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BosR (BB0647) governs virulence expression in *Borrelia burgdorferi*

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Summary

Borrelia burgdorferi (*Bb*), the Lyme disease spirochete, encodes a potential ferric uptake regulator (Fur) homologue, BosR (BB0647). Thus far, a role for BosR in *Bb* metabolism, gene regulation, or pathogenesis has not been determined, largely due to the heretofore inability to inactivate *bosR* in low-passage, infectious *Bb* isolates. Herein, we report the generation of the first *bosR*-deficient mutant in a virulent strain of *Bb*. Whereas the *bosR* mutant persisted normally in ticks, the mutant was unable to infect mice, indicating that BosR is essential for *Bb* infection of a mammalian host. Moreover, transcriptional profiling of the *bosR* mutant showed that a number of genes were either positively or negatively influenced by BosR deficiency, suggesting that BosR may function both as a global repressor and activator in *Bb*. Strikingly, our study showed that BosR controls the expression of two major virulence-associated *Bb* lipoproteins, OspC and DbpA, likely via an influence on the alternative sigma factor, RpoS. This study thus not only has elucidated another key virulence gene of *Bb*, but also provides new insights into a previously unknown layer of gene regulation governing RpoS in *Bb*.

Introduction

Lyme disease is the most common arthropod-borne disease in the United States. *Borrelia burgdorferi* (*Bb*), the causative agent of Lyme disease, survives in nature through a complex life cycle including an arthropod vector (*Ixodes* tick) and a variety of mammalian hosts (Burgdorfer *et al.*, 1982; Steere *et al.*, 1983). In order to maintain its life cycle in nature, *Bb* must adapt to and transit between these two disparate environments by altering its gene expression profile in response to various environmental stimuli. Studies have shown that certain signals, including temperature, pH, cell density and other unknown host factors, modulate *Bb* gene expression (Akins *et al.*, 1998; Anguita *et al.*, 2003; Brooks *et al.*, 2003; Burtnick *et al.*, 2007; Caimano *et al.*, 2005; Caimano *et al.*, 2007; Hyde *et al.*, 2007; Lybecker and Samuels, 2007; Ojaimi *et al.*, 2003; Revel *et al.*, 2002; Schwan, 2003; Schwan *et al.*, 1995; Singh and Girschick, 2004; Stevenson *et al.*, 1995; Tokarz *et al.*, 2004; Yang *et al.*, 2000). Moreover, *Bb* controls its major outer membrane lipoproteins, such as outer surface (lipo)protein C (OspC) and decorin-binding (lipo)protein A (DbpA), through a central regulatory pathway consisting of a putative response regulator Rrp2 and two alternative sigma factors RpoN and RpoS (Boardman *et al.*, 2008; Caimano *et al.*, 2004; Caimano *et al.*, 2007; Hubner *et al.*, 2001; Lybecker and Samuels, 2007; Ouyang *et al.*, 2008; Smith *et al.*, 2007; Yang *et al.*, 2003a; Fisher *et al.*, 2005).

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In addition to the Rrp2-RpoN-RpoS pathway, the *Bb* genome also encodes a putative transcriptional regulator, BB0647 (Boylan *et al.*, 2003; Fraser *et al.*, 1997; Katona *et al.*, 2004). This protein has been predicted to belong to the ferric uptake regulator (Fur) family. However, its role in *Bb* biology has remained obscure. In a wide variety of microorganisms, Fur functions principally as a global repressor to control gene expression in response to iron availability (Carpenter *et al.*, 2009; Lee and Helmann, 2007). When intracellular iron supply is abundant, Fur forms a complex with its co-factor (Fe^{2+}), and the complex binds to a Fur box (with a consensus sequence of GATAATGATAATCATTATC) (Escolar *et al.*, 1999; Lee and Helmann, 2007) located in the promoter regions of Fur-regulated genes, thereby blocking gene transcription. In contrast, when the iron supply is limited, Fur dissociates from Fe^{2+} and the Fur box, leading to de-repression. Fur also can serve as an activator to positively regulate genes, probably via an indirect effect in which Fur represses RhyB, a small regulatory RNA that blocks gene expression by binding to and degrading target mRNAs (Lee and Helmann, 2007). In addition, Fur can regulate gene expression in its apo form without binding to co-factors. Not surprisingly, by sensing the intracellular iron levels, Fur modulates the expression of genes involved in iron acquisition. Fur also controls genes unrelated to iron transport. For example, in *E. coli*, the Fur- Fe^{2+} complex represses the expression of many 'non-iron' genes, such as *cyoA*, *flbB*, *fumC*, *gpmA*, *metH*, *nohB*, *nrdH*, *purR* and *sodA*, with functions in respiration, flagellar chemotaxis, the TCA cycle, glycolysis, methionine biosynthesis, phage-DNA packaging, DNA synthesis, purine metabolism, and redox stress resistance (Lee and Helmann, 2007). Moreover, some Fur homologues, such as the regulators that sense zinc (Zur), manganese (Mur), nickel (Nur) and peroxide stress (PerR), also regulate the expression of genes involved in the uptake of zinc, manganese, or nickel, as well as the peroxide stress response (Jacquamet *et al.*, 2009; Lee and Helmann, 2007).

Given the notion that *Bb* may not accumulate or rely on iron (Posey and Gherardini, 2000), it seems unlikely that BB0647 regulates iron homeostasis in this pathogen. However, BB0647 may regulate *Bb* genes involved in non-iron functions, such as the acquisition of metal ions (e.g., zinc and manganese), or even oxidative stress responses. In a previous report, by employing a *lacZ* reporter vector (*napAP/O-lacZ*, generated by fusing the *bb0690* [*napA/dps*] promoter to a promoterless *lacZ* gene), and an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible BB0647 expression construct (pJAB3), Boylan *et al.* (Boylan *et al.*, 2003) reported that BB0647 (expressed from pJAB3) activated transcription of *napAP/O-lacZ* in *E. coli* and proposed that BB0647 positively regulated *napA/dps* expression in *Bb*. *napA/dps* is a homologue of *dps* (DNA-binding protein from starved cells) implicated in being important for *Bb* to protect itself against oxidative stress (Boylan *et al.*, 2003; Li *et al.*, 2007). Thus, BB0647 was presumed to regulate genes involved in the oxidative stress response in *Bb* and, consequently, BB0647 was renamed as BosR (*Borrelia* oxidative stress regulator) (Boylan *et al.*, 2003). However, this regulation effect has not yet been widely corroborated, mainly due to the inability to obtain a *bosR*-deficient mutant in low-passage (LP), infectious strains of *Bb*. To date, the only reported *bosR* mutant was created in the high-passage (HP), non-infectious *Bb* isolate CHP100 (Seshu *et al.*, 2004). Although interesting data were acquired by using this latter mutant, the data obtained may be equivocal for several reasons. First, the HP, noninfectious *Bb* isolate CHP100 was deficient in a few plasmids (Seshu *et al.*, 2004). It also contained unidentified mutations (Seshu *et al.*, 2004). Third, the strain differs dramatically from LP, infectious *Bb* in resistance to oxidizing agents, in which CHP100 is nearly 10^5 -fold more sensitive to *t*-butyl peroxide (Seshu *et al.*, 2004). Fourth, the *bosR* allele (*bosRR39K*) in the HP strain possessed a critical point mutation relative to the wild-type (WT) *bosR*, leading to a substantial alteration in the biochemical activity of BosR (Seshu *et al.*, 2004). All of these observations suggest that the functions of HP BosRR39K and WT BosR are distinctly different. As such, studies on BosRR39K might not be applicable to WT BosR. To more

directly explore the role of BosR in *Bb*, we created a *bosR*-deficient mutant in virulent *Bb* strain B31MI. This allowed us to assess the role(s) of BosR in the tick/mammalian life cycle of *Bb*, including its effects on *Bb*'s ability to colonize ticks, infect mice, and cause disease. The mutant also was strategic for revealing other *Bb* genes controlled by BosR, thereby establishing it as another global regulator of virulence expression in *Bb*.

Results

bosR is co-transcribed with *bb0646* and *bb0648*

In the *Bb* B31 genome, *bb0646*, *bosR*, and *bb0648* are proposed to be oriented in the same transcriptional direction (Fraser *et al.*, 1997); *bosR* is separated from *bb0648* and *bb0646* by 100 bp and 3 bp, respectively (Fig. 1A). *bosR* encodes a Fur homologue, whereas *bb0646* is predicted to encode a hypothetical protein belonging to the α/β hydrolase family (Fraser *et al.*, 1997). In addition, *bb0648* encodes a putative serine/threonine kinase (Fraser *et al.*, 1997). To determine whether *bb0646*, *bosR*, and *bb0648* constitute a single transcript, RNA was isolated from low-passage B31, reverse-transcribed to cDNA, and analyzed by PCR amplification using specific primers (Table S1). As shown in Fig. 1B, a fragment was amplified using the primer pair spanning the junction of either *bb0646/bosR* (lane 4) or *bosR/bb0648* (lane 6). Further, an amplicon spanning *bb0646*, *bosR*, and *bb0648* was amplified using primers complementary to *bb0646* or *bb0648* (lane 7). Positive controls were conducted by using genomic DNA (Fig. 1B, lanes 3 and 8) or cDNA (lane 5) as templates, and PCR amplification using RNA only as the template (lane 2) was conducted as a negative control. These data support that these three genes likely form an operon and are co-transcribed as a single transcriptional unit.

Inactivation and complementation of *bosR* in *Bb*

A *bosR* mutant was constructed by introducing the suicide plasmid pOY24 into strain B31. Through allelic exchange, a 469-bp internal fragment of *bosR* was replaced with the 1143-bp *PflgB*-kan cassette (Fig. 2A). The insertion and the orientation of the *PflgB*-kan cassette within the disrupted *bosR* were confirmed by sequence analysis. The *PflgB*-kan cassette was inserted in *bosR* in the same direction as *bosR* transcription, in order to allow *bb0646* expression from *PflgB* (Fig. 2A). Following transformation, two kanamycin-resistant transformants, OY10/D12 and OY10/H3, were isolated. To complement the *bosR* mutant, the suicide vector pOY83 was created by linking the *bb0649-bosR* fragment to the *PflgB-aadA* cassette (Fig. 2A). In pOY83, the *PflgB-aadA* cassette was located between *bosR* and *bb0646* in the same direction as *bosR-bb0646* transcription, which also allows *bb0646* expression from *PflgB* (and thus the same level of expression of *bb0646* in the *bosR* mutants and the complemented strains). Upon electroporating pOY83 into OY10/D12 or OY10/H3, two corresponding complemented clones, OY34/E6 and OY34/C4, were created. The inactivation and complementation of *bosR* in these strains were confirmed using PCR amplification (Fig. 2B). Using primers ZM25F and ZM25R, a 531-bp fragment was amplified in both the WT B31 and the complemented strains, whereas a 1205-bp fragment was amplified in the *bosR* mutants. This is consistent with the replacement of a 469-bp internal fragment of *bosR* with the 1143-bp *PflgB*-kan cassette.

To detect the expression of BosR in WT B31, the *bosR* mutants and the complemented strains, RT-PCR employing primers specific for *bosR* was performed to detect *bosR* transcripts. As expected, *bosR* transcripts were detected in both B31 and the complemented strains, but not in the mutants (Fig. 2C). This observation was further confirmed by detecting BosR using immunoblot employing a specific polyclonal antibody against BosR, which demonstrates that the mutant does not express the BosR protein (Fig. 2D). In addition, we also examined the expression of *bb0646* and *bb0648* in the *bosR* mutants and

complemented strains using RT-PCR. Results showed that transcripts of both genes were readily detected in all *Bb* isolates (Fig. S1). As intended from the cloning and recombination strategy employed, the expression of *bb0646* in the *bosR* mutants was desired and promoted via the insertion of *PflgB* just upstream of *bb0646* (Fig. 2A).

To confirm that all essential plasmids, such as lp25, lp28-1, lp54, lp36 and cp26 (Jewett *et al.*, 2007; Labandeira-Rey and Skare, 2001; Purser and Norris, 2000; Stewart *et al.*, 2005), were retained in the *bosR* mutant and complemented strains, PCR-based plasmid profiling (Purser and Norris, 2000) was performed. As shown in Fig. S2, the *bosR* mutant OY10/H3 lost only cp9 and cp32-9, but retained all essential plasmids required for *Bb* virulence. In addition, the *bosR* mutant clone OY10/D12 and the complemented strains (OY34/E6 and OY34/C4) also contained the same plasmid profile as that of OY10/H3 (data not shown). No obvious differences were observed among WT B31, the *bosR* mutants, and the complemented strains when spirochete morphology and motility were examined using dark-field microscopy. The effect of the inactivation of *bosR* on *Bb in vitro* growth was assessed by determining the growth curves of *Bb* strains grown in Barbour-Stoenner-Kelly (BSK)-II medium. As shown in Fig. S3, no discernable effect on growth was observed when *bosR* was inactivated. The *bosR* mutants displayed similar growth to WT B31 and the complemented strains.

***bosR* is required by *Bb* to infect mice via needle inoculation**

To investigate the functional importance of *bosR* to *Bb* biology, C3H/HeN mice were challenged intradermally via needle inoculation with WT B31, *bosR* mutants, or complemented strains. After four weeks, mice were sacrificed and assessed for *Bb* infection by culturing mouse skin, heart and joint specimens in BSK-II medium. Cultures were monitored continually for 4 weeks for spirochete growth. Whereas motile spirochetes were recovered from all cultures from mice inoculated with 10^4 spirochetes per mouse of WT B31 or the complemented strains (OY34/E6 and OY34/C4), no bacterial growth was observed in cultures from mice infected with 10^7 spirochetes per mouse of the *bosR* mutants (OY10/D12 and OY10/H3) (Table 1). These data suggest that *bosR* is essential for *Bb* to establish infection in mice.

***bosR* is not essential for *Bb* persistence in ticks**

The inability to infect mice with a *bosR* mutant via needle inoculation precluded us from assessing whether the mutant could be naturally acquired by feeding ticks. Thus, as a surrogate system to examine the mutant's ability to colonize ticks, sustain itself in tick midguts, and be transmitted to naïve mice, *Ixodes scapularis* nymphs were first loaded with the *bosR* mutant via a widely used microinjection technique (Pal *et al.*, 2004). We initially examined the survival of spirochetes in injected unfed ticks using an immunofluorescence assay (IFA), which revealed that spirochetes were readily detected in ticks 4 days after injection with all three isolates (WT B31, the *bosR* mutant OY10/H3, or the complemented strain OY34/C4) (Fig. S4). We then investigated the survival of *Bb* isolates during tick engorgement. For each isolate, 36 microinjected nymphs were allowed to feed on 3 naïve mice. Three ticks from each group were forcibly removed after 24h and 72h of feeding, whereas the remaining nymphs were allowed to feed to repletion (collected at 24h post-feeding). To determine the spirochete burden in ticks, nymphs were then analyzed by detecting *Bb flaB* transcripts using quantitative RT-PCR (qRT-PCR) and data were normalized using tick β -actin. As shown in Fig. 3A, regardless of the *Bb* isolates used, comparable levels of spirochetes were detected in ticks (collected after 72h of feeding) injected with WT B31, the *bosR* mutant OY10/H3, or the complemented strain OY34/C4. Similar results were obtained for ticks collected at 24h feeding or 24h post-feeding, showing that the spirochete burden in ticks injected with the *bosR* mutant was not significantly below

the spirochete burden in ticks injected with either WT B31 or the complemented strain (data not shown). These data demonstrated that the *bosR* mutant was able to colonize and survive in ticks and that *bosR* likely is dispensable for *Bb* to infect and survive in ticks.

The *bosR* mutant is defective in establishing tick-borne murine infection

We then assessed whether the *bosR* mutant was able to be transmitted from ticks to naïve murine hosts. To achieve this, nymphs were microinjected with either WT B31, the *bosR* mutant OY10/H3, or the complemented strain OY34/C4 and the nymphs were allowed to feed on naïve mice. One week after tick feeding, mice were sacrificed and infection was determined by culture and qRT-PCR. As shown in Table 2, no spirochetes were cultivated from multiple samples of mice infested with nymphs harboring the *bosR* mutant. In contrast, spirochetes were recovered from tissues from mice infested with ticks containing WT B31 or the complemented strain. This was further confirmed by qRT-PCR analyses, which demonstrated that *bosR* mutants remained virtually undetectable in mice (Fig. 3B). The combined data indicate that a *bosR* mutant is unable to establish infection in mice when transmitted by ticks.

Identification of BosR-regulated genes in *Bb* using microarray analysis

Although the above data that *bosR* plays an important role in *Bb* virulence were compelling, the precise function of this protein remains unknown. Inasmuch as it was plausible that *bosR* encodes some type of regulatory protein, we sought to assess the potential effect of *bosR* deficiency on the spirochete transcriptome. A 70-mer B31-based oligonucleotide array (Ouyang *et al.*, 2008; Terekhova *et al.*, 2006) was employed to compare the gene expression profiles in the *bosR* mutant relative to WT B31. Our data showed that 199 genes were differentially regulated from 2.09- to 116.7 fold by BosR (relative to the mutant deficient in *bosR*, 137 and 62 genes were up- or down-regulated, respectively, in WT B31) (Table S2 and Table S3). Of these genes, 67 (34%), 23 (12%) and 19 (10%) genes were located on the chromosome, lp54, or lp28-2, respectively (Fig. 4); 119 (60%) genes had unknown function, categorized as either hypothetical (77 genes) or hypothetically conserved (42 genes), and 80 (40%) genes had some predicted functions.

To validate the results of the microarray analysis, qRT-PCR was utilized to examine expression of 19 genes differentially regulated by BosR. As shown in Fig. S5, a strong and significant linear correlation ($r=0.94$) was found when the log-transformed ratios determined by qRT-PCR and microarray were compared. When examining the absolute levels of gene expression for each given gene, similar trends were observed between these experimental approaches (Table 3). These data indicate that the differences observed in mRNA expression levels obtained by qRT-PCR correlated well with those obtained from microarray-based transcriptome analysis.

Notably, our transcriptome comparison revealed that genes activated by the alternative sigma factor RpoS in *Bb*, such as *ospC*, *dbpB*, *dbpA* and *bb0844*, and even *rpoS* itself, were up-regulated in WT B31 relative to the *bosR* mutant (Table S2). When comparing the *bosR* microarray data with our previous transcriptional profiling data in one *rpoS* mutant (Ouyang *et al.*, 2008), we found that, of those 137 genes up-regulated in WT B31, 87 genes including *ospC*, *dbpB*, *dbpA* and etc, were also affected by RpoS, whereas 50 genes were only regulated by BosR, but not by RpoS. Moreover, 9 Erp outer surface lipoproteins encoding genes (Fraser *et al.*, 1997), including *bbf01*, *bbl39* (*erpA*), *bbo39* (*erpL*), *bbo40* (*erpM*), *bbq47* (*erpX*), *bbr40* (*erpH*), *bbr41* (*ospE*), *bbr42* (*erpY* or *ospF*), and *bbs41* (*erpG* or *ospG*), were found to be induced by BosR. These Erp proteins have been proposed to bind to the complement inhibitory factor H proteins in a variety of mammalian hosts and are important for *Bb* to establish infection (Alitalo *et al.*, 2001;Brissette *et al.*, 2008;Brissette *et*

al., 2009; Bykowski *et al.*, 2007; Coleman *et al.*, 2008; Hartmann *et al.*, 2006; Hefty *et al.*, 2001; McDowell *et al.*, 2003). Additionally, of the 62 genes down-regulated in WT B31 relative to the *bosR* mutant, only 5 genes, including *bb0076* (*ftsY*), *bb0646*, *bba62*, *bba74* (*oms28*) and *bbu05*, were affected by both BosR and RpoS, whereas a majority (57/62) of genes were affected only by BosR, not by RpoS. Of these 57 genes repressed only by BosR, superoxide dismutase A (*bb0153*, *sodA*) and *bb0690* (*napA/dps*) are important for *Bb* to protect itself against oxidative stress (Esteve-Gassent *et al.*, 2009; Li *et al.*, 2007). Additional genes of interest in this category include: (i) *bb0763*, which encodes the response regulator Rrp2; (ii) *bb0184* encoding a putative carbon storage regulator; (iii) many genes involved in physiological responses: *bb0240* (*glpF*), *bb0401* (encodes a putative glutamate transporter), *bb0812* (*dfp*, encodes a pantothenate metabolism flavoprotein), *bb0730* (*pgi*, encodes a glucose-6-phosphate isomerase), *bb0152* (*nagB*, encodes a glucosamine-6-phosphate isomerase), and oligopeptide ABC transporter encoding genes *bb0333* (*oppC-1*) and *bb0335* (*oppF*).

BosR controls the expression of OspC and DbpA in *Bb*

The above microarray analysis suggested that *ospC*, *dbpA* and *rpoS* were regulated by BosR. To further substantiate the role of BosR in the regulation of *Bb* genes, WT B31, the *bosR* mutants and the complemented strain were cultured at 37°C in BSK-H media at pH6.8 or pH7.6. pH6.8 was chosen for one of the *Bb* culture conditions because the RpoS regulon is highly expressed under this condition (Ouyang *et al.*, 2008; Yang *et al.*, 2000). Cells were harvested at late-log phase and whole-cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. As shown in Fig. 5A, when *Bb* was grown at pH6.8, the expression of *ospC* essentially was abolished in the *bosR* mutants. This result was further confirmed using immunoblot analysis, showing that the expression of *ospC* was dramatically diminished in *bosR* mutants (Fig. 5B). Moreover, immunoblot data also revealed that the expression of another major outer membrane lipoprotein in *Bb*, DbpA, was abolished when *bosR* was inactivated (Fig. 5B). To verify the reduction of OspC and DbpA expression in the *bosR* mutant was due solely to the inactivation of *bosR*, we investigated the expression of both OspC and DbpA in the complemented strains (OY34/E6 and OY34/C4). As shown in Fig. 5A and 5B, the expression of both OspC and DbpA were fully restored when the *bosR* mutation was complemented. Similar data were also obtained when *Bb* was grown in BSK-H at pH7.6 (data not shown). These data indicate that BosR plays a prominent role in controlling the expression of major outer membrane lipoproteins, such as OspC and DbpA.

Previous studies suggested that both OspC and DbpA were controlled by the central Rrp2-RpoN-RpoS pathway (Boardman *et al.*, 2008; Caimano *et al.*, 2004; Caimano *et al.*, 2007; Fisher *et al.*, 2005; Hubner *et al.*, 2001; Lybecker and Samuels, 2007; Ouyang *et al.*, 2008; Smith *et al.*, 2007; Yang *et al.*, 2003a). More specifically, RpoS directly controls OspC through an RpoS-dependent promoter (Eggers *et al.*, 2004; Yang *et al.*, 2005), although it is still unclear how RpoS controls DbpA. In this study, we examined the expression of RpoS in the *bosR* mutants and the complemented strains. Immunoblotting revealed that the expression of RpoS was abolished in the *bosR* mutants but restored in the complemented strains (Fig. 5B). These data were further verified by RT-PCR analysis (Fig. S6). In addition, we also examined the expression of Rrp2 and RpoN in *Bb* variants. Consistent with the notions that both Rrp2 and RpoN are constitutively expressed in *Bb*, expression of Rrp2 (Fig. 5B, Fig. S6) and RpoN (Fig. S6) were not significantly altered when *bosR* was inactivated.

Discussion

Although BosR has been the focus of a number of previous studies (Boylan *et al.*, 2003; Katona *et al.*, 2004; Seshu *et al.*, 2004), prior attempts to disrupt *bosR* within a low-passage,

infectious strain of *Bb* were unsuccessful (Seshu *et al.*, 2004). In this study, we successfully created a *Bb* mutant lacking BosR in low-passage, virulent *Bb* strain B31. Inasmuch as *bosR* constitutes an operon with *bb0648* and *bb0646*, three critical strategies were employed to ensure that the phenotypes of the *bosR* mutant observed in this study were due solely to the inactivation of *bosR* (and not indirect effects). First, in the *bosR* mutants, the *PflgB*-Kan cassette was inserted into *bosR* in the direction of *bosR*-*bb0646* transcription, thereby promoting the transcription of *bb0646* from *PflgB*. Second, in the complemented strains, the *PflgB*-*aadA* cassette was inserted between *bosR* and *bb0646* and oriented in the same direction as *bb0646* transcription, which allowed *bb0646* expression (from *PflgB*, and at the same level in both the *bosR* mutant and the complemented strain). Finally, the *bosR* mutation was complemented in *cis* using a construct harboring the native promoter of *bosR*, thereby facilitating a similar WT-level of expression of BosR in the complemented strain.

Bb growth *in vitro* was not affected by *bosR* inactivation. Similarly, *bosR* was dispensable for *Bb* persistence in ticks. However, our data revealed that inactivation of *bosR* rendered *Bb* completely noninfectious for mice, irrespective of needle inoculation or tick challenge, whereas genetic complementation fully restored the WT phenotype. This demonstrated that BosR is essential for *Bb* infectivity of a mammalian host. In this regard, an association between *fur* mutation and decreased bacterial virulence has been observed for many other pathogens (Carpenter *et al.*, 2009), such as *Campylobacter jejuni*, *Listeria monocytogenes*, *Helicobacter pylori*, *Staphylococcus aureus*, *Actinobacillus pleuropneumoniae*, *Bacillus cereus*, and *Vibrio cholerae*, although the precise underlying mechanism(s) remain unclear. Given that iron is essential to the physiology and pathogenesis of these bacteria, aberrations in iron homeostasis are strongly implicated. Alternatively, a decrease in the virulence of *fur* mutants might also result from the variation in expression of specific virulence determinants. Because *Bb* does not appear to accumulate iron when cultivated *in vitro*, and no iron uptake systems have been predicted or identified in *Bb* (Posey and Gherardini, 2000), it seems less likely that the loss of infectivity for mice by the *bosR* mutant is due to a generalized effect in *Bb* on iron homeostasis. It is thus more plausible that other unknown virulence factors controlled by BosR contribute to *Bb*'s mammalian infectivity phenotype.

Microarray analysis was employed to identify *Bb* genes potentially regulated, either directly or indirectly, by BosR. Our data revealed that BosR influences the expression of two essential *Bb* outer membrane lipoproteins, OspC and DbpA, which was substantiated by SDS-PAGE and immunoblotting. It is well established that OspC and DbpA are important for *Bb* virulence (Blevins *et al.*, 2008; Grimm *et al.*, 2004; Pal *et al.*, 2004; Shi *et al.*, 2008; Stewart *et al.*, 2006; Tilly *et al.*, 2006; Weening *et al.*, 2008), and they both are governed by the central Rrp2-RpoN-RpoS pathway (Boardman *et al.*, 2008; Burtneck *et al.*, 2007; Caimano *et al.*, 2004; Caimano *et al.*, 2007; Fisher *et al.*, 2005; Hubner *et al.*, 2001; Lybecker and Samuels, 2007; Ouyang *et al.*, 2008; Smith *et al.*, 2007; Yang *et al.*, 2003a). In this pathway, under stimulation by various environmental factors including pH, temperature, cell density, and other unknown mammalian factors, the putative response regulator Rrp2 is likely activated via phosphorylation (via an unidentified histidine kinase). Activated Rrp2 then activates RpoN which, in turn, promotes the expression of the alternative sigma factor RpoS. RpoS then allows the expression of a number of virulence-associated proteins, including OspC and DbpA. Additionally, in response to changes in temperature, a small RNA molecule, DsrA_{Bb}, also activates RpoS (Lybecker and Samuels, 2007). In our current study, the expression of OspC and DbpA was markedly decreased or abolished in the *bosR* mutant, suggesting that expression of both OspC and DbpA are activated by BosR. Thus far, it remains unknown how BosR controls the expression of OspC and DbpA. Although it remains possible that BosR controls *ospC* and *dbpA* directly, BosR more likely is somehow involved in regulating the expression of RpoS which, in turn, influences *ospC* and *dbpA*, inasmuch as the expression of RpoS was also abolished in the

bosR mutant. BosR may influence *rpoS* directly by binding to a sequence proximal to the *rpoS* minimal promoter (Burtnick *et al.*, 2007; Smith *et al.*, 2007), or alternatively influence the phosphorylation of Rrp2 by modulating the unknown cognate histidine kinase required for Rrp2 activation. In addition, BosR may also be involved in the pathway(s) of environmental signals that induce the Rrp2-RpoN-RpoS pathway. All of these possibilities warrant further investigation. Regardless, this is the first study to reveal another important layer of gene regulation, which somehow involves BosR, exerted on the central Rrp2-RpoN-RpoS pathway. Interference with the putative regulatory activity of BosR may lead to new strategies to interrupt the spirochete's life cycle.

Previous studies have indicated that the RpoS regulon is induced at higher temperature (35°C or 37°C), but is repressed at lower temperature (23°C) (Burtnick *et al.*, 2007; Caimano *et al.*, 2004; Caimano *et al.*, 2007; Yang *et al.*, 2000). Nonetheless, BosR is expressed efficiently at both 23°C or 35°C (Katona *et al.*, 2004), suggesting that BosR may also participate in gene regulation by means other than inducing the RpoS regulon. In agreement with this possibility, transcriptome profiling of the *bosR* mutant revealed that, in addition to the genes regulated by RpoS, other genes were found to be induced only by BosR. Moreover, an additional group of 62 genes was found to be down-regulated in WT B31 (relative to the *bosR* mutant), suggesting that these genes are repressed by BosR. Among these genes, a majority were repressed only by BosR, but not by RpoS. However, considering that the fold-changes in the expression of these genes were modest (<4-fold), it remains possible that BosR may not regulate these 62 genes; the modest changes in the expression levels of these genes could be due to subtle, but generalized, metabolic effects. On the other hand, the *in vitro* culture conditions for *Bb* used in this study may not accurately mimic the *in vivo* conditions that *Bb* encounters in its life cycle. Under the culture conditions employed in this study (37°C), or in mammalian hosts, BosR may function in *Bb* principally as an activator to induce the RpoS regulon. However, under other conditions, such as when *Bb* is cultivated at 23°C or when *Bb* encounters a metal deficiency or oxidative stress, BosR may adopt another molecular configuration or cooperative structure that exerts different regulatory effects on those 62 genes. Future studies on the structural aspects of BosR may be strategic for addressing these possibilities.

The 62 genes repressed by BosR included *sodA* and *napA/dps*. In *Bb*, *sodA* ostensibly encodes a superoxide dismutase A; *napA/dps* has been annotated as a neutrophil activating protein A (Fraser *et al.*, 1997) that has homology to the Dps proteins. In many bacteria, Dps proteins protect DNA during starvation and oxidative stress. Studies have showed that NapA/Dps is important for spirochete persistence in ticks during the inter-molt period (Li *et al.*, 2007), and SodA is required by *Bb* to contend against oxidative stress (Esteve-Gassent *et al.*, 2009). Moreover, others have reported that purified recombinant BosR binds to the promoters of *sodA* and *napA/dps* (Boylan *et al.*, 2003; Katona *et al.*, 2004; Seshu *et al.*, 2004). These independent observations thus provide credence for the validity of our microarray data. Boylan *et al.* (Boylan *et al.*, 2003) reported that LacZ expression from the *napA/dps* promoter-*lacZ* transcriptional fusion vector was activated by hyper-expression of BosR in *E. coli*, suggesting that BosR activates *napA/dps*. However, our data showed that the expression of *napA/dps* was up-regulated in the *bosR* mutant (Table 3, Table S3, and Fig. 5B), suggesting that *napA/dps* is actually repressed, rather than induced, by BosR, under the experimental conditions examined. The explanation for this apparent discrepancy remains unclear. One possibility is that BosR may adopt different configurations in *E. coli* vs. *Bb*. In other Fur homologues, the proteins typically contain two metal binding sites in the C-terminal domain, consisting of a Zn²⁺ structural site and a regulatory site usually occupied by Fe²⁺ (Lee and Helmann, 2007). In *E. coli*, BosR might prefer iron as a co-factor and regulate genes directly or indirectly. In *Bb*, BosR may utilize other metal ions, such as zinc or manganese, as cofactors. Of note, *Bb* appears not to accumulate iron (Boylan *et al.*, 2003;

Posey and Gherardini, 2000), but does accumulate manganese and zinc, probably through the manganese transporter BmtA (Ouyang *et al.*, 2009) and one or more unidentified zinc transporters, respectively. Or, BosR may influence *Bb* gene expression in its apo form. Given these possibilities, *E. coli* may not be an ideal heterologous system for studying *Bb* gene expression and regulation, especially for those genes affected by BosR.

Our study clearly demonstrated that BosR influences *Bb* virulence. In addition to the key lipoproteins OspC and DbpA, a large number of genes with unknown functions and genes involved in a variety of putative functions were also positively or negatively regulated by BosR. It is plausible that some of these BosR-regulated genes also are important for *Bb* virulence. Thus, BosR likely plays multifaceted roles in *Bb* pathogenesis by coordinating the synthesis of *Bb* virulence factors with the appropriate *in vivo* signals, and in *Bb* global gene regulation.

Our study prompts a number of new and important questions. First, given the fact that *bb0646*, *bosR*, and *bb0648* form an operon, what roles do the other two members of the *bosR* operon play in *Bb* pathogenesis and gene regulation? Inactivation of each gene of the operon may shed additional light on this issue. Second, it is unclear how BosR exerts its regulatory effect in *Bb*. Does it employ manganese or zinc as a co-factor, as previously assumed (Boylan *et al.*, 2003; Posey and Gherardini, 2000), or does it regulate gene expression in its apo form? Finally, it remains unknown how *bosR* is expressed in *Bb*. *bosR* was reported to be expressed in *Bb* grown at either 23°C or 35°C, suggesting that expression of BosR is not influenced by temperature (Katona *et al.*, 2004). However, the expression of BosR was induced when *Bb* was grown under anaerobic conditions, inferring that the dissolved CO₂ level in the medium influences *bosR* expression (Hyde *et al.*, 2007). How may dissolved CO₂ influence BosR expression? Does *Bb* express *bosR* in response to other environmental stimuli, such as intracellular metal levels, pH, or cell density? Continued efforts are warranted to address these salient questions.

Experimental procedures

Strains and culture conditions

Infectious *Bb* strain B31MI (Fraser *et al.*, 1997) was used as the WT strain (referred as B31) throughout this study. Isogenic *Bb* strains, including *bosR* mutants and *bosR*-complemented strains, were generated as described below. *Bb* was routinely cultured at 37°C and 5% CO₂ in either BSK-II medium or BSK-H medium (Sigma) (Pollack *et al.*, 1993) supplemented with 6% rabbit serum (Pel-Freeze). When appropriate, supplements were added to media at following concentrations: kanamycin, 160 µg/ml; streptomycin, 150 µg/ml. *E. coli* was grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates.

Construction of *bosR* mutants and complemented strains

bosR was inactivated through homologous recombination using a suicide vector (pOY24). All constructs were confirmed using PCR amplification, restriction digestion, and sequence analysis. The 1261-bp 5' arm for creating pOY24 was PCR-amplified using primers ZM22.2F and ZM22R, whereas the 1579-bp 3' arm was amplified using ZM23F and ZM23R (Table S1). The 5' DNA fragment was cloned into pGEM-Teasy vector (Promega), yielding pOY18. After digestion with *Bss*HIII, the 3' arm was ligated into pOY18 that was digested with *Asc*I, creating pOY22. The *PflgB*-Kan cassette, excised from pJD55 (Frank *et al.*, 2003; Stewart *et al.*, 2001; Blevins *et al.*, 2008) using *Asc*I, was then ligated into pOY22 at the *Asc*I site. In the resulting construct, pOY24, the *PflgB*-Kan cassette was inserted into *bosR* in the same direction as transcription of *bb0648-0646* (Fraser *et al.*, 1997), which allows the expression of *bb0646* from the *PflgB*. *Bb* transformation was performed as

previously described (Yang *et al.*, 2005). Transformants were selected using kanamycin and confirmed by PCR amplification.

To complement the *bosR* mutation, a suicide plasmid (pOY83) was constructed. Briefly, the 3772-bp DNA (*bb0649-bosR*) containing *bosR* and 3241-bp upstream of *bosR*, was PCR-amplified using primers ZM82 and ZM83, digested with *Xma*I, and ligated into pJD54 (Frank *et al.*, 2003; Stewart *et al.*, 2001), which yielded pOY82. Next, the *bb0649-bosR-PflgB-aadA* cassette was excised from pOY82 using *Asc*I, and ligated into pOY22, creating pOY83. The resulting construct, pOY83, was electroporated into *Bb* OY10, which created OY34. Transformants were selected using 150 µg/ml of streptomycin, and all transformants and constructs were confirmed by PCR. Plasmid profiling for all *Bb* strains were performed as previously described (Purser and Norris, 2000).

Recombinant BosR expression and generation of rat polyclonal anti-BosR antibody

Recombinant BosR was produced in *E. coli* using the bacterial expression vector pPROEX-HTB (Invitrogen). Briefly, *bosR* was amplified using primers ZM26F and ZM26R. The resultant PCR fragment contained a *Bam*HI site at the 5'-end and an *Eco*RI site at the 3'-end. After digestion with *Bam*HI and *Eco*RI, the PCR product was ligated into the corresponding polylinker sites of the vector pPROEX-HTB, creating pOY21. The resulting construct, pOY21, was then transformed into *E. coli* strain BL21-DE3. After induction with 1mM IPTG (Sigma), recombinant BosR was purified using a Ni-NTA spin column according to the manufacturer's instruction (Qiagen). Rat polyclonal antibody against the purified BosR, Ab-BosR, was generated as previously described (Yang *et al.*, 2003b).

SDS-PAGE and immunoblot analysis

SDS-PAGE and immunoblot analysis were carried out as previously described (Yang *et al.*, 2003b). Briefly, a volume of whole cell lysate equivalent to 4×10^7 bacteria was loaded per lane on a 12.5% acrylamide gel. Resolved proteins were either stained with Coomassie brilliant blue or transferred to nitrocellulose membrane for immunoblot analysis. BosR was detected using the anti-BosR rat polyclonal antibody, Ab-BosR. Rrp2, RpoS, OspC, DbpA and NapA/Dps were detected using anti-Rrp2 monoclonal antibody 5B8-100-A1, anti-RpoS monoclonal antibody 6A7-101, anti-OspC monoclonal antibody 1B2-105A, anti-DbpA monoclonal antibody 6B3, or a polyclonal antibody against NapA/Dps, respectively. To confirm equal loading of bacteria in each lane, immunoblotting for the flagellar core protein (FlaB) was performed using a chicken IgY anti-FlaB antibody. Immunoblots were developed colorimetrically using 4-chloro-1-naphthol as the substrate or by chemiluminescence using ECL Plus Western Blotting Detection system (Amersham Biosciences).

Microarray and qRT-PCR analyses

To compare gene expression profiles between *Bb* WT strain B31 and its isogenic *bosR* mutant OY10/H3, microarray analysis was performed essentially as described (Ouyang *et al.*, 2008). Briefly, B31 and OY10/H3 were grown in triplicate in BSK-H medium at 37°C and 5% CO₂. Spirochetes were harvested when the bacterial growth reached a density of 5×10^7 cells per ml. Total RNA was isolated using Trizol (Invitrogen) according to the instructions. After genomic DNA was digested using RNase-free DNase I (GenHunter Technology), RNA was further purified using RNeasy Mini Kit (Qiagen). cDNA was synthesized from 10 µg of total RNA using Amino Allyl cDNA Labeling Kit (Ambion) and then applied to the pre-hybridized 70-mer *Bb* array slides. Hybridization and slide scanning on an Axon 4000B microarray scanner (using GenePix Pro 6.1; Molecular Devices) were performed as previously described (Ouyang *et al.*, 2008). The data were analyzed using the professional microarray data analysis program Acuity 4.0 according to the manufacturer's

instructions (Molecular Devices). Briefly, raw data were first normalized using a ratio-based normalization method to equalize the means and medians of the features to 1. Additionally, the features that were designated by the software as “bad”, “absent”, or “not found” were also excluded from further analysis. Statistical analyses were performed using a one-sample *t*-test in the Acuity program. Differentially expressed genes were identified by both fold change (≥ 2 -fold) and statistical significance ($P < 0.05$). Fully processed and the raw microarray data from the microarray experiments were deposited in the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number of GSE17422. qRT-PCR was employed to validate selected data from microarray experiments, using the relative quantification method ($\Delta\Delta C_T$) as described (Ouyang *et al.*, 2008).

Bb infection of mice via needle inoculation

The infectivity of the *bosR* mutants and complemented strains was assessed using the murine needle-challenge model of Lyme borreliosis (Akins *et al.*, 1998; Barthold *et al.*, 1993). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at UT Southwestern Medical Center. Prior to infection, bacteria were enumerated by dark-field microscopy. C3H/HeN mice (Charles River Laboratories) were infected via intradermal injection with various concentrations of *Bb*. At 4 weeks post inoculation, mice were sacrificed and skin, heart, and joint tissues were collected and cultured in BSK-II medium supplemented with $1\times$ *Borrelia* antibiotic mixture (BAM, Sigma). The outgrowth of spirochetes in these cultures was assessed using dark-field microscopy.

Microinjection of Bb into nymphal ticks and transmission studies

I. scapularis nymphs were reared and maintained at the University of Maryland, College Park. A microinjection procedure was used to introduce spirochetes into the gut of *I. scapularis* nymphs as previously described (Pal *et al.*, 2004). Briefly, B31, OY10/H3 and OY34/C4 grown in BSK-II medium containing appropriate antibiotics were harvested by centrifugation and injected into nymphs ($\sim 4.2 \times 10^4$ spirochetes/tick). For each *Bb* strain, approximately 50 ticks were injected. After injection, 36 ticks were placed onto naïve C3H/HeN mice (3 animals/group, 12 ticks/mouse). After 24h or 72 h of feeding, 3 ticks were forcibly collected at each time-point, and at 24h post-feeding, 6 additional ticks were collected. The spirochete burden in ticks was analyzed by qRT-PCR as described previously (Coleman *et al.*, 2008), and statistical analysis was performed by the Student's *t*-test. At day 4 after injection, the remaining unfed ticks were dissected and spirochetes were detected in the gut using IFA confocal microscopy. To examine whether injected ticks were able to transmit *Bb*, tick-infested mice were sacrificed at 7d post-feeding and murine tissue samples including skin, heart, and spleen were collected. Transmission of *Bb* from microinjected ticks to mice were determined by recovering spirochetes from murine tissues cultured in BSK-II medium and by assessing *Bb flaB* transcripts in infected murine skin and heart samples using qRT-PCR (Coleman *et al.*, 2008). Two independent experiments were performed.

Immunofluorescence assays

Spirochetes were detected in ticks using confocal IFAs as detailed (Pal *et al.*, 2001). Briefly, nymphal guts were isolated from ticks, air-dried, and fixed in acetone for 10 min. Slides were blocked at room temperature for 30min with blocking solution (PBS/0.05% Tween 20 with 5% goat serum) in a humidified chamber, and then incubated for 1 h with BacTrace fluorescein isothiocyanate (FITC)-conjugated goat anti-*Bb* antibody (KPL Inc.). After two washes in PBS/0.05% Tween 20, slides were counterstained with 20 μ g/ml of propidium iodide in PBS for 5 min, washed twice with PBS/0.05% Tween 20, and then treated with the

SlowFade Antifade Kit (Invitrogen). Samples were imaged using a Zeiss LSM 510 scanning laser confocal microscope equipped with an argon/krypton laser.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>Bb</i>	<i>Borrelia burgdorferi</i>
BSK medium	Barbour-Stoenner-Kelly medium
Dbp	decorin-binding protein
HP	High-passage
IFA	immunofluorescence assay
LP	low-passage
Osp	outer surface protein
qRT-PCR	quantitative RT-PCR
RT	reverse transcriptase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
WT	wild-type

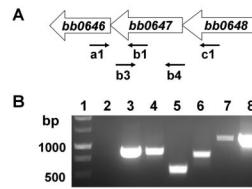


Fig. 1. *bb0646*, *bb0647* (*bosR*), and *bb0648* are co-transcribed. (A) Schematic representation of the putative *bb0646-bb0648* operon in *Bb*. (B) Results from RT-PCR. Lane 1, molecular weight markers; lane 2, primer pair a1 and b1 in control PCR using RNA as template (no reverse transcriptase [RT]); lane 3, primer pair a1 and b1 in ordinary PCR using genomic DNA as template; lane 4, primer pair a1 and b1; lane 5, primer pair b3 and b4; lane 6, primer pair b3 and c1; lane 7, primer pair a1 and c1; lane 8, primer pair a1 and c1 using genomic DNA as template. Arrows indicate the approximate positions of the oligonucleotide primers used for subsequent PCR analyses.

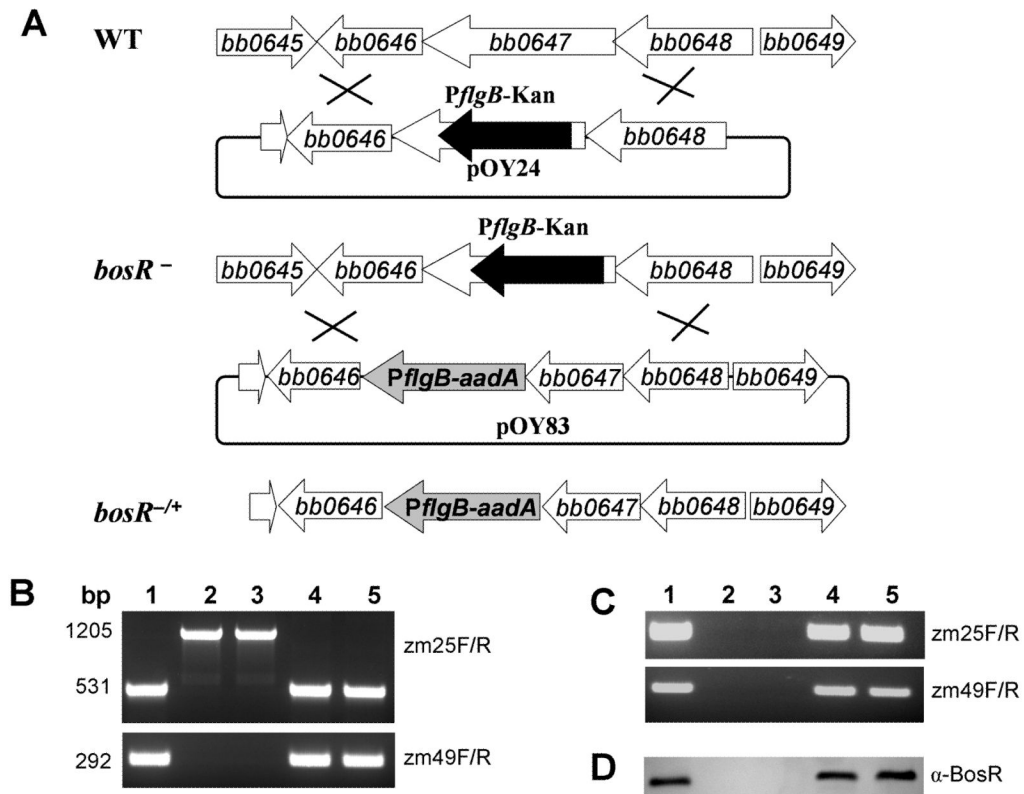
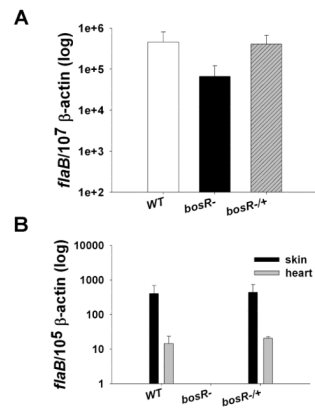


Fig. 2. Construction of *bb0647* (*bosR*) disruption mutants (*bosR*⁻) and complemented strains (*bosR*^{-/+}). (A) Schematic representation of the *bb0645-bb0649* genes in the *Bb* chromosome, insertion of the *PflgB-kan* gene cassette by homologous recombination, and the relevant complemented strain. (B) PCR analysis of WT B31, *bosR* mutants, and the complemented strains. The *bosR*-specific primer pairs used in PCR are indicated on the right. Lane 1, WT B31; lane 2, *bosR*⁻ OY10/D12; lane 3, mutant OY10/H3; lane 4, *bosR*^{-/+} OY34/E6; lane 5, *bosR*^{-/+} OY34/C4. RT-PCR (C) and immunoblot analyses (D) were employed to determine the expression of BosR. α -BosR: rat polyclonal antibody against BosR. Lanes and primer pair designations are as in (B).

**Fig. 3.**

Survival of the *bosR* mutant in ticks. *I. scapularis* nymphs microinjected with various *Bb* strains were allowed to feed on naive mice. Ticks were collected at various times; infected mice were sacrificed at 7d post-feeding and multiple mouse tissues also were collected. Spirochete burdens in ticks after 72h of feeding (A) or mouse samples collected at 7d post-feeding (B) were examined using qRT-PCR. The experiments were replicated twice, and bars represent the mean measurements \pm SEM from six representative qPCR measurements.

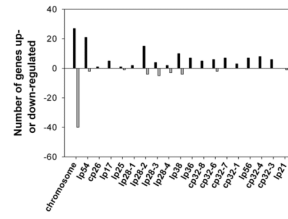


Fig. 4. Genomic distribution of genes (see Table S2 and S3) differentially regulated by BosR. Black bars indicate the numbers of genes that are activated by BosR, whereas grey bars indicate the number of genes down-regulated.

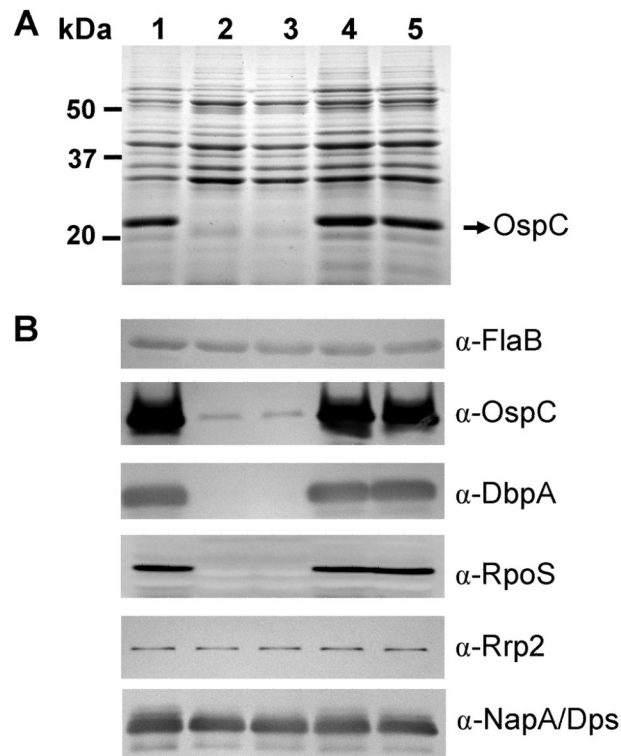


Fig. 5. BosR controls the expression of OspC and DbpA in *Bb*. *Bb* was inoculated into BSK-H medium (pH6.8) at 1000 spirochetes/ml and grown at 37°C. Spirochetes were collected at late-log phase. A volume of whole-cell lysates equivalent to 4×10^7 cells was loaded per gel lane and *Bb* gene expression was assessed by SDS-PAGE (A) and immunoblot (B) analyses. Approximate molecular masses are indicated at the left in kDa. The arrow in (A) indicates the position of OspC in SDS-PAGE. Specific antibodies, indicated as α -, used in the immunoblot (B) are indicated on the right. Lane 1, WT B31; lane 2, *bosR*⁻ OY10/D12; lane 3, mutant OY10/H3; lane 4, *bosR*^{-/+} OY34/E6; lane 5, *bosR*^{-/+} OY34/C4.

Table 1

Infectivity of *B. burgdorferi* B31 clones in mice.^a

Strain, clone	Description	Dose	No. of cultures positive / total No. of specimens examined				No. of mice infected / total No. of mice
			Heart	Joint	Skin	All sites	
B31	wild type <i>B. burgdorferi</i>	10 ⁴	6/6	6/6	6/6	18/18	6/6
OY10/D12	B31, <i>bosR</i> ^{-b}	10 ⁷	0/4	0/4	0/4	0/12	0/4
OY10/H3	B31, <i>bosR</i> ⁻	10 ⁷	0/7	0/7	0/7	0/21	0/7
OY34/E6	OY10/D12 with pOY83, <i>bosR</i> ^{-/+b}	10 ⁴	3/3	3/3	3/3	9/9	3/3
OY34/C4	OY10/H3 with pOY83, <i>bosR</i> ^{-/+}	10 ⁴	3/3	3/3	3/3	9/9	3/3

^aData were collected from two independent experiments.

^b*bosR*⁻: *bosR* mutant; *bosR*^{-/+}, *bosR* complemented strain.

Table 2

Recovery of *B. burgdorferi* from mice infected by microinjected ticks^a

Strain, clone	No. of cultures positive / total No. of specimens examined				No. of mice infected / total No. of mice
	Heart	spleen	Skin	All sites	
wild type <i>B. burgdorferi</i> B31	3/5	3/6	4/4	10/15	5/6 ^b
OY10/D12, <i>bosR</i> ^{-c}	0/6	0/6	0/6	0/18	0/6
OY34/E6, <i>bosR</i> ^{-hc}	5/5	4/6	5/6	15/17	5/6 ^b

^aData were acquired from two independent experiments. For each strain, totally 6 mice were infected and 18 samples were collected^bCulture data from several samples were not determined due to contamination^c*bosR*⁻: *bosR* mutant; *bosR*^{-/+}, *bosR* complemented strain

Table 3

Validation of microarray results using quantitative RT-PCR

Gene	Function	WT/ <i>bosR</i> mutant	
		microarray	qRT-PCR
<i>bbb19</i>	outer surface protein C (<i>ospC</i>)	87.76	38.96
<i>bbg27</i>	conserved hypothetical protein	35.84	42.86
<i>bba24</i>	decorin binding protein A (<i>dbpA</i>)	14.54	42.86
<i>bbo39</i>	ErpL protein (<i>erpL</i>)	7.04	9.38
<i>bba48</i>	hypothetical protein	5.14	1.25
<i>bbe31</i>	antigen, P35, putative	4.37	4.05
<i>bbj26</i>	ABC transporter, ATP-binding protein	4.32	4.11
<i>bb0565</i>	purine-binding chemotaxis protein (<i>cheW-2</i>)	3.46	4.29
<i>bbf01</i>	ErpD protein, putative	3.02	8.82
<i>bbr41</i>	conserved hypothetical protein	2.87	2.24
<i>bb0071</i>	RNA polymerase sigma factor (<i>rpoS</i>)	2.81	3.66
<i>bb0690</i>	neutrophil activating protein (<i>napA</i>)	^a -2.11	-1.57
<i>bb0763</i>	response regulatory protein (<i>rrp-2</i>)	-2.16	-1.32
<i>bb0104</i>	periplasmic serine protease DO (<i>htrA</i>)	-2.18	-1.49
<i>bb0153</i>	superoxide dismutase (<i>sodA</i>)	-2.25	-1.37
<i>bba62</i>	lipoprotein	-2.33	-2.07
<i>bb0032</i>	hypothetical protein	-2.53	-2.04
<i>bb0401</i>	glutamate transporter, putative	-2.72	-1.15
<i>bb0646</i>	hydrolase, alpha/beta fold family	-3.03	-2.29

^a indicates the fold change of genes down-regulated in WT B31 relative to the *bosR* mutant.