the seroconversion of inoculated mice to *B. burgdorferi* proteins and attempted to reisolate spirochetes from ear biopsy tissue. We found that none of the mice inoculated with *S9Δbbd18*, *S9Δbbd18*/lp17::kan, or *S9Δbbd18*/pBSV2*NP-*bbd18* seroconverted and that no

spirochetes were reisolated from ear biopsy cultures, whereas wt S9 resulted in infection by both measures, as expected (Table 4-7). The lack of infectivity in mice inoculated with *S9Δbbd18* was surprising since mice infected with *S9/pGCB473*, which lacks *bbd18*, had seroconverted to *B. burgdorferi* by 3 weeks and spirochetes were isolated from all ear tissues from these mice at 8 weeks (Table 4-5). The fact that mice inoculated with *S9Δbbd18/lp17::kan* or *S9Δbbd18/pBSV2*NP-bbd18* were also not seropositive and no spirochetes were reisolated from ear tissues (Table 4-7) indicated that neither complementing strategy restored the ability to infect mice. This inability to complement the infectivity defect of *S9Δbbd18* suggests that other mutations occurred in *S9Δbbd18* in addition to the insertion of *flgBp-aacC1* into the *bbd18* ORF. We did not detect any obvious changes in *S9Δbbd18* that would account for its lack of infectivity, as it retained the full plasmid content of wt S9 and had a similar protein profile as analyzed by Coomassie staining (Figure 4-6). We have attempted to reconstruct another *bbd18* mutant in an infectious background using both the linearized allelic exchange construct (pOK12-*bbd18*-KO) and genomic DNA from *S9Δbbd18*, but so far have been unsuccessful.

The secondary mutations that occurred in *S9Δbbd18* and the fact that we have been unable to isolate a second *bbd18* mutant are not surprising considering the difficulty in obtaining the first *bbd18* mutant in S9. It is noteworthy, though, that using the same allelic exchange construct, pOK12-*bbd18*-KO, we readily generated a *bbd18* mutant in the non-infectious *B. burgdorferi* clone A34 (53) during the first round of transformations and this was easily repeated. Additionally, displacing full-length lp17 from S9 with pGCB473, the lp17 truncation that is missing *bbd18*, was relatively easy compared to the allelic exchange that resulted in *S9Δbbd18*. These

Table 4-7: The *bbd18* mutant and its complemented clones are non-infectious in mice by needle inoculation

strain	No. of sero-positive mice/no. of injected mice ^a	No. of mouse reisolates/no. of injected mice
S9	5/5	5/5 ^b
S9 Δ <i>bbd18</i>	0/10	0/10 ^c
S9 Δ <i>bbd18</i> /lp17::kan	0/10	0/10 ^d
S9 Δ <i>bbd18</i> /pBSV2*NP- <i>bbd18</i>	0/5	0/5 ^d

^a seroconversion to *B. burgdorferi* proteins was assessed at 3 weeks post-inoculation

^b reisolation from ear, bladder, and joint for each mouse, mice were euthanized at 6 weeks post-inoculation

^c total of reisolation from ear, bladder, and joint for 5 mice euthanized at 6 weeks post-inoculation and reisolation from ear tissues only from 5 mice euthanized at 5 weeks post-inoculation

^d reisolation from ear only, mice were euthanized at 4-5 weeks post-inoculation

Figure 4-6: Coomassie staining does not reveal any differences in total protein content between S9 and S9 Δ *bbd18*

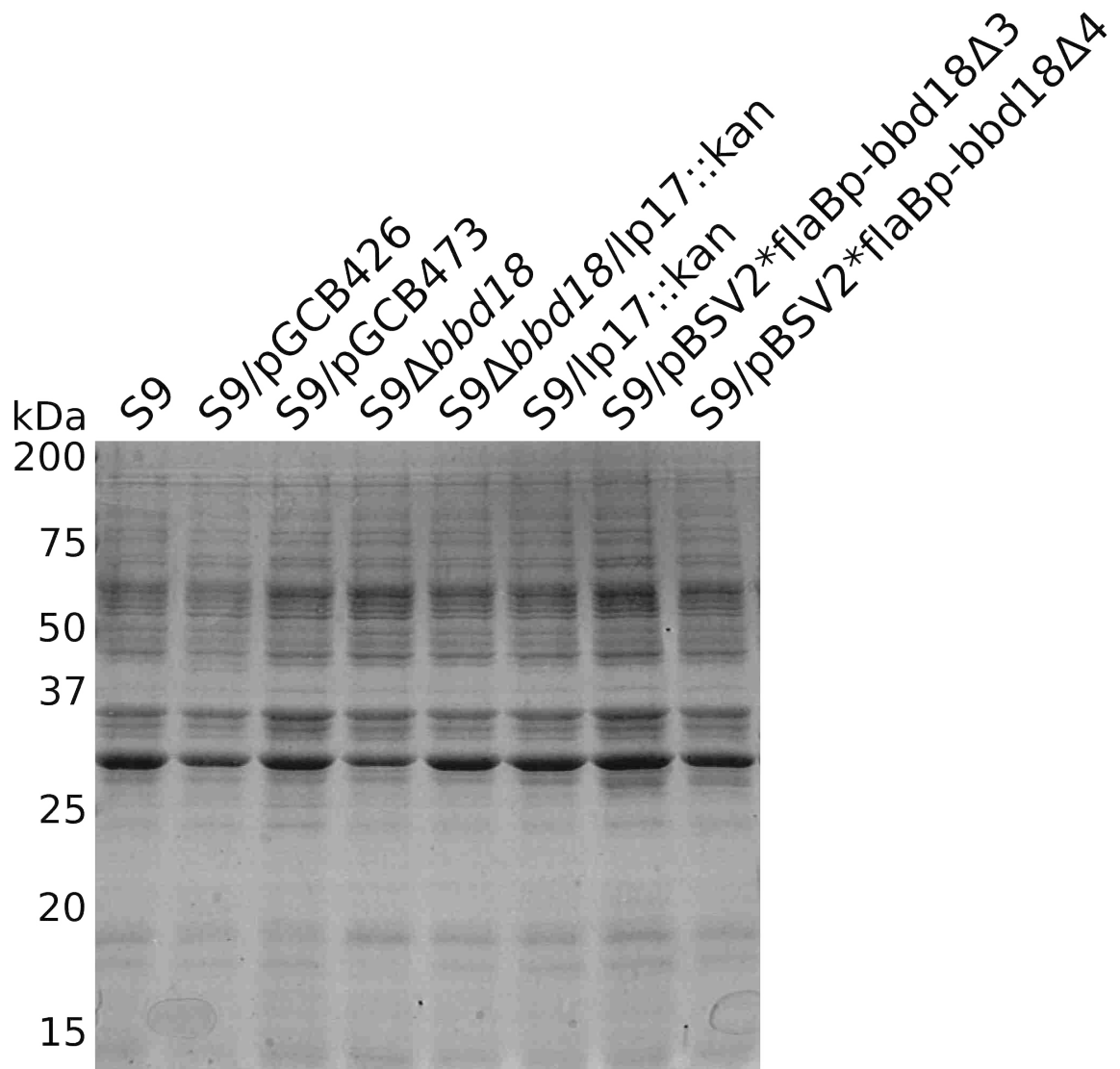


Figure 4-6 legend: Coomassie blue stained gel of S9 clones. Whole cell lysates of S9 and S9 derivatives, as indicated, were separated on a 12.5% acrylamide gel and then stained with Coomassie blue to visualize protein. Numbers on the left refer to protein size in kDa determined by the mobility of protein standards.

data suggest that there is some undefined but critical aspect of the retention of the *bbd18* locus and/or the BBD18 protein within the context of an infectious strain, which carries other lp17 genes.

We have shown that *bbd18* likely plays a role during the *B. burgdorferi* infectious cycle, as spirochetes that constitutively express *bbd18* are non-infectious and do not upregulate OspC within feeding ticks. Spirochetes lacking a large segment of lp17, including *bbd18*, were able to establish infection but were attenuated for colonization of the mice bladder and joint. However, we were unable to identify a specific contribution of *bbd18* to this phenotype, as *B. burgdorferi* containing pGCB426, which retains a wt *bbd18* gene, had a similar phenotype, and secondary mutations in *S9Δbbd18* prevented mouse infection even when the *bbd18* mutation was complemented with a wt *bbd18* gene. The fact that the only *bbd18* mutant that we recovered in an infectious background had additional mutations might intrinsically suggest the importance of *bbd18* during the infectious cycle, as these additional changes may be required in order for *bbd18* to be disrupted. To further understand what role BBD18 might be playing during the infectious cycle, we also used *in vitro* assays to investigate the mechanism of how BBD18 represses OspC.

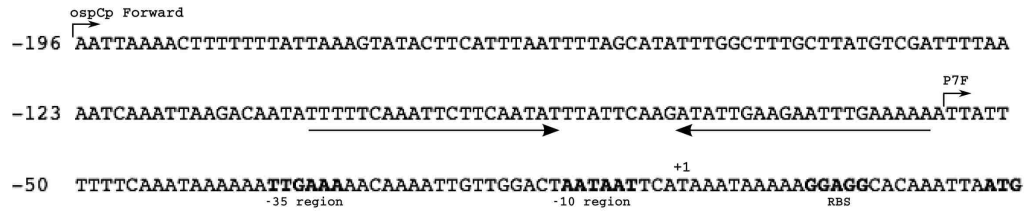
The putative *ospC* operator is not necessary for repression by BBD18.

Previous work by Xu and colleagues (28) identified a putative operator upstream of the *ospC* promoter that was necessary for the downregulation of *ospC* in response to immune pressure. Thus, the simplest mechanism we imagined for how BBD18 might repress *ospC* would be BBD18 binding to this putative operator sequence. To test this, we wanted to determine whether the operator sequence was

required for repression of *ospC* by BBD18. Typically, wt *B. burgdorferi* clones do not produce OspC *in vitro* unless grown to high density or otherwise manipulated. On the other hand, the high passage *B. burgdorferi* clone B312 (Table 4-1 and (30, 32, 38)) produces abundant OspC protein *in vitro* without manipulation, which makes it an ideal clone with which to investigate the *ospC* promoter sequences required for repression by BBD18. We also utilized the *lacZ* reporter system that was developed for *B. burgdorferi* (32) to aid in these experiments. We expressed *lacZ*^{Bb*} in B312 using either a full-length *ospC* promoter that includes the entire inverted repeat (putative operator sequence) or a minimal *ospC* promoter, P7F, which lacks the putative operator sequence (Figure 4-7A). Xu *et al.* demonstrated that this minimal *ospC* promoter was sufficient for inducing OspC in *B. burgdorferi*, but that these spirochetes were unable to downregulate *ospC* *in vivo* (28). In B312, both versions of the OspC promoter led to expression of *lacZ* as detected by X-gal in solid media (Figure 4-7B, top panels). We then co-expressed *bbd18* in these clones using a constitutive promoter on a compatible shuttle vector. BBD18 was able to repress *lacZ* expression from either *ospC* promoter regardless of whether the putative operator sequence was present or not (Figure 4-7B, bottom panels). These data indicate that BBD18 does not require the putative operator sequence for repression of *ospC*. Furthermore, similar experiments using *lacZ* expressed from an *ospC-flaB* fusion promoter, which was created by fusing the core *ospC* promoter (-35 region up to the +1) and the *flaB* promoter 5' untranslated region (+1 to the ATG translation start, including the RBS) demonstrated that only the core *ospC* promoter (-35 and -10 regions) was required for repression of *ospC* by BBD18 (Figure 4-7B, right panels). These findings indicate that BBD18 is an operator-independent

Figure 4-7: The putative *ospC* operator is not required for repression by BBD18.

A



B

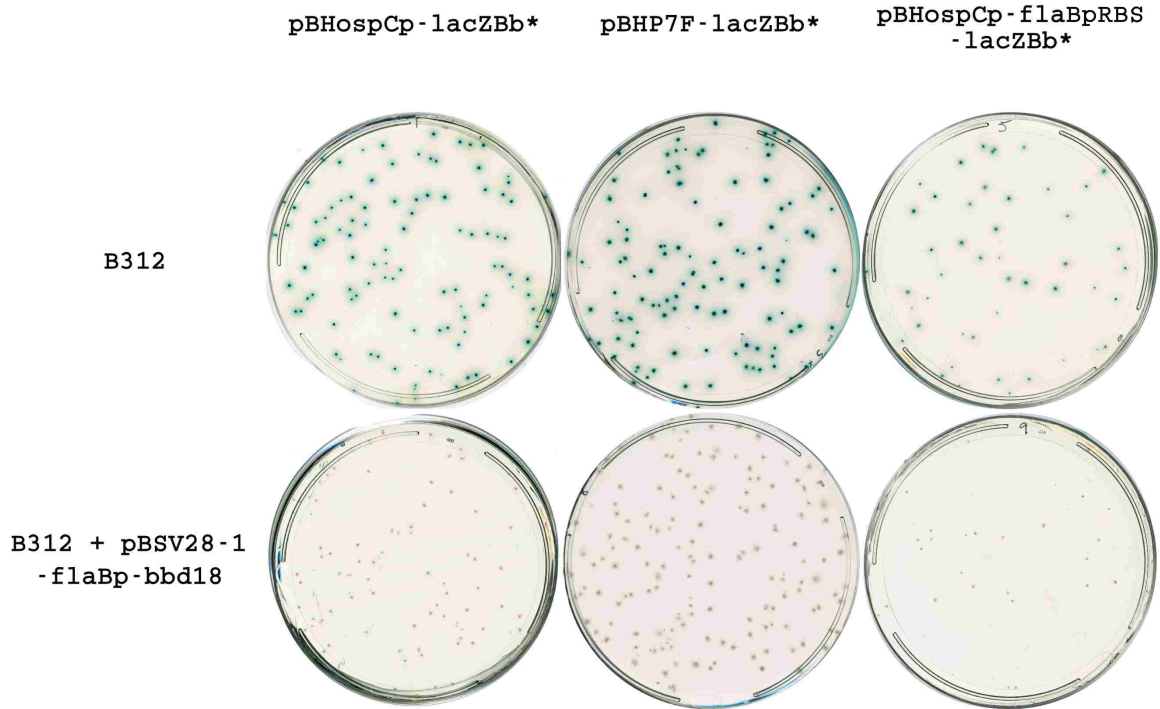


Figure 4-7 legend: **A.** The *ospC* promoter and upstream regulatory regions. Two large inverted repeat sequences (putative operator, indicated by a pair of divergent arrows), the -35 and -10 regions, the ribosome-binding site (RBS) and the start codon ATG (in bold) of *ospC* are all indicated. The transcriptional start site (54) is designated as +1. The 5' ends of primers used to construct the *ospC-lacZ* reporter genes with or without the putative operator (*ospCp* forward and P7F, respectively; Table 4-2) are indicated with arrows above the sequence. This diagram is closely modeled after a figure by Xu *et al.* (28). **B.** *lacZBb* expression in *B. burgdorferi* from a variety of *ospC* promoters assessed by X-gal in solid media. BSK solid medium without phenol red was used to grow B312/pBHospCp-*lacZBb** (left), B312/pBHP7F-*lacZBb** (middle), or B312/pBHospCp-flaBpRBS-*lacZBb** (right) with (bottom) or without (top) pBSV28-1-flaBp-bbd18, as indicated. After colony formation, X-gal was added. β -galactosidase activity (blue color) was only detected in colonies without BBD18 (top), indicating that BBD18 was able to repress *lacZBb* expression from the *ospC* promoter whether or not the operator sequence was present and without the *ospC* 5' untranslated region.

manner, suggesting that BBD18 is not the *ospC*-specific *in vivo* repressor (28). Since our previous experiments in *E. coli* (Figure 3-3) indicated an indirect mode of *ospC* repression by BBD18, we questioned whether BBD18 might be working upstream of *ospC* possibly through the alternative sigma factor, RpoS, which is required for *ospC* expression in *B. burgdorferi* (20, 25).

Inactivation of RpoS in B312 abrogates OspC production

Since B312 is a high passage strain and produces OspC *in vitro* without manipulation unlike wt virulent *B. burgdorferi*, we wanted to confirm that OspC expression in B312 is indeed dependent on RpoS as described for low-passage virulent *B. burgdorferi* (20, 25). In order to accomplish this, we inactivated *rpoS* in clone B312 by the same allelic exchange construct used to inactivate *rpoS* in wt *B. burgdorferi* (37). Although we had tried to obtain transformants in B312 with various allelic exchange constructs in the past, we had not succeeded. In this attempt, we first transformed B312 with a shuttle vector harboring a constitutively expressed *recA* gene, as Dr. Frank Gherardini (RML, NIAID, NIH) had suggested that this might increase the frequency of homologous recombination (and consequently allelic exchange) in *B. burgdorferi* (personal communication with Dr. Frank Gherardini). To this end, we transformed the B312 derivative containing *recA* (B312/pBSV2G-flaBp-*recA*, Table 4-1) with the *rpoS*::kan inactivation construct (37). This strategy was successful and we were able to isolate a B312 transformant in which *rpoS* had been inactivated. We then analyzed the B312Δ*rpoS* clone for the presence of OspC by immunoblot (Figure 4-8). We detected OspC in wt B312, as expected, and in B312/pBSV2G-flaBp-*recA*, but not in the B312 *rpoS* mutant in. These data confirm that OspC expression in B312 is RpoS-dependent (Figure 4-8)

Figure 4-8: OspC synthesis in B312 depends on RpoS

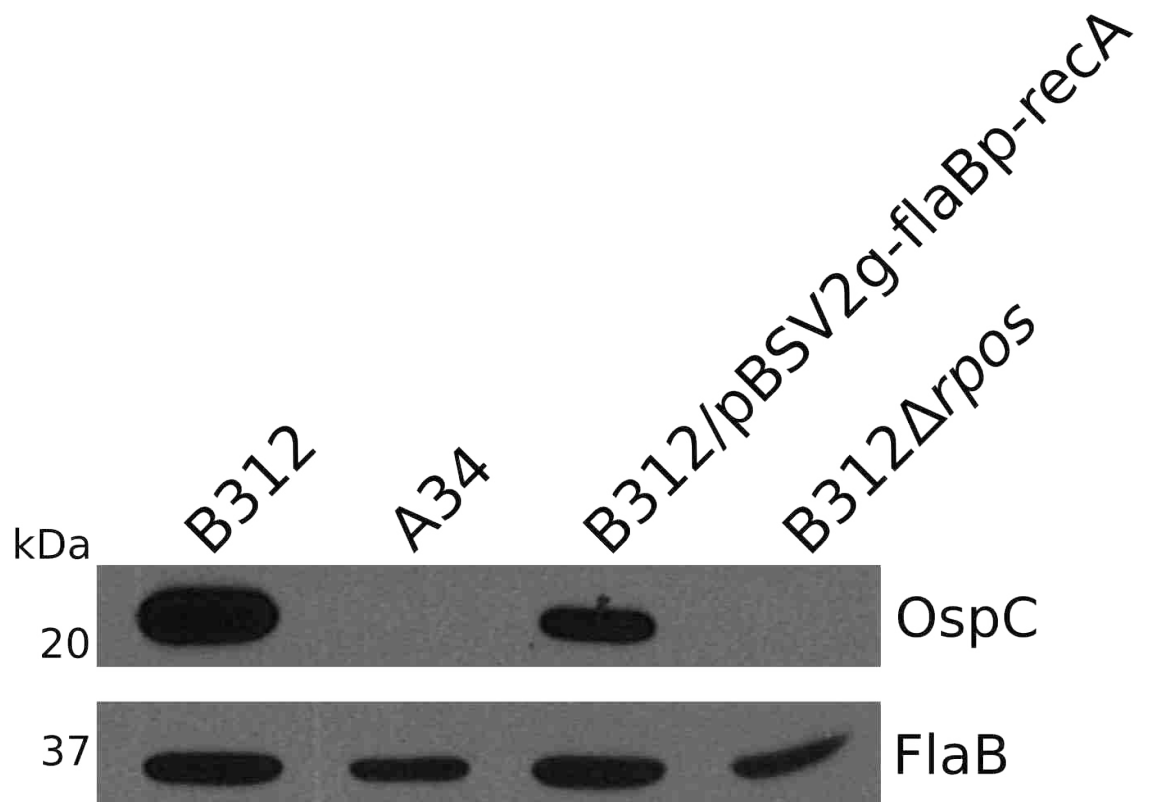


Figure 4-8 legend: OspC and FlaB Immunoblots of *B. burgdorferi*. Whole cell lysates of B312, A34 (negative control), B312/pBSV2G-flaBp-recA (parent of B312 Δ rpos), and B312 Δ rpos were analyzed with antisera recognizing OspC and FlaB, which was used as an internal control for protein loading. Numbers at the left refer to protein size in kDa relative to the mobility of protein standards.

and validate the utility of B312 in the analysis of *ospC* regulation by BBD18.

BBD18 affects other RpoS-dependent genes.

During the infectious cycle, spirochetes within feeding ticks induce the Rrp2-RpoN-RpoS cascade and RpoS is directly responsible for the upregulation of *ospC* (20, 25) and numerous other genes, including *dbpA* (20, 25) and *bba66* (46) We hypothesized that if BBD18 were acting upstream of *ospC*, perhaps on RpoS, then BBD18 would also affect the production of other RpoS-regulated proteins, such as DbpA and BBA66. Thus, we wanted to determine if BBD18 has an effect on these other RpoS-dependent genes by constitutively expressing BBD18 in an infectious *B. burgdorferi* clone and analyzing the protein content by immunoblot. Unlike the high-passage non-infectious clone B312 that produces OspC *in vitro* without manipulation, the production of RpoS and thus OspC, DbpA, and BBA66 in an infectious *B. burgdorferi* clone like S9 must be induced. This can be achieved by mimicking some of the environmental changes that are found within feeding ticks, such as the change in temperature (7, 17, 55) and pH (17, 56). Since we wanted to assess the ability of BBD18 to repress the production of OspC, DbpA, and BBA66, we used temperature-shifted *B. burgdorferi* cultures to ensure that the necessary signals for RpoS induction were present. Spirochetes were harvested from cultures grown at 25° or from cultures that were shifted to 35°. Whole cell lysates were assessed for the production of OspC, DbpA, BBA66, or RpoS using specific antisera for each (Figure 4-9A). Of note, multiple antibodies against RpoS were tested, but only the one used in Figure 4-9A (a gift from Dr. Frank Gherardini, RML, NIAID, NIH) detected a protein that migrated at the estimated size of RpoS, 31 kDa. Because this antibody also reacted with proteins of other sizes, a larger portion of the RpoS

Figure 4-9: BBD18 represses additional RpoS-dependent genes

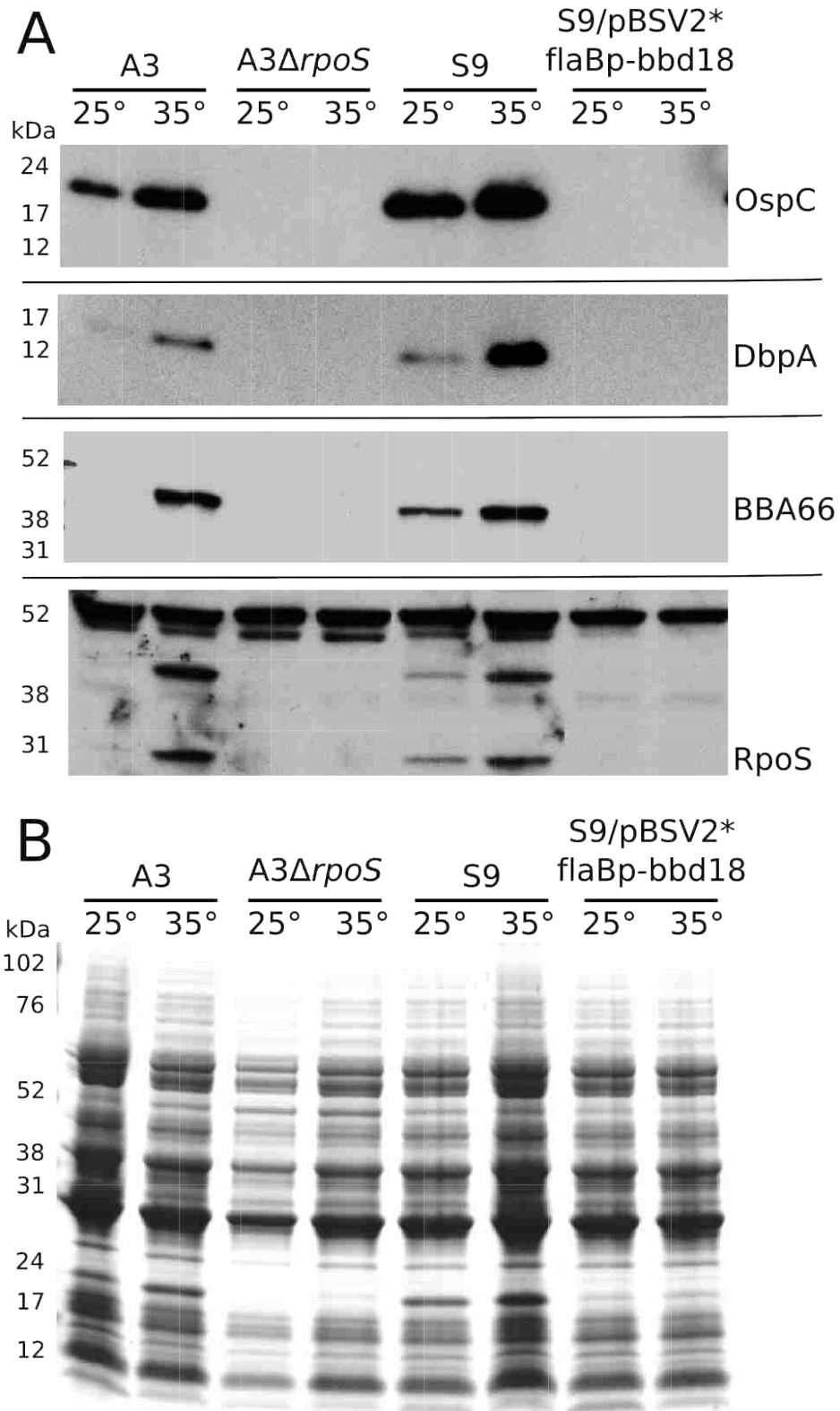


Figure 4-9 legend: **A.** OspC, DbpA, BBA66, and RpoS immunoblots of whole-cell lysates from A3, A3 Δ rpoS, S9, or S9/pBSV2*flaBp-bbd18 that were temperature-shifted from 25° to 35°C to induce the Rrp2-RpoN-RpoS regulon, as indicated. *B. burgdorferi* were harvested in equal number from cultures growing at 25° or 35°C and analyzed on immunoblots with antisera recognizing OspC, DbpA, BBA66, or RpoS. Numbers at the left refer to protein size in kDa relative to the mobility of protein standards. RpoS and the RpoS-dependent proteins OspC, DbpA, and BBA66 were induced in A3 and S9 when cultures were harvested at 35°C, but are not found in the *rpoS* mutant (A3 Δ rpoS) or S9/pBSV2*flaBp-bbd18 at either temperature. **B.** Coomassie blue stain gel of samples from A. A duplicate gel of samples from A was stained with Coomassie blue to illustrate comparable protein loading. Numbers at the left refer to protein size in kDa relative to the mobility of protein standards. These experiments were conducted by Dr. Daniel Dulebohn.

immunoblot is shown. While A3 and S9 both produced RpoS and subsequently OspC, DbpA, and BBA66 when grown at 35°, these proteins were not detected at either temperature in lysates from the *rpoS* mutant (*A3ΔrpoS*, (37)) or in S9 harboring a constitutively expressed *bbd18* (*S9/pBSV2*flaBp-bbd18*, Figure 4-9A). A duplicate gel of the samples shown in Figure 4-9A was stained with Coomassie blue to illustrate protein loading (Figure 4-9B). The absence of OspC, DbpA, and BBD18 from *S9/pBSV2*flaBp-bbd18* indicates that BBD18 repression affects multiple RpoS-dependent genes and not just OspC. The lack of detectable RpoS protein in *S9/pBSV2*flaBp-bbd18* also supports the interpretation that BBD18 affects RpoS, which is the mechanism through which BBD18 represses OspC, DbpA, and BBA66. Ongoing experiments are directed at identifying the mechanism of BBD18 repression of RpoS (Dr. Daniel Dulebohn)

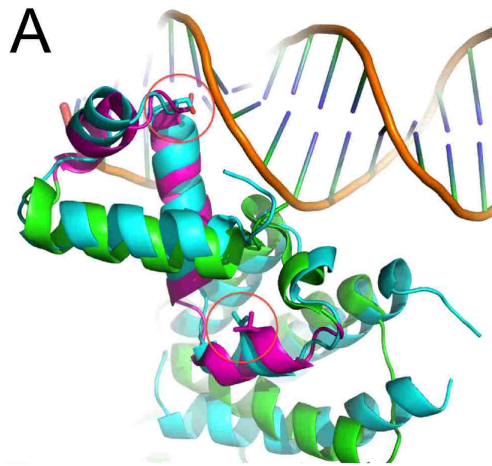
BBD18 structural prediction and site-directed mutagenesis

Although BBD18 is highly conserved in Lyme disease and relapsing fever spirochetes, we were unable to find any appreciable homologs or analogs outside of the *Borrelia* genus. The predicted BBD18 protein is a basic protein with many charged and aromatic residues, and structural predictions suggest a primarily alpha-helical structure, not incompatible with nucleic acid interaction. With limited information as to the function of BBD18, we contacted the Office of Cyberinfrastructure and Computational Biology (OCCB), NIAID, NIH for their expertise. Xavier Ambroggio, a Computational Structural Biologist at OCCB, used the primary amino acid sequence of BBD18 in a Position-Specific Iterated BLAST (PSI-BLAST, NCBI) search to create a limited structural model of BBD18 based on weak similarity with the DNA-binding motif of the plasmid partitioning genes ParB and

SopB (Figure 4-10A). The alignment of the BBD18, ParB, and SopB primary amino acid sequences within this structural motif is provided in Figure 4-10B. Although this is a limited model and the structural prediction only accounts for 34 of the 220 residues in the predicted BBD18 protein, it is the only structural model available. Thus, we hypothesized that if BBD18 were indeed interacting with nucleic acids through this putative motif, we might be able to abrogate repressor function by site-directed mutagenesis within this region. Using this structural prediction (Figure 4-10A and B) and an alignment of the primary amino acid sequence of BBD18 in Lyme disease spirochetes and its counterpart in relapsing fever spirochetes (unpublished sequences, Dr. Tom Schwan, RML, NIAID, NIH), we designed four BBD18 variants. Each variant had multiple amino acid substitutions (see Materials and Methods and Figure 4-10C), most within the putative nucleic acid binding domain (Figure 4-10A and B), although 2 aspartic acids residues outside of this region, which were absolutely conserved in all sequences analyzed, were also changed. A partial amino acid alignment of wt BBD18 and these 4 site-directed mutants is provided in Figure 4-10C. Synthetic genes encoding these BBD18 variants were obtained from GenScript and cloned into a shuttle vector, creating pBSV2*flaBp-bbd18 Δ 1-4, respectively (Table 4-1).

We then wanted to test whether these *bbd18* variants were similar to wt *bbd18* in their ability to repress *ospC* in *B. burgdorferi*. We again utilized the high-passage *B. burgdorferi* clone B312, which produces substantial OspC *in vitro*. After stably introducing each of the *bbd18* variants into B312, we analyzed whole cell lysates for the presence of OspC. As expected, B312 (which lacks lp17 and thus *bbd18*) produces significant OspC (Figure 4-10D, lane 1) while there is no detectable

Figure 4-10: Putative DNA-binding motif of BBD18



B

BBD18/56 - 89	NIKALSDPNFSSSTNIAMNCINTFKLIVDIVNMQT
SopB/178 - 211	NISALADAENISRKIITRCINTAKLPKSVVALFS
ParB/167 - 200	SQKDIAAKEGLSQAKVTRALQAASAPEELVALFP

C

BBD18 - wt	NIKALSDPNFSSSTNIAMNCINTFKLIVDIVNMQTGENYDYD
BBD18 Δ 1	NI A ALSDPNF S ATNIAM N AINTF A LIVDIVNMQTGENY A YD
BBD18 Δ 2	AAA ALSDPNF AATA AAA AANCINTFKLIVDIVNMQTGENYDYD
BBD18 Δ 3	NI E ALSDPNFSSSTNIAMNCINTF E LIVDIVNMQTGENY R YR
BBD18 Δ 4	NIKALSDPNF S ATNIAMNCINTFKLIVDI A NMQTGENYDYD

D

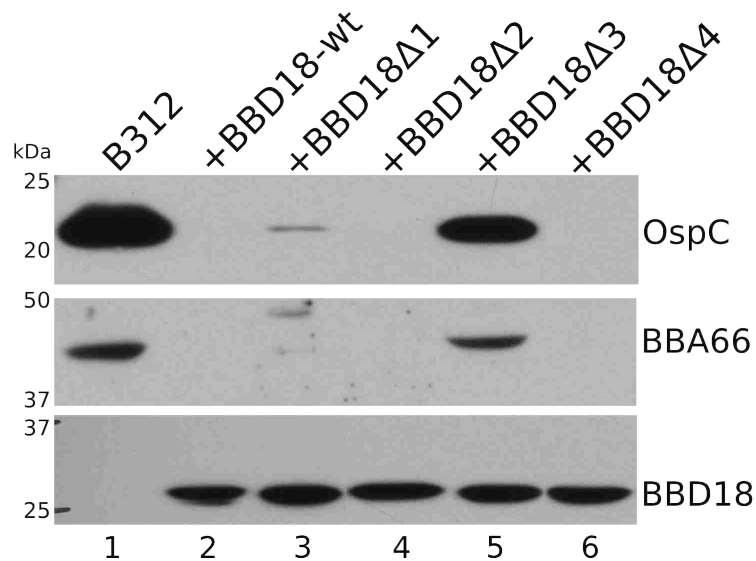


Figure 4-10 legend: **A.** The structural alignment of BBD18 (magenta) and the DNA-binding motif of SopB (green) and ParB (cyan) created by Xavier Ambroggio (OCCB, NIAID, NIH). Image created in Pymol and includes the DNA double helix as reference. The circled residues, serine (top circle, position 67 in BBD18) and valine (bottom circle, position 85 in BBD18) are conserved in all three proteins (see B). **B.** The primary amino acid alignment of the residues included in the structural mapping in A. The protein text colors correspond to the ribbon colors in A. The serine and valine residues conserved in BBD18, SopB, and ParB are shown in red text. **C.** The amino acid alignment of wt BBD18 (position 56-96) and the four BBD18 variants, which were created based on the structural model (A and B) and amino acid conservation within *Borrelia* species. Residue changes compared to wt BBD18 are indicated in red text. Note these changes include residues that are seven amino acids downstream of the carboxy terminal end of the structural model in A. Residues not listed in this partial alignment were not altered from those in wt BBD18. **D.** OspC (top), BBA66 (middle), and BBD18 (bottom) immunoblots of B312 harboring wt *bbd18* or *bbd18* variants (listed in C) expressed from the *flaB* promoter on a shuttle vector. Whole cell lysates of B312 without a shuttle vector (lane 1), B312/pBSV2**flaBp*-*bbd18* (wt, lane 2), or B312 harboring the *bbd18* variants, as indicated (lanes 3-6) were analyzed by immunoblot with antisera recognizing OspC (top), BBA66 (middle), or BBD18 (bottom). The size (kDa) based on the mobility of protein standards is denoted on the left and lane numbers are indicated on the bottom.

OspC in B312 harboring a shuttle vector carrying a constitutive wt *bbd18* gene (Figure 4-10D, lane 2). BBD18 Δ 2 and BBD18 Δ 4 were also able to fully repress OspC production in B312 (Figure 4-10D, lanes 4 and lane 6, respectively), indicating that the respective mutations in these *bbd18* variants did not abrogate BBD18 repressor activity. It was somewhat surprising that *bbd18* Δ 4 was still able to repress OspC because *bbd18* Δ 4 encoded a change in S67 and V85, the only two absolutely conserved residues in the motif shared by BBD18, SopB and ParB (Figure 4-10A, circled and Figure 4-10B in red). In contrast, BBD18 Δ 3 had a striking loss in repressor function compared to wt BBD18, as evident by the abundant synthesis of OspC protein when this BBD18 variant was introduced into B312 (Figure 4-10D, lane 5). OspC was also detected in B312 harboring pBSV2**flaBp-bbd18* Δ 1 (Figure 4-10D, lane 3), although to a lesser extent than with BBD18 Δ 3. We also investigated the effect of the *bbd18* variants on the production of another RpoS-dependent gene, *bba66*. Although BBA66 production was less than that of OspC in B312, it followed a similar pattern as OspC, where BBA66 was detected in wt B312 and when *bbd18* Δ 3 was expressed (Figure 4-10D, lanes 1 and 5, respectively). There is a faint band corresponding to BBA66 in B312/pBSV2**flaBp-bbd18* Δ 1 (Figure 4-10D, lane 3), but BBA66 is not detected in clones harboring the wt BBD18 or other *bbd18* variants. Using the BBD18-antibody, we were able to detect a BBD18 protein in all of the B312 clones harboring a *bbd18* gene expressed on a shuttle vector (Figure 4-10D, lanes 2-6).

Closer examination of the amino acid changes that resulted in loss of repression by BBD18 identified four residues that may be involved in BBD18 function; K58, K79, D94, and D96 were changed in the *bbd18* variants that had

reduced or absent repressor activity when expressed in B312 (Figure 4-10D). However, single mutations for each of these residues seem to have no effect, as each of these single residue *bbd18* variants was still able to fully repress OspC in B312 (Figure 4-11 and data not shown). A caveat to these studies is that since we are expressing the variants from a strong constitutive promoter, a slight phenotype might be masked in our qualitative OspC repressor assay. Regardless, the identification of a *bbd18* variant (*bbd18Δ3*) that produces BBD18 protein but does not repress OspC or BBA66, provides us with a tool to further investigate the function of BBD18.

***B. burgdorferi* that constitutively express *bbd18Δ3* remain infectious in mice**

The difficulty in inactivating *bbd18* in an infectious background led us to question whether *bbd18* might have a function other than regulating RpoS and RpoS-dependent genes, none of which are essential for *in vitro* growth. Since we have identified a *bbd18* variant that does not repress OspC (*bbd18Δ3*), we wanted to determine if constitutively expressing this repressor-inactive form of BBD18 in virulent *B. burgdorferi* would render them non-infectious, as we had observed with wt *bbd18*. To this end, we transformed S9 with both pBSV2*flaBp-*bbd18Δ3* or pBSV2*flaBp-*bbd18Δ4* as a positive control, since BBD18Δ4 retained repressor activity (Figure 4-10C and D).

Groups of 5 mice were inoculated with wt S9, S9/pBSV2*flaBp-*bbd18Δ3*, or S9/pBSV2*flaBp-*bbd18Δ4*. At 3 weeks, we found that mice infected with both S9 and S9/pBSV2*flaBp-*bbd18Δ3* had seroconverted to *B. burgdorferi* proteins, while mice infected with S9/pBSV2*flaBp-*bbd18Δ4* were not infected (Table 4-8). The infectivity of S9 constitutively expressing *bbd18* variants was inversely correlated

Figure 4-11: Single and double residue site-directed mutants of BBD18 retain the ability to repress OspC.

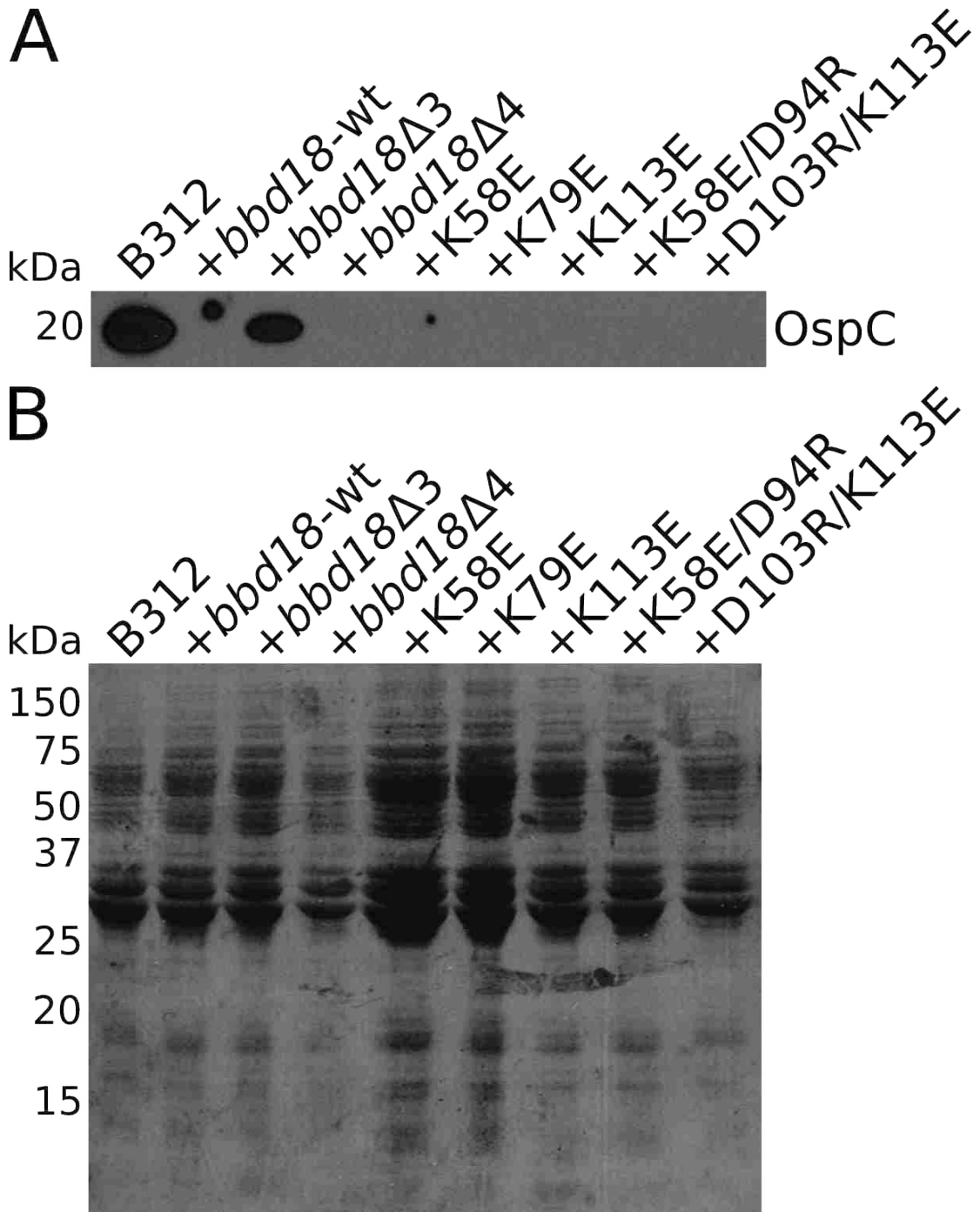


Figure 4-11 legend: **A.** OspC immunoblot of B312 containing single and double residue variants of *bbd18*. Whole cell lysates of B312 harboring *bbd18* variants, as indicated, were analyzed with antisera recognizing OspC. The estimated size of OspC relative to the mobilities of standards is indicated on the left. **B.** A duplicate gel of the samples from A was stained with Coomassie blue to assess the total protein in each sample. The size (kDa) based on the mobility of protein standards is denoted on the left.

Table 4-8 *B. burgdorferi* that constitutively express *bbd18Δ3* remain infectious in mice

strain	No. of sero-positive mice/no. of injected mice ^a	No. of ear biopsy reisolates/no. of injected mice ^b	No. of mouse reisolates/no. of injected mice (ear, bladder, joint) ^c
S9	4/4	4/4	4/4, 4/4, 4/4
S9/pBSV2*flaBp-bbd18Δ3	4/5	1/5	4/5, 4/5, 4/5
S9/pBSV2*flaBp-bbd18Δ4	0/5	0/5	0/5, 0/5, 0/5

^a seroconversion to *B. burgdorferi* proteins was assessed at 3 weeks post-inoculation

^b ear biopsies were taken at 3 weeks post-inoculation

^c mice were euthanized at 32 days post-inoculation

with their *in vitro* ability to repress OspC; BBD18Δ3 does not repress OspC (Figure 4-10D) and does not render S9 non-infectious when it is overexpressed (Table 4-8), whereas the opposite is true for BBD18Δ4. This experiment served as an important control for previous experiments with constitutively expressed wt BBD18 (Table 4-3) in that inappropriate expression of an irrelevant or non-functional protein does not intrinsically render *B. burgdorferi* non-infectious, since constitutive expression of only the BBD18 proteins with repressor activity (i.e. wt *bbd18* or *bbd18*Δ4) resulted in a non-infectious phenotype. Although these data do not rule out the possibility of a secondary function of BBD18, they do indicate that repression of RpoS-dependent genes is the reason that *B. burgdorferi* constitutively expressing *bbd18* are non-infectious in the mouse model. To our knowledge, this is the first report of a plasmid-encoded gene in *B. burgdorferi* that regulates RpoS, and thus, we conclude that BBD18 is a novel participant in the regulation of the complex Rrp2-RpoN-RpoS cascade, which is integral to the natural infectious cycle of *B. burgdorferi*.

Discussion

The complex life cycle of *B. burgdorferi* requires the spirochete to sense environmental changes and subsequently adjust gene expression and protein profiles as it transits from the tick vector to the mammalian host and vice versa. The best-studied example of differential gene expression in *B. burgdorferi* is the reciprocal expression of OspA and OspC during tick feeding (7). This groundbreaking observation led to the identification of a global regulatory cascade that includes a response regulatory protein, Rrp2 (57-59), and two alternative

sigma factors, RpoN, and RpoS (19, 20). It has been shown that the Rrp2-RpoN-RpoS cascade is responsible for the upregulation of genes necessary during the mammalian phase of the spirochete's enzootic cycle (20, 24, 25, 46, 60), including OspC (20, 23), a critical virulence factor during early infection (8, 11). Spirochetes lacking a functional OspC protein are non-infectious in mice (9, 10), but *B. burgdorferi* that constitutively express OspC are recognized and subsequently cleared by the mammalian adaptive immune response (12, 14, 15). Although the strict regulation of OspC has been well documented, the underlying mechanisms are still not entirely understood.

Previously we identified a plasmid-encoded protein, BBD18 that repressed expression of OspC in the high-passage non-infectious *B. burgdorferi* clone B312 (Chapter 3, (30)) Here, we have investigated the *in vivo* effect of constitutively expressing *bbd18* or deleting *bbd18* in low-passage, virulent *B. burgdorferi*. By expressing *bbd18* with a constitutive promoter, we confirmed that BBD18 can suppress the induction of OspC during tick feeding (Figure 4-3). This is significant since spirochetes within the tick midgut require OspC to be transmitted and subsequently establish infection in the mammalian host (8). Taking into account this essential role of OspC, it is not surprising that S9 harboring a constitutively expressed *bbd18* were not infectious by needle inoculation (Table 4-3) and tick bite (Table 4-4). This outcome suggests that BBD18 is important during infection, but does not elucidate its *in vivo* function. To this end, we undertook to inactivate *bbd18*. Based on the ability of BBD18 to repress OspC, we expected a *bbd18* mutant to establish infection in mammals but not persist if BBD18 were the critical *in vivo* *ospC* repressor for evasion of host immunity (12, 14). After many attempts (~2

years of effort by multiple people), we finally isolated a *bbd18* mutant in the virulent clone S9 (*S9Δbbd18*, see Figure 4-5). *S9Δbbd18* was non-infectious when inoculated into mice, but did not regain infectivity when *bbd18* was restored on full-length lp17 or a shuttle vector; this indicated that other secondary mutations were probably responsible for the non-infectious phenotype of *S9Δbbd18* (Table 4-7).

During the course of our studies, a report by Casselli *et al.* demonstrated that spirochetes containing a truncated version of lp17 in which *bbd16-25* had been deleted (and importantly, *bbd18*) were infectious in mice by needle inoculation. We repeated similar experiments using two different lp17 truncations that retain or lack *bbd18*. Similar to Casselli *et al.*, we found that *B. burgdorferi* lacking *bbd18* (and a large portion of lp17) was infectious by needle inoculation (Table 4-5). Liang and colleagues reported that downregulation of OspC in response to immune pressure occurs from day 17 onward (12, 14). However, both Casselli *et al.* and our lab found that *B. burgdorferi* containing lp17 truncations, which do not retain *bbd18*, were able to persist at least 8 weeks (~56 days) post-inoculation (Table 4-5). The ability of these BBD18 mutants to persist in mice beyond the point at which OspC would typically be downregulated indicates that *bbd18* is likely not the *in vivo* OspC repressor. In addition, these data imply that *bbd18* is not critical for *B. burgdorferi* survival or infection within mice. Thus, it remains unclear why inactivation of *bbd18* in wt *B. burgdorferi* presented such a challenge and why, once isolated, the *bbd18* mutant had accumulated secondary mutations that rendered it non-infectious in mice. Furthermore, the ease with which we were able to obtain a *bbd18* mutant in a high-passage, non-infectious *B. burgdorferi* clone also suggests the complex role of *bbd18* in a wt background.

The ability of constitutively expressed BBD18 to repress OspC and thereby prevent infection in mice, the strict conservation of *bbd18* in both Lyme disease and relapsing fever spirochetes, combined with the lack of an apparent role for BBD18 in mammalian infection, made us question the role of BBD18 and where BBD18 might function during the infectious cycle. To address the first question, we investigated the mechanism of BBD18 repression of OspC. The simplest mechanism to invoke is a direct interaction of BBD18 with the *ospC* promoter, which would prevent the transcription of *ospC*. Xu and colleagues described a putative operator site upstream of the traditional *ospC* promoter that is necessary for the downregulation of *ospC in vivo*. The persistence in mice of spirochetes of S9 lacking *bbd18* (S9/pGCB473) suggested that *bbd18* was not playing a role in the downregulation of OspC *in vivo*, but we wanted to identify the requisite *ospC* promoter elements for repression by BBD18. To this end, we utilized the *B. burgdorferi lacZ* reporter system (Chapter 2 and (32)) in combination with the high-passage *B. burgdorferi* clone B312, which produces abundant OspC *in vitro* (30, 32, 38). When BBD18 was introduced into B312 carrying *lacZBb** constructs with different versions of the *ospC* promoter, we found that BBD18 repression of *ospC* did not require the putative operator sequence (Figure 4-7). Furthermore, we determined that only the -35 and -10 regions of the *opsC* promoter were required for repression by BBD18 (Figure 4-7B).

It is well documented that the expression of *ospC in vivo* is dependent on the alternative sigma factor RpoS (17, 20, 23, 25) and we confirmed that this was also the case in the high-passage clone B312 (Figure 4-8). Since only the -35 and -10 regions of the *ospC* promoter (the region where RpoS and the RNA polymerase

holoenzyme bind) were necessary for BBD18-mediated repression of *ospC*, we wished to determine if the regulatory activity of BBD18 was through RpoS. Therefore, Dr. Daniel Dulebohn analyzed lysates of S9 carrying or lacking a constitutively expressed *bbd18* gene. He found that while RpoS and subsequently OspC, DbpA, and BBA66 were induced in response to a temperature-shift to 35°C, spirochetes harboring a constitutively expressed *bbd18* failed to make any of these proteins (Figure 4-9). These data indicate that BBD18 is likely repressing OspC (as well as DbpA and BBA66) through its effect on RpoS. Preliminary data by Dr. Dulebohn are most consistent with a post-transcriptional mechanism, suggesting that BBD18 either prevents translation of the *rpoS* transcript or targets degradation of RpoS protein.

The *B. burgdorferi* genome does not encode many commonly known genetic regulators (61). However, in recent years a number of regulatory elements that intersect with the central Rrp2-RpoN-RpoS pathway have been identified, including DsrA (18), BosR (62-64), and Hfq (65). DsrA is a small non-coding RNA that binds to a region upstream of the *rpoS* ORF, which leads to a conformational change. This conformational change in the *rpoS* transcript frees the Shine-Dalgarno site, allowing translation to occur (18). Lybecker and colleagues later identified a *B. burgdorferi* homolog of the RNA chaperone Hfq, which is typically required for the activity of sRNAs. Indeed, they found that the *B. burgdorferi* Hfq bound DsrA and the *rpoS* transcript (65).

BosR (BB0647) was originally annotated as a Fur (Ferric uptake regulator) homolog in *B. burgdorferi* (61) but was renamed after Boylan *et al.* determined that it activated the oxidative stress response in *B. burgdorferi*; BosR stands for Borreli

oxidative stress regulator (62). Two parallel studies using low-passage *B. burgdorferi* found that BosR was required for efficient RpoS production and thus the upregulation of *ospC* and other genes necessary for mammalian infectivity (63, 64). The inactivation of *dsrA* or *hfq* had similar effects on RpoS, and the *hfq* and *bosR* mutants were non-infectious in mice by needle inoculation.

It is interesting that the phenotype of *B. burgdorferi* overexpressing *bbd18* is similar to *B. burgdorferi* in which DsrA, BosR, or Hfq have been inactivated. Although it has been shown that BosR is required for transcription of *rpoS* (63, 66), DsrA and Hfq are involved in translational regulation of *rpoS* (18, 65). BBD18 might counteract the effects any of these factors, preventing production of RpoS protein.

To our knowledge, only the *bosR* mutant has been examined in ticks. Ouyang *et al.* detected similar numbers of spirochetes in *I. scapularis* nymphs after they were microinjected with wt *B. burgdorferi* or the *bosR* mutant and subsequently fed on naïve mice (63). This is again similar to phenotype of S9 harboring a constitutively expressed *bbd18*, where there was not a significant difference in spirochete load in larval or nymphal ticks (Figure 4-2). The lack of a detectable phenotype in ticks infected with spirochetes that either lack *bosR* or that inappropriately express *bbd18* is not surprising since *rpoS* is not expressed by *B. burgdorferi* in flat ticks (22). In addition, neither RpoN, nor RpoS is required for persistence within ticks (60) In fact, this lack of *rpoS* in flat ticks indirectly suggests a possible role for BBD18 in the transition from mammal to tick, in that BBD18 could repress the production of RpoS protein in feeding ticks as spirochetes are acquired from an infected host before *rpoS* transcription is downregulated by other mechanisms. Since RpoS is also required for the repression of *ospA* as spirochetes

transmit to the mammalian host (22), the repression of RpoS by BBD18 as *B. burgdorferi* transition back to the tick vector would allow OspA to be de-repressed, resulting in the spirochetes' effective colonization of the tick midgut (67). The ability of *B. burgdorferi* lacking *bbd18* to infect mice also indirectly supports a role for BBD18 in the tick vector rather than the mammalian host. However, we did not succeed in an initial attempt to test this hypothesis due to a technical problem with larval tick feeding.

Although *B. burgdorferi* lacking *bbd18* were infectious in mice, Casselli *et al.* reported that their *B. burgdorferi* clone harboring a truncated form of lp17 did not colonize the mouse bladder. We found that spirochetes carrying two different lp17 truncations, one of which retained *bbd18*, did not efficiently colonize the mouse bladder or joint tissue (Table 4-5), likely ruling out a role for BBD18 in dissemination and colonization of distal tissues. The number of differentially regulated genes encoded on lp17 (22, 59, 68), the high conservation of most lp17 genes among *B. burgdorferi* strains (69), and the fact that lp17 is rarely lost, all point to the importance of additional genes on lp17 during the infectious cycle.

Our finding that BBD18 represses the production of RpoS in wt *B. burgdorferi* is interesting in that two groups previously implicated the Rrp2/RpoN/RpoS in regulating *bbd18*. Caimano and colleagues reported that microarray analysis of spirochetes grown in dialysis membrane chambers (DMCs) within a rat peritoneum suggested that *bbd18* was repressed by RpoS, but this was not confirmed with qRT-PCR (22), and *bbd18* was not listed as being repressed by RpoS or RpoN in a previous microarray report (60). In a separate study, microarray data indicated that *bbd18* was repressed by Rrp2 but not RpoS (68). Considering the inverse roles of

BBD18 and Rrp2 on RpoS activity, it is possible to imagine a scenario in which Rrp2 represses BBD18 in order to activate RpoS. *rpoS* is transcribed through the coordinated roles of Rrp2 and the alternative sigma factor RpoN. Since it is widely accepted that phosphorylation of Rrp2 serves as the trigger for spirochetes transitioning into the mammalian-phase of the life cycle (57), it would make sense that Rrp2 would also repress expression of *bbd18*, preventing BBD18 from repressing the translation of *rpoS*. However, if we entertain the initial suggestion that RpoS is responsible for the repression of BBD18, the question becomes how would *B. burgdorferi* produce enough RpoS protein in the presence of BBD18, if BBD18 can prevent the production or accumulation of RpoS. Additional studies are necessary to investigate the regulation and timing of *bbd18* expression. Undoubtedly, regulation in *B. burgdorferi* is complicated, but BBD18 in concert with BosR, DsrA, Hfq, Rrp2, and RpoN might help modulate specific levels of RpoS needed throughout the *B. burgdorferi* life cycle.

In order to gain a better understanding of a possible role for BBD18 during the infectious cycle, we wanted to investigate how BBD18 might function. Since we were unable to find any homologs for BBD18 outside of the *Borrelia* genus, we collaborated with Xavier Ambroggio, a Computational Structural Biologist from the Office of Cyberinfrastructure and Computational Biology (NIAID, NIH). He created a partial model for BBD18 through a structural alignment with the DNA-binding motifs of ParB and SopB from *E. coli*, with which they share weak similarity. ParB and SopB are best known for their roles in the partitioning of plasmids and chromosomes and are highly conserved in both Gram-positive and Gram-negative bacteria (reviewed in (70)). In addition, it has been shown that ParB and SopB also

function as autoregulators controlling their own transcription (71, 72). Given the data suggesting that BBD18 represses *rpoS* post-transcriptionally, we could imagine a BBD18 domain that interacts with nucleic acids. To test the hypothesis that BBD18 repression requires this putative DNA-binding domain, we designed synthetic *bbd18* variants with discrete amino acid substitutions within this region and determined if the variants retained repressor activity of OspC. We found one variant, BBD18 Δ 3, that was not able to repress OspC (Figure 4-10D). Four amino acids substitutions distinguish BBD18 Δ 3 from wt BBD18 (Figure 4-9C). It seems unlikely that mutating four random residues within the entire BBD18 protein would have a similar effect on OspC repression, which leads us to tentatively conclude that there is some validity to this structural prediction of BBD18. We anticipated that we could further delineate the amino acids that were critical for BBD18 function by mutating each of these four residues individually, but this experiment did not yield any additional information, as individual or double amino acid substitutions did not abrogate repressor activity (Figure 4-11 and data not shown). One caveat is that these *bbd18* variants were expressed from the constitutive *flaB* promoter and a slight defect in BBD18 function may be compensated by the amount of the variant protein produced.

We also attempted to infect mice with spirochetes that overexpress the BBD18 variants. We predicted that *B. burgdorferi* overexpressing a BBD18 variant that could not repress OspC would be infectious in mammals. We also hypothesized that we might be able to identify a secondary function of BBD18 within the life cycle of *B. burgdorferi* by using a *bbd18* variant that lacked the ability to repress *ospC* but still attenuated infectivity in mice. However, we found that the ability to prevent

infection of mice was strongly correlated with the *in vitro* repressor activity of BBD18 (Tables 4-3 and 4-8). These data also suggest that BBD18 does not have a critical unrelated function *in vivo*. This experiment also served as an important control for the possibility that overexpressing any protein in *B. burgdorferi* above its normal levels might attenuate the infectivity of the spirochetes. However, these data argue against that interpretation, as overexpressing a non-repressing variant (*bbd18*Δ3) did not intrinsically alter the infectivity of *B. burgdorferi*.

We have provided evidence that BBD18 can alter the infectivity of *B. burgdorferi* by repressing critical virulence factors like OspC through its affect on RpoS. To our knowledge, BBD18 is the first example of a plasmid-encoded gene that affects the central Rrp2-RpoN-RpoS regulon. Given this new information, it is interesting that Lyme disease and relapsing fever spirochetes, which display strikingly different life cycles and infect different vectors, both retain *bbd18*. Thus, we propose that the conserved BBD18 protein assists in the precise gene regulation that is critical for the survival and infection of spirochetes within the Lyme disease and relapsing fever families. Ongoing experiments will determine the specific target of BBD18 and define the conditions in which *bbd18* is expressed.

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APPENDIX A: Library screen for the *ospA* repressor

Introduction

While OspC is upregulated as *Borrelia burgdorferi* establishes infection in the host (see introduction and chapters 1-3), OspA is produced when *B. burgdorferi* colonizes the tick (1), where it is required for colonization and thus survival during the infectious cycle (2). However, if present in the mammalian host, OspA is a potent antigen and stimulates the production of bactericidal antibodies. In fact, recombinant OspA is the only vaccine ever licensed to prevent Lyme disease in humans by blocking transmission from the tick vector (3)

Although OspA is strictly repressed when spirochetes are transmitted from the tick to the mammalian host, the specific mechanisms and *B. burgdorferi* genes controlling *ospA* expression have not been identified. Others have shown that *ospA* is regulated at the level of transcription (4), suggesting that the *B. burgdorferi* genome encodes a specific *ospA* repressor.

In order to identify the genes necessary for repression of *ospA*, I designed an expression library screen using an *ospA* promoter-*lacZ* reporter construct. Although most of the randomly cloned inserts would not affect *lacZ* expression from the *ospA* promoter, those inserts encoding proteins that normally repress *ospA* during the infectious cycle should repress the production of β -galactosidase.

I created a complete *B. burgdorferi* genomic DNA library containing small (100-300bp) inserts under expression of the constitutive *flaB* promoter and introduced this library into the *B. burgdorferi* clone that expresses *lacZ* from the *ospA* promoter. Expression of these library clones failed to affect *lacZ* expression

from the *opsA* promoter or endogenous OspA production in *B. burgdorferi*. Multiple attempts were made to construct a middle-sized insert (300-850bp) and a large-sized insert (850bp-2kb) library, but technical difficulties prevented successful completion and the project was not continued.

Materials and Methods

All enzymes were purchased from New England Biolabs (NEB, Ipswich, MA) and all chemicals and other materials were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Bacterial Strains and Growth Conditions

BSKII medium (5) for culture of *B. burgdorferi* was made with CMRL 1060 lacking phenol red (US Biologicals, Swampscott, MA). *B. burgdorferi* strain B31-A34, (Table A-1, referred to as A34 from now on)(6) was grown to mid log phase, enumerated using dark-field microscopy with a Petroff-Hausser counting chamber and prepared for electroporation as described previously (7). Transformants were selected using gentamicin at a concentration of 40µg/mL and/or kanamycin at 200µg/mL. All *B. burgdorferi* cultures were grown at 35°C and plates were incubated under 2.5% CO₂.

The *Escherichia coli* strain Top10 (Invitrogen, Carlsbad, CA) was used for routine cloning and propagation of the libraries. *E. coli* was plated on LB agar (kanamycin at 30 µg/mL , or gentamicin at 5 µg /mL) and liquid cultures were grown in LB broth supplemented with the appropriate antibiotics (kanamycin at 100µg/mL or gentamicin at 10 µg/mL).

Table A-1: Strains and Plasmids used in Appendix A

Name	Description	Reference
<i>B. burgdorferi</i> strains		
B31-A3	Infectious derivative of wild-type B31-MI, lacks cp9	(8)
B31-A34	derivative of non-infectious clone B31-A that also lacks lp56; high transformation efficiency	(6)
A34-XAZ1	derivative of A34 in which the <i>ospA</i> promoter- <i>lacZBb</i> * cassette was inserted into cp26 by allelic exchange; gentamicin resistant	this study
plasmids		
pBH-lacZBb*	promoter-less <i>Borrelia</i> codon-optimized <i>lacZBb</i> gene on the shuttle vector pBSV2G with BspHI restriction site added upstream of <i>lacZBb</i>	(9)
pBHospAp-lacZBb*	pBH-lacZBb* with the <i>ospC</i> promoter driving expression of <i>lacZBb</i>	this study
pXAZ1	allelic exchange vector to add <i>ospAp-lacZBb</i> * and <i>flgBp-aacC1</i> between <i>resT</i> and <i>chbC</i> on cp26	(10); this study
pBSV2*flaBp	derivative of <i>B. burgdorferi</i> shuttle vector pBSV2 that contains the constitutive <i>flaB</i> promoter followed by a unique EcoRI site; vector used to build <i>B. burgdorferi</i> expression libraries	(11); Amit Sarkar,, unpublished

ospA promoter-lacZ fusion on cp26

The *ospA* promoter was amplified from B31-A3 (Table A-1) genomic DNA (gDNA) using forward primer **CGCGGATCCCCTGAAAGTCCCAAACTGGG** and reverse primer **CCCCCTCATGATATATTCTCCTTTTAT**, which added BamHI and BspHI sites (in bold), respectively, to the 5' and 3' ends, and cloned into pCR2.1 (Invitrogen). The insert, confirmed by sequencing, was digested with BamHI and BspHI and ligated into appropriately digested pBH-lacZBb* (9), creating pBHospAp-lacZBb* (Table A-1). To construct an allelic exchange vector that would mediate insertion of the *ospAp-lacZBb* fusion into cp26, the fragment containing the *ospA* promoter driving *lacZBb* and the *flgBp-aacc1* (conferring gentamicin resistance) was excised from pBHospAp-lacZBb* (Table A-1) with restriction enzymes MluI and KpnI, and the overhangs were filled-in or removed, respectively, with T4 DNA polymerase. This fragment was ligated into the Topo-XL-based plasmid used to make pKK83 (10), which contains the intergenic region between *chbC* and *resT* and a unique BglII site. The resulting plasmid, termed pXAZ1 (Table A-1), was used to transform *B. burgdorferi* clone A34 (Table A-1).

Library construction

Genomic DNA was isolated from a mid-log phase culture of B31-A3 (Table A-1), a low-passage infectious clone of *B. burgdorferi* that retains all plasmids except cp9 (8), using the Genomic DNA Kit (Qiagen, Valencia, CA). gDNA was partially digested for 1 hour with dilutions (1, 1:2, 1:4, 1:8, and 1:16) of the restriction enzyme Tsp5091. Tsp5091 recognizes the site AATT (5'-3') and therefore cuts the AT-rich *B. burgdorferi* genome frequently. To terminate restriction endonuclease activity, EDTA was added to the samples. Small aliquots were analyzed on an

agarose gel to visualize the extent of digestion (Figure A-1). All samples were combined and separated on a 1X TAE 0.8% agarose gel using 1X TAE buffer containing 1mM guanosine 5'-triphosphate. DNA bands corresponding to 100-300bp, 300-850bp, and 850bp-2kb were purified using the MinElute Gel Extraction Kit (Qiagen). The shuttle vector pBSV2*flaBp (Amit Sarkar, unpublished, (11)) was digested with EcoRI, end phosphates removed with Antarctic Phosphatase, and purified from an agarose gel as described above. 500ng of linearized vector and equivalent molar amounts of insert DNA fragments were used for ligations (1:1 ratio of vector to insert). For example, for 500ng of the 6700bp vector, 15ng of the 100-300bp fragments were used. 1/10 of the ligation mix was used to transform electrocompetent Top10 *E. coli* (Invitrogen). Aliquots of transformed *E. coli* were plated on LB-agar containing kanamycin to determine the number of transformants in the library and to screen for presence of inserts by PCR. Plasmid DNA from the complete library was used to transform A34-XAZ1 (Table A-1), the derivative of *B. burgdorferi* A34 carrying the *ospA* promoter-*lacZ* fusion on cp26.

Sequencing DNA inserts

For both *E. coli* and *B. burgdorferi* colonies, library clone inserts were amplified directly from colonies PCR using a *flaB* promoter 3' out primer (TTTTATCATGGAGGAATGATATATGATTATC) and a library reverse primer (ACTTTATGCTTCCGGCTCGT). PCR reactions were diluted 1:10 and used directly as templates for Sanger sequencing with the same primers. The *B. burgdorferi* genes corresponding to the insert sequences were identified using BLAST (NCBI) against the *B. burgdorferi* B31 genome in order to assess the variability of inserts present within the library.

Screening for β -galactosidase

Approximately 400 μ L 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; Roche Diagnostics, Indianapolis, IN) dissolved in DMSO (10mg/mL) was spread on *B. burgdorferi* plates after colony formation. Plates were incubated overnight at 37° before assessing β -galactosidase activity.

Electrophoresis and Immunoblotting

Endogenous OspA production in *B. burgdorferi* was assessed using SDS-PAGE as previously described (12). Briefly, *B. burgdorferi* was harvested from late-log phase cultures grown in BSKII and washed twice in HEPES-NaCl (HN) buffer (50mM each; pH 7.6) before lysis in Laemmli sample buffer containing 2- β -mercaptoethanol. Approximately 10⁷ bacteria were loaded per lane in a 10% acrylamide gel. Protein gels were stained with Coomassie Brilliant Blue (Bio-Rad) or prepared for immunoblotting.

For Western Blots, proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad Trans blot Transfer Medium, Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20). Monoclonal mouse anti-OspA (H5332) (13) at 1:200, monoclonal mouse anti- β -galactosidase (Santa Cruz Biotechnology Inc.) at 1:100, and monoclonal mouse anti-FlaB (H9724) (14) at 1:200 were used to probe membranes. Primary antibodies were diluted in TBST with or without 5% milk. A horseradish-peroxidase conjugated secondary anti-mouse antibody was diluted 1:10,000 in TBST with 5% milk. Immunoreactivity was visualized using the Supersignal West Pico Chemiluminescence substrate (Thermo Scientific) and X-ray film (Labscientific Inc., NJ).

Results

ospAp-lacZBb* fusion in B. burgdorferi

I have previously demonstrated that the *lacZ* gene, which codes for β -galactosidase activity, accurately reflects endogenous gene expression in *B. burgdorferi* (15). Thus, I decided to utilize this genetic tool in my library screen for the *ospA* repressor. To stably introduce an *ospA* promoter-*lacZ* fusion into *B. burgdorferi* without the possibility of the fusion gene being lost, I created a suicide vector to insert *ospAp-lacZBb* along with the *flgBp-aacc1* cassette (conferring gentamicin resistance) by allelic exchange between the *resT* and *chbC* genes on cp26. Foreign DNA has been inserted into this locus before (10) without affecting *in vitro* growth or infectivity during the mouse-tick cycle. By inserting the *ospA* promoter-*lacZ* fusion into the endogenous cp26 plasmid, I was free to use a stable and highly transformable cp9 based shuttle vector (pBSV2) for library construction. Furthermore, cp26 is presumably at a lower copy number than pBSV2, which should minimize any gene dosage effects of the hypothetical repressor on the *ospA* promoter. To ensure that I would get a high frequency of transformation with the libraries plasmids produced in *E. coli*, I used the *B. burgdorferi* high passage clone, A34 (Table A-1) (6), which lacks many plasmids and importantly two restriction modification systems that severely reduce transformation frequency (16-18). A34 was transformed with the allelic exchange vector pXAZ1 (Table A-1), adding *ospAp-lacZBb** to cp26 (see above). One transformant, which showed β -galactosidase activity on solid medium when screened with X-gal, was chosen to take forward. This clone, referred to as A34-XAZ1 (Table A-1), exhibited stable and consistent β -galactosidase activity and did not display any growth defects in liquid or solid

media.

Plasmid expression libraries in *E. coli*

A derivative of the *B. burgdorferi* shuttle vector, pBSV2, which replicates in both *E. coli* and *B. burgdorferi* and has the constitutive *flaB* promoter followed by a unique EcoRI site (Amit Sarkar, unpublished (11)), was used to construct the *B. burgdorferi* expression libraries. This plasmid, pBSV2**flaB*p (Table A-1) was linearized with EcoRI and the terminal phosphates removed to minimize empty vector self-ligation. To prepare the *B. burgdorferi* inserts, genomic DNA was isolated from a low-passage infectious clone, B31-A3 (Table A-1), which retains all of the endogenous plasmids (except cp9) and presumably encodes the *ospA* repressor. gDNA was partially digested with dilutions of Tsp5091 (Figure A-1). DNA digested with Tsp5091 has EcoRI-compatible 3' overhangs, but Tsp5091 lacks the C/G in the recognition sequence (i.e. the 5'-3' recognition site for EcoRI is G^εAATTC, while Tsp5091 recognizes ^εAATT, where ^ε represents the cleavage site), making Tsp5091 restriction sites more frequent in the A/T rich *B. burgdorferi* genome. Vector and insert DNA were separated on agarose gels using TAE buffer containing guanosine 5'-triphosphate to minimize damage to DNA during UV-exposure. Digested genomic DNA was divided into 3 different size fragments to create 3 different expression libraries. The small insert size (100-300bp) was chosen to include DNA encoding any small proteins or RNAs that might repress the *ospA* promoter while eliminating any degraded DNA smaller than 100bp. These smaller fragments will likely ligate into the vector more efficiently but will require more clones to obtain full *B. burgdorferi* genome coverage. A middle-sized insert of 300-850bp was picked to cover entire or mostly complete open reading frames (ORFs), as the average ORF

Figure A-1: Partial digestion of B31-A3 gDNA

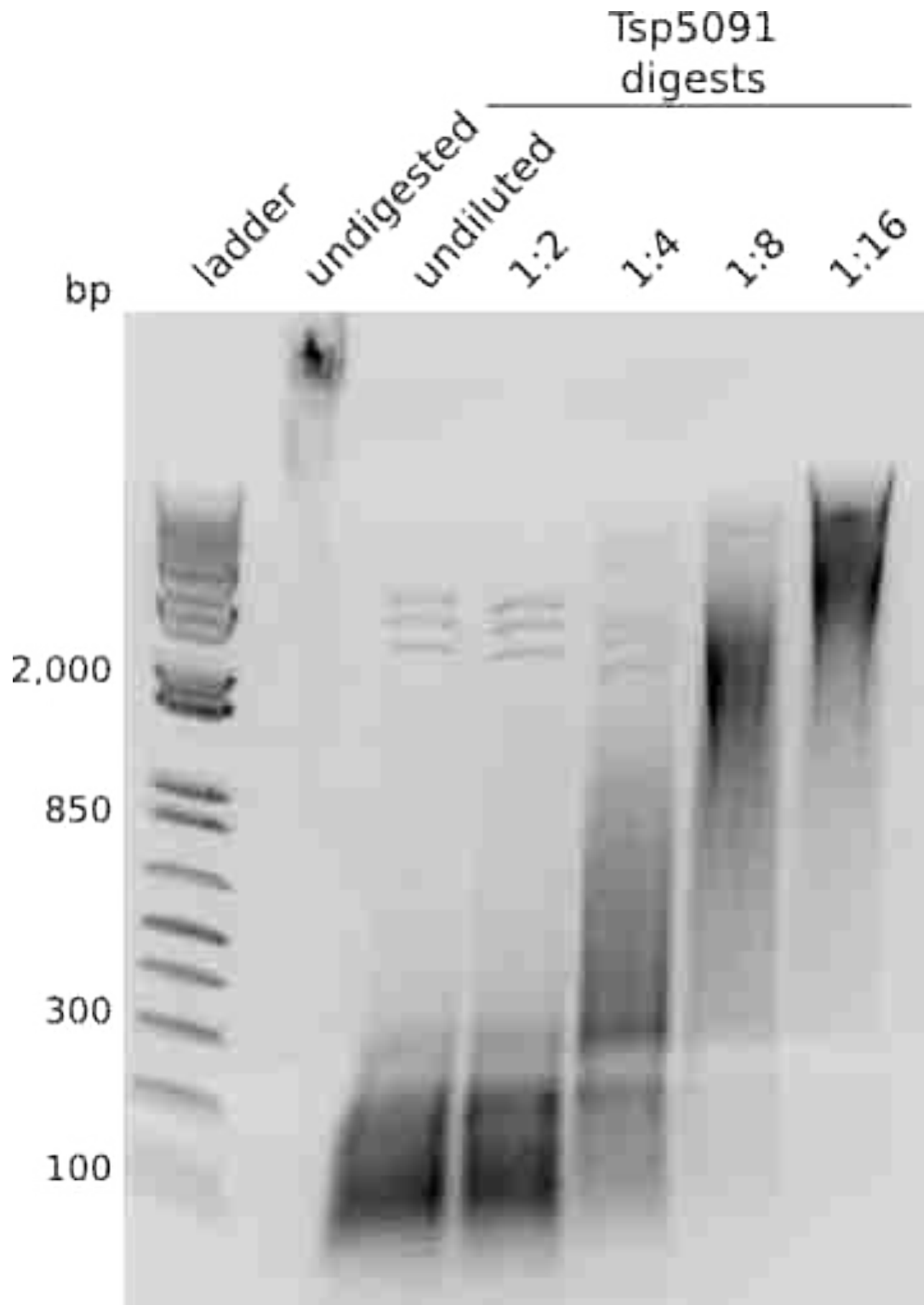


Figure A-1 legend: A 1% TAE agarose gel was used to separate partial digestion of B31-A3 gDNA. Lanes are as indicated. Dilutions of Tsp5091 (1:2, 1:4, 1:8, 1:16) or undiluted Tsp5091 were used in restriction digest reactions for 1 hour, which randomly cut the gDNA to varying degrees compared to undigested gDNA (lane 2). The 1kB plus DNA ladder from Invitrogen (lane 1, ladder) was used as a guide in purification of the small-, medium-, and large-sized inserts from the agarose gel to be used in library construction. The three distinct bands present in the undiluted, 1:2 and 1:4 TSp5091 digests lanes represent the A/T-rich region of nonrandom repeats from lp21, which contains few restriction sites (19).

size in *B. burgdorferi* is 507bp for the plasmids and 992bp for the chromosome (20). Lastly, a large-sized insert (850bp-2kB) was chosen to cover any large ORFs and to provide additional genome coverage. Equivalent amounts of gel-extracted vector and insert DNAs were used for ligations. *E. coli* transformed with ligations were combined and propagated to produce plasmid DNA after aliquots were plated to assess genome coverage and insert sequences. For the small sized insert library, 3,000-20,000 transformants were recovered from 1/10 of the ligation mix. Of these clones, 80-90% contained inserts. After 5 *E. coli* transformations (approximately 42,000 independent transformants), 3x coverage of the *B. burgdorferi* genome was obtained.

Introducing and characterizing the libraries in A34-XAZ1

Although *E. coli* has been used to study gene regulation of *B. burgdorferi* genes, I wanted to screen for the *ospA* repressor in its native bacterium to facilitate expression and folding of the putative repressor and to control for any accessory cofactors required for repression of *ospA*. 10-20ug of plasmid DNA from the complete small-size insert library in *E. coli* was used to transform A34-XAZ1, the *B. burgdorferi* clone containing the *ospA* promoter-*lacZ* fusion on cp26 (Table A-2). Transformed *B. burgdorferi* was allowed to recover in 10mL BSK II overnight before the addition of antibiotics. The following day, half of the culture was used to make a permanent glycerol stock and the remaining half was plated in solid media containing antibiotics. X-gal was added to plates a few days after colonies were visible. Although some colonies remained white after treatment with X-gal, those clones still produced OspA protein when lysates were probed with an anti-OspA antibody. Random colonies were screened by PCR to determine the proportion of

Table A-2: Representative inserts in small library *Borrelia burgdorferi* transformants

<u>clone</u>	<u>alignment</u>	<u>size-bp</u>	<u>gene*</u>
1	lp38	94	
	chromosome	216	diphosphate--fructose-6-phosphate 1-phosphotransferase
2	lp28-2	137	
3	chromosome	206	molecular chaperone DnaK
	cp32-7	126	
4	chromosome	233	protein kinase C1 inhibitor (pkcI)-Mg ²⁺ transport protein (mgtE)
5	chromosome	109	hypothetical protein
6	chromosome	155	hypothetical protein
7	lp25	74	
	lp36	72	
8	chromosome	168	purine-binding chemotaxis protein (cheW-2)
9	chromosome	184	N-acetylmuramoyl-L-alanine amidase, putative
10	chromosome	318	PTS system, maltose and glucose-specific IIABC component
11	lp28-2	291	
12	chromosome	113	hypothetical protein
13	chromosome	117	xylulokinase (xylB)
14	chromosome	251	hypothetical protein
15	chromosome	205	phnP protein (phnP)
16	cp32-8	160	
	cp32-7	160	
	cp32-3	160	
	cp32-1	160	
17	chromosome	163	3-hydroxy-3-methylglutaryl-CoA synthase
	chromosome	134	50S ribosomal protein L9
18	chromosome	149	hypothetical protein
19	chromosome	127	membrane-associated protein p66
20	chromosome	188	flagellar MS-ring protein
21	chromosome	147	DNA-directed RNA polymerase subunit beta'
22	chromosome	135	lysyl-tRNA synthetase
23	chromosome	181	50S ribosomal protein L5
24	lp25	159	
25	chromosome	179	ribonuclease Z
26	chromosome	173	hypothetical protein
27	chromosome	211	ATP-binding protein (ylxH-2)
28	lp28-2	194	
	lp28-3	194	
	lp28-4	180	
	lp38	180	
	lp36	60	
29	lp36	100	
	lp56	99	
	lp21	74	
30	chromosome	151	elongation factor Tu

31	chromosome	89	chromate transport protein, putative - hypothetical protein
32	lp28-1	93	different portions of lp28-1 with variable identity- vlsE?
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	
	lp28-1	93	

* inserts that mapped to regions without annotated genes are left blank

clones containing an insert and the resulting PCR fragments were subsequently sequenced to identify the gene(s) covered (Table A-2). The identities of these inserts were random and covered both the chromosome and plasmid-encoded genes. Some sequenced inserts displayed homology to regions on several plasmids (see clones 7, 16, 28, and 29 in Table A-2), indicating genes in a paralogous family that are present on many of the *B. burgdorferi* plasmids, whereas others had homology to 2 different regions within the genome, indicating the presence of 2 distinct inserts (Table A-2, clones 1 and 3), and one clone (Table A-2, clone 32) mapped to different sites on the same plasmid, presumably indicating several copies of the same gene or silent cassettes. Many of the plasmid borne sequences mapped to unannotated regions, in which the gene column was left empty in Table A-2. An average of 4,000 independent clones arose from each *B. burgdorferi* transformation. Sequencing results (Table A-2) implied that clones were both random and variable, so after 10 successful transformations, I assumed at least 3X genome coverage in *B. burgdorferi* had been achieved for the small-insert library.

Expansion of the middle-sized insert library

A similar protocol was used to create the middle-sized insert library. The initial characterization of the library inserts in *E. coli* indicated that 3X coverage of the *B. burgdorferi* genome had been obtained. However, after transforming the library DNA into A34-XAZ1 (Table A-1), transformants primarily contained two distinct inserts. These inserts mapped to two regions in the *B. burgdorferi* chromosome, spanning base pairs 454832-455306 and 887773-888449. The first mapped to an internal 3'-5' region of gyrase B (*gyrB*). The later region mapped to phosphomannomutase (*cpsG*) and excinuclease ABC subunit B (*uvrB*), including the

intergenic region between the two genes. I hypothesized that expression of these particular inserts provided *B. burgdorferi* with a selective advantage and that resulted in the higher transformation frequencies of these particular library clones, but this was not the case, as neither of these clones transformed into *B. burgdorferi* more efficiently than the empty pBSV2*flaBp shuttle vector. I subsequently discovered that the plasmid DNA isolated after the expansion of this library in *E. coli* predominately contained those two specific inserts. Several attempts were taken to remake this library but none produced 3X genome coverage. Often, the vector remained uncut or self-ligated without an insert fragment.

Large-sized insert library

Similar difficulties were encountered when making the large-sized insert library. After transforming the ligation mix into *E. coli*, it was determined that less than 50% of clones screened by PCR contained inserts. Although 3X *B. burgdorferi* genome coverage was eventually achieved, the high proportion of empty vector hindered any attempts to sub-clone this library into *B. burgdorferi*.

Conclusions

I was able to demonstrate the feasibility of creating a *B. burgdorferi* expression library in *E. coli* and subsequently moving it into *B. burgdorferi* to screen for repression of *ospA*. However, inserts in this initial library were small in size and none of the small-insert clones repressed *lacZ* from the *ospA* promoter. I intended to construct three different genomic libraries, but technical difficulties prevented the completion of the middle-sized and large-sized insert libraries. The most prevalent complication was the high rate of recovering pBSV2*flaBp (Table A-1) without an

insert. This was due to both undigested vector coming through and re-ligation of the empty shuttle vector, despite using new EcoRI enzyme and Antarctic phosphatase to remove end phosphates, respectively. Additionally, the middle-sized library upon expansion in *E. coli* primarily contained two distinct inserts. Although these two inserts did not transform more efficiently into *B. burgdorferi*, it is likely that they were favored in *E. coli* and thus out-competed other *B. burgdorferi* inserts during overnight growth, since characterization of the library directly after transformation revealed random inserts of varying sizes had been ligated into the pBSV2*flaBp shuttle vector. This was unfortunate due to time-limitation but a few changes in the protocol might make this project successful.

First, in order to assure that the vector was efficiently digested and to minimize re-ligation, I would use a linearized vector made available by a commercial retailer. In addition or alternatively, I would use a counterselectable marker to eliminate the recovery of empty shuttle vector. *rpsL* has been described as a counterselectable marker for use in *B. burgdorferi* (21), as well as in other bacteria including *E. coli* (22). The use of streptomycin and a sensitive *rpsL* gene would limit the recovery of the empty shuttle, which would greatly aid in the recovery of a complete large-sized insert library. Additionally, using a host other than *E. coli* to propagate the expression libraries could possibly reduce any preferential expansion of certain *B. burgdorferi* genes in *E. coli*. Perhaps a more promising approach would be to use a constitutive *B. burgdorferi* promoter, which is not recognized in *E. coli*, to express the *B. burgdorferi* gDNA inserts, although, I am not aware of any *B. burgdorferi* promoters that satisfy these criteria. Lastly, I subcloned the libraries created in *E. coli* to screen for the *ospA* repressor directly in *B.*

burgdorferi. I chose to use *B. burgdorferi* B31 clone A34 because it is highly transformable with shuttle vectors, i.e. I could achieve full-library coverage easier than if I had used a low-passage infectious clone. However, A34 does not retain all of the plasmids nor has it been demonstrated that A34 can repress OspA, thus, it might not be the best background for screening my expression libraries for the putative *ospA* repressor. During this project, a clone of *B. burgdorferi* that is both infectious and is highly transformable with shuttle vectors was identified. This clone, S9 (23), lacks linear plasmid 56 (lp56) and the gene *bbe02*, both of which encode restriction modification genes (16-18). S9 has been taken through the entire infectious cycle of *B. burgdorferi* and thus produces and then represses OspA at the appropriate times. Using S9 as the background to screen for the *ospA* repressor would ensure that all factors necessary for repression are available.

Although my attempts at identifying an *ospA* repressor were unfruitful, defining the mechanisms involved in OspA regulation remains a significant area of interest. Knowing how OspA is repressed would open a new door to understanding how *B. burgdorferi* senses and adapts to the different environments during its infectious cycle and could perhaps shed light on how other organisms regulate their gene expression.

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