The Fur homologue BosR requires Arg39 to activate rpoS transcription in Borrelia burgdorferi and thereby direct spirochaete infection in mice

Laura I. Katona

INTRODUCTION

Lyme disease is caused by the spirochaete Borrelia burgdorferi (Benach et al., 1983; Burgdorfer et al., 1982; Steere et al., 1983). Infection occurs in humans when the spirochaete is transmitted by an infected tick during tick feeding. Normally, however, the spirochaete completes its life cycle in the wild, passing back and forth between an Ixodid tick vector and various vertebrate hosts (e.g. mice, deer). This requires that the spirochaete survive and multiply in two very different environments. To do this, B. burgdorferi employs a separate set of genes for each of these environments. Determining how the spirochaete turns these genes on and off is currently an area of intense research (Radolf et al., 2012; Samuels, 2011).

In B. burgdorferi, many of the genes required for mammalian infection are under the control of the alternative sigma factor RpoS (Radolf et al., 2012). Transcription of rpoS in B. burgdorferi is complex. Under certain growth conditions (i.e. higher temperatures, low cell density), the spirochaete produces a long rpoS transcript from an RpoD (σ70)-dependent promoter; however, under other growth conditions (i.e. acid pH, higher temperatures, high cell density), the spirochaete produces a short rpoS transcript from an RpoN (σN)-dependent promoter (Hübner et al., 2001; Lybecker & Samuels, 2007; Lybecker et al., 2010; Samuels, 2011). Translation of the long transcript (but not the short transcript) also requires the assistance of a small RNA (Samuels, 2011).

Production of the short rpoS transcript is tightly regulated, requiring both the alternative sigma factor RpoN (Hübner et al., 2001) and the Fur homologue BosR (Hyde et al., 2009; Ouyang et al., 2009). To initiate transcription, RpoN must first be activated by the response regulator Rrp2 (Yang et al., 2003) which, in turn, must first be phosphorylated (Xu et al., 2010). The binding site for RpoN within the rpoS promoter is known (Smith et al., 2007; Studholme & Buck, 2000). However, even though Rrp2 has a predicted DNA-binding domain, it appears that Rrp2 acts on RpoN without the benefit of binding DNA (Blevins et al., 2009; Burtnick et al., 2007; Yang et al., 2003). In vitro, BosR binds multiple sites within the rpoS promoter (Ouyang et al., 2011, 2014, 2015). Presumably, in vivo, BosR must bind one or more of these sites to activate rpoS transcription.

Originally, BosR was annotated as Fur (ferric uptake regulator) because it showed homology to the FUR family of DNA-binding proteins (Fraser et al., 1997). Boylan et al. (2003) renamed it BosR (Borrelia oxidative stress regulator)

Abbreviations: kan, kanamycin resistance; qRT-PCR, quantitative real-time PCR; str, streptomycin resistance.

Seven supplementary tables, nine supplementary figures and supplementary methods are available with the online Supplementary Material.
and put forward the idea that BosR’s function in *B. burgdorferi* was to regulate the oxidative stress response. Genes which may be regulated by BosR in this fashion include *napA* (renamed *bicA*), *sodA* and *cdr* (Boylan et al., 2003, 2006).

Although BosR may be involved in the oxidative stress response (Hyde et al., 2009, 2010), it appears its main function is to act as a global regulator. To prepare the spirochaete for mammalian infection, BosR upregulates transcription of *rpoS* (Hyde et al., 2009, 2010; Ouyang et al., 2009, 2011) and also downregulates transcription of various tick-phase genes (e.g. *ospA* and *ospD*), which need only be expressed in the tick and which if allowed expression in the mammal could lead to immune clearance (Shi et al., 2014; Wang et al., 2013). When BosR activates *rpoS*, it binds the direct repeat (DR) sequence TAAATT-ATTATAA (Wang et al., 2013); however, when BosR represses *ospA* and *ospD*, it binds a variant of the Per box sequence TTATAAT-ATTATAA (Wang et al., 2013). How BosR can bind two very different sequences and generate two very different outcomes is currently unclear.

*B. burgdorferi* strain B31 was isolated in 1981 from a tick collected on Shelter Island (Burgdorfer et al., 1982). Genome sequencing of strain B31 identified a *fur* homologue (bb0647) and determined that residue 39 of the predicted protein was an arginine (Fraser et al., 1997). Since then, clonal isolates of strain B31 have been found that contain a single nucleotide change in *bosR*, making residue 39 of BosR a lysine (unpublished data; Seshu et al., 2014). This residue is highly conserved as arginine in virtually all FUR proteins. With BosR, substitution of this arginine with lysine affects the protein’s ability to bind DNA *in vitro* (Seshu et al., 2004). Not known is what effect this substitution has on the protein’s ability to function *in vivo* during infection (Hyde et al., 2006; Seshu et al., 2004).

Here, strains of *B. burgdorferi* were constructed that expressed either BosR or BosRR39K. Mice became infected by the strains expressing BosR but not by the strains expressing BosRR39K. This was, at least in part, because the spirochaetes expressing BosR activated *rpoS* transcription while the spirochaetes expressing BosRR39K did not. The R39K mutation affected the ability of BosRR39K to bind the *rpoS* promoter *in vitro*. It is therefore possible that BosRR39K failed to activate *rpoS* transcription because it could not bind the *rpoS* promoter *in vivo*. This study shows that BosR needs Arg39 to upregulate *rpoS* and in so doing support mammalian infection.

**METHODS**

**Bacterial strains and culture conditions.** Table S1 (available in the online Supplementary Material) lists the *B. burgdorferi* strains used in this study. Strain B31-A3 was kindly provided by Patricia Rosa and strain B31-F was kindly provided by Justin Radolf. Spirochaetes were routinely cultured at 33 °C in BS-K-II (Barbour, 1984) or BS-K-H medium (Sigma) supplemented with 6 % rabbit serum. To upregulate *rpoS*, spirochaetes were cultured at 37 °C in BS-K-II or BS-K-H medium supplemented with 6 % rabbit serum and adjusted to pH 6.8. Spirochaetes were enumerated by dark-field microscopy.

**BosR disruption mutants and complemented strains.** bosR disruption mutants and complemented strains were constructed as detailed in the Supplementary Material.

**Mouse infections.** C3H/HeN mice (Taconic) were infected by intradermal injection at 8 weeks of age. Ears, heart and bladder were collected 21 days post-infection, and the tissues were cultivated at 33 °C in BSK-H medium containing 6 % rabbit serum with or without 50 μg rifampicin ml⁻¹. Outgrowth of spirochaetes in these cultures was assessed by dark-field microscopy at weekly intervals for up to 1 month at which time the infections were deemed negative if no spirochaetes were detected. All animal procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University.

**Quantitative real-time PCR (qRT-PCR).** qRT-PCR was carried out on a model 7500 machine (Applied Biosystems) using the relative quantification method (AAG) with *flaB* as endogenous control. RNA was extracted with TRI-Reagent LS ( Molecular Research Center) and further purified on an RNasey spin column (Qiagen). cDNA was synthesized by using random hexamers with the Superscript III first strand synthesis system (Invitrogen). qRT-PCR primers (Table S2) were designed with ABI Primer Express software. ABI Power SYBR Green PCR master mix was used with 200 nM primers.

**Gel shift assays.** Gel shift assays were carried out as previously described (Katona et al., 2004). Briefly, recombinant BosR (or BosRR39K) was mixed with target DNA in 20 mM Bistris/borate (pH 7.5) buffer containing 1.5 mM DTT, 1.3 mM MgCl₂, 53 mM KCl, 3.3 % glycerol and 133 μg BSA ml⁻¹, incubated for 20 min at room temperature, and separated on a 6 % nondenaturing gel. The DNA was stained with SYBR Green I ( Molecular Probes) and the gel was scanned in blue fluorescence mode on a Storm 860 imager (Amersham-Pharmacia Biotech).

**Target DNA.** Target DNA was generated by PCR from cloned sequences. For *P_rpoS*, a 1.6 kb region containing the *rpoS* gene plus upstream sequence was PCR-amplified from strain B31-A3 genomic DNA with primers BB0771-SphI F and BB0771-xhoI R and inserted into the *Sphi*-XhoI site of pST-Blue1 (Novagen), giving pLK43/1. The *P_rpoS* target DNA was then amplified by PCR from pLK43/1 using primers BB0771p F1 and BB0771p R1. For BbDR, equal molar amounts of the synthesized single strands DRF2 and DRR2 were mixed, heated for 5 min at 95 °C, and slowly cooled to room temperature. Next, the duplex oligonucleotide was inserted into the EcoRV site of pST-Blue1, giving pLK45/16. The BBDR target DNA was then amplified by PCR from pLK45/16 using primers T7 promoter and U-19mer. Table S2 lists all primer sequences.

**Recombinant BosR and BosRR39K.** Recombinant proteins were prepared as previously described (Katona et al., 2004). Briefly, the His-tagged proteins were overexpressed in *Escherichia coli* BL21(DE3) from pLK1/5 or pLK42/2, purified by zinc chelate chromatography, and then treated with thrombin to free the N-terminal His tags. pLK42/2 was constructed by PCR-amplifying the bosRR39K gene from pLK38/2 using primers FLAF and FRR (Table S2) and inserting it into the NdeI–XhoI site of pET28a (Novagen). pLK1/5 was from an earlier study (Katona et al., 2004).

**Tricine SDS-PAGE and Western blotting.** Tricine SDS-PAGE was carried out as previously described (Katona et al., 2004). Western blots of recombinant proteins were probed with mouse anti-His-1 monoclonal antibody (Sigma) to detect the N-terminal His tags. Western blots of *Borrelia* lysates were probed with mouse anti-flaB.
CB1 monoclonal antibody (Coleman & Benach, 1989), rat anti-OspC (kindly provided by Justin Radolf) or rat anti-BosR. Rat anti-BosR was raised against purified recombinant BosR that retained the N-terminal His tag. All blots were developed with IRDye-conjugated secondary antibodies (Rockland or LI-COR) and scanned on an Odyssey infrared imager (LI-COR). Odyssey 2-colour protein markers were used.

Statistics. Fisher’s exact test was used to measure statistical significance in the mouse studies and Student’s t-test was used to measure statistical significance in the qRT-PCR studies.

RESULTS

*bosR* disruption mutants are generated in strain B31-A3

In previous studies, knockouts of *bosR* were generated in strains B31-MI (Ouyang et al., 2009), ML23 (Hyde et al., 2009) and 297 (Ouyang et al., 2011). Strains B31-MI and 297 are infectious in mice (Ouyang et al., 2009, 2011); however, strain ML23 lacks the lp25 linear plasmid and therefore is not infectious in mice (Hyde et al., 2009). Here, strain B31-A3 was chosen for study. Strain B31-A3 is a clonal isolate of strain B31-MI that retains infectivity (Elias et al., 2002).

Two sets of *bosR* mutants were generated (Fig. S1a). A kanamycin resistance (kan) cassette was introduced by allelic exchange in the forward orientation to yield the L-series mutants and in the reverse orientation to yield the K-series mutants. The kan cassette had a *flgB* promoter to drive constitutive expression of the kanamycin resistance gene and a T7 terminator to avoid overexpression of downstream genes. A total of 58 transformants were identified: 28 with the kan cassette in the forward orientation and 30 with the kan cassette in the reverse orientation. Clones L9 and K18 were selected for further study. Both clones contained the full complement of linear and circular plasmids originally identified by Elias et al. (2002) in strain B31-A3 (data not shown). PCR products of a size consistent with the introduction of a kan cassette were obtained for both clones (Fig. S1b). Sequence analysis of these PCR products confirmed that *bosR* was disrupted and that no unwanted mutations were incorporated.

The complemented strains are engineered to express either BosR or BosRR39K

Repeated attempts were made to complement the *bosR* mutants in *trans* by introducing a pKFSS1 shuttle vector harbouring *bosR*; however, no transformants were identified. Li et al. (2007) succeeded in complementing their *dps* mutant by inserting *dps* with its promoter into the intergenic region between *bb0445* and *bb0446*. This approach offered several advantages: the location on the main chromosome ensured that the gene was present as a single copy and because the gene was located downstream of both *bb0445* and *bb0446*, transcriptional read-through from the inserted sequences was less of a concern. Here, their approach was adopted, but with some modifications. Li et al. (2007) inserted *dps* downstream of *bb0445* and followed it with a streptomycin resistance (str) cassette. This arrangement, however, made the inserted gene susceptible to transcriptional read through from promoters upstream of *bb0445*. Here, *bosR* along with its promoter was inserted downstream of the str cassette and a T7 terminator was then added to the cassette to prevent read through from the cassette’s *flgB* promoter.

Three complemented strains were constructed for each *bosR* mutant strain: a mock complement that contained only the str cassette, a *bosR* complement that contained the str cassette plus a WT *bosR* gene, and a *bosRR39K* complement that contained the str cassette plus an R39K-mutated *bosRR39K* gene (Fig. S2a). Multiple transformants were identified. A single clone was selected from each set and assayed by PCR to determine that it contained the desired insert (Fig. S2b). All of the selected clones were then screened for plasmid content and found to have the same profile as strain B31-A3 (data not shown). DNA sequence analysis confirmed the identity of the inserts and established that no unwanted mutations were incorporated. Clones KN1 (mock complement), KN107 (*bosR* complement) and KN203 (*bosRR39K complement) were from *bosR* mutant K18. Clones LN1 (mock complement), LN101 (*bosR* complement) and LN202 (*bosRR39K complement) were from *bosR* mutant L9.

*bosR* is expressed at near WT levels in both of the *bosR*-complemented strains

Ouyang et al. (2009) reported that the region *bb0648-bb0647-bb0646* functioned as an operon with transcription initiating from a promoter upstream of *bb0648*. Because genes within an operon can often be transcribed from their own promoters as well, qRT-PCR analyses were carried out on the *bosR*-complemented strains to determine if *bosR* was being transcribed. For these studies, the spirochaetes were cultured at 33 °C and the RNA was isolated during early exponential phase, mid-exponential phase and stationary phase. As expected, the *bosR*-mutant strains (L9 and K18) produced no *bosR* transcript (Table S3). However, the *bosR*-complemented strains (LN101 and KN107) each produced nearly the same level of *bosR* transcript as the WT strain during all three stages of growth (Table S3). Therefore, the sequence located immediately upstream of *bosR* does appear to function as a promoter to allow transcription independent of the operon.

qRT-PCR analyses were also carried out on the gene located immediately downstream of *bosR*. Compared with the WT strain, the two *bosR*-mutant strains each showed a lower level of expression of *bb0646* during early exponential phase and mid-exponential phase, and a much lower level of expression during stationary phase (Table S3). This decreased expression of *bb0646* was not due to the loss of *bosR* expression, because both of the *bosR-
complemented strains also showed decreased expression of *bb0646* (Table S3). Interestingly, the orientation of the kan cassette did not seem to matter: *bosR* mutant L9 (forward orientation) and *bosR* mutant K18 (reverse orientation) showed the same decreased expression of *bb0646* (Table S3). Thus, if the T7 terminator embedded within the kan cassette was responsible for terminating transcription of *bb0646* from an upstream promoter, then it did so from either orientation.

From these data, it was clear that the *bosR*-mutant strains showed two defects: loss of *bosR* expression and reduced expression of *bb0646*. The reduced expression of *bb0646* due to disruption of *bosR* is a cis effect since complementation of *bosR* did not restore *bb0646* expression. Neither of these defects, however, affected the ability of the spirochaetes to grow in culture (Fig. S3 and data not shown).

The complemented strains express comparable levels of *bosR* and *bosRR39K* transcript

Seshu et al. (2004) reported that strain CHP100 encodes a variant of the *bosR* gene. This variant, *bosRR39K*, is also present in strain B31-F (unpublished observation). Strains CHP100 and B31-F were both derived from high-passage strain B31: CHP100 in the Skare laboratory (Hyde et al., 2006; Seshu et al., 2004) and B31-F in the Radolf laboratory (Eggers et al., 2002). Other mutations in the *bosR* gene have not been identified.

Seshu et al. (2004) further reported that BosRR39K and BosR did not function the same in *vitro* or *in vivo*. However, because their studies were carried out with non-infectious isolates, it remained unclear if BosRR39K, like BosR, could support infection in mice. Here, spirochaetes were engineered to express *bosR* or *bosRR39K* in the infectious background of strain B31-A3. According to the qRT-PCR analyses, both of the *bosRR39K*-complemented strains (LN202 and KN203) produced transcript at a level comparable to that of the *bosR*-complemented strains (Table S3). Also, both of the *bosRR39K*-complemented strains expressed decreased levels of *bb0646* (Table S3). Thus, the *bosR* and *bosRR39K*-complemented strains seemed to differ only in their expression of *bosR* versus *bosRR39K*.

Mice become infected by spirochaetes expressing BosR but not BosRR39K

Others have reported that *bosR*-knockouts of *B. burgdorferi* are not infectious in mice (Hyde et al., 2009; Ouyang et al., 2009, 2011). Here, the WT strain B31-A3 was infectious at a dose of 20,000 spirochaetes per mouse, whereas neither of the *bosR*-mutant strains (L9 and K18) was infectious at this dose or at a higher dose of 100,000 spirochaetes per mouse (Table 1 and data not shown).

Ouyang et al. (2009) also reported that their *bosR*-complemented strain regained its infectious phenotype. Here, both of the *bosR*-complemented strains (LN101 and KN107) were infectious; however, the frequency of infection was about half that of the WT strain (Table 1). In part, this may have been because the *bosR*-complemented strains expressed slightly less *bosR* transcript than the WT strain (Table S3). Also, the defect in *bb0646* expression may have been a contributing factor (Table S3). Shaw et al. (2012) found that while a *bb0646* knockout was infectious in mice, its ability to infect was attenuated. *bb0646* encodes an enzyme that has lipase activity (unpublished observation; Shaw et al., 2012). It is thus possible that the spirochaete uses this enzyme to help promote infection.

Regardless of the reason for the reduced infectivity of the *bosR*-complemented strains, the results still show that expression of *bosR* from the *bb0445*–*446* intergenic site allowed the spirochaete to regain infectivity. In contrast, Table 1 shows that neither of the *bosRR39K*-complemented strains (LN202 or KN203) was infectious at the dose of 20,000 spirochaetes per mouse. Thus, while the *bosR*-complemented strains infected 10 out of 20 mice, the *bosRR39K*-complemented strains infected 0 out of 20 mice (Table 1). According to Fisher’s exact test, there is a highly significant difference between these two frequencies of infection (*P*<0.001).

Taken together, the data show that under this one set of infection conditions, the spirochaetes expressing BosR were capable of infecting mice whereas the spirochaetes expressing BosRR39K were not.

OspC is detected in spirochaetes expressing BosR but not BosRR39K

Others have suggested that the reason why their *bosR*-knockout strains were not infectious in mice was, at least in part, because they failed to produce sufficient OspC (Hyde et al., 2009; Ouyang et al., 2009). To test this, WT strain B31-A3, *bosR* mutant L9, mock complement LN1, *bosR* complement LN101 and *bosRR39K* complement LN202 were each cultured under conditions previously shown to upregulate expression of OspC *in vitro* (Yang et al., 2000). Under these conditions (37°C, pH 6.8), OspC was expressed at high levels in both the WT strain and *bosR* complement, but not in the *bosR* mutant, mock complement or *bosRR39K* complement (Fig. 1). The level of BosR, or BosRR39K, present in the samples was also determined. The *bosR* mutant and mock complement showed no protein; however, the WT strain, *bosR* complement and *bosRR39K* complement each showed about the same level of protein (Fig. 1). Thus, the *bosRR39K* complement did not fail to express OspC only because the BosRR39K was not expressed or not stable. A second experiment gave similar results (data not shown).

Transcripts of ospC and rpoS are detected by qRT-PCR in spirochaetes expressing BosR

OspC expression in *B. burgdorferi* is regulated at the level of transcription by the alternative sigma factor RpoS.
Both Ouyang et al. (2009) and Hyde et al. (2009) reported that their bosR-knockout strains produced less RpoS than their WT strains during growth in culture. Ouyang et al. (2009) also reported that their bosR-knockout strain produced less rpoS transcript. Here, the WT strain, bosR mutant L9 and bosR complement LN101 were each cultured as before and the RNA was isolated for qRT-PCR during late-exponential phase. The results in Table 2 show that the level of ospC transcript was greatly decreased in the bosR-mutant strain compared with the WT strain (125-fold), thus confirming that BosR was needed to see upregulation of ospC.

As noted earlier, Borrelia rpoS can be transcribed from an RpoD-dependent promoter to yield a long transcript or from an RpoN-dependent promoter to yield a short transcript (Lybecker & Samuels, 2007; Smith et al., 2007). Here, two sets of qRT-PCR primers were designed to allow detection of both transcripts (Figs. 2 and S4). The first set of primers (Fig. 2, primers 1 and 2) recognized sites near the 3' end of both long and short transcripts and therefore yielded data on the level of total rpoS transcript (i.e. the sum of long and short transcripts). The second set of primers (Fig. 2, primers 3 and 4) recognized sites near the 5' end of the long transcript (but not the short transcript) and therefore yielded data on the level of long rpoS transcript only. qRT-PCR analysis with primers 1 and 2 showed that the level of total rpoS transcript was decreased twofold in the bosR-mutant strain compared with the WT strain (Table 2, Experiment I – rpoS). However, qRT-PCR analysis with primers 3 and 4 showed that the level of long rpoS transcript was increased 2.1 fold in the bosR-mutant strain compared with the WT strain (Table 2, Experiment I – rpoS-L). Thus, BosR appears to have repressed long rpoS transcription while at the same time activated total rpoS transcription.

### Table 1. Infectivity of B. burgdorferi clones in mice

Mice were infected by intradermal injection of 2 × 10⁸ spirochaetes.

<table>
<thead>
<tr>
<th>Strain Description</th>
<th>No. of cultures positive/total no. of cultures</th>
<th>No. of mice infected/total no. of mice*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skin</td>
<td>Bladder</td>
</tr>
<tr>
<td>A3 WT</td>
<td>17/18</td>
<td>17/18</td>
</tr>
<tr>
<td>L9 bosR mutant of A3</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>K18 bosR mutant of A3</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>LN1 Mock complement of L9</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>KN1 Mock complement of K18</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>LN101 bosR complement of L9</td>
<td>4/10</td>
<td>4/10</td>
</tr>
<tr>
<td>KN107 bosR complement of K18</td>
<td>6/10</td>
<td>6/10</td>
</tr>
<tr>
<td>LN202 bosRR39K complement of L9</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>KN203 bosRR39K complement of K18</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

*P < 0.001 by Fisher’s exact test for the following comparisons: (i) bosR mutants L9 and K18 vs WT A3, (ii) bosR complements LN101 and KN107 vs mock complements LN1 and KN1, and (iii) bosRR39K complements LN202 and KN203 vs bosR complements LN101 and KN107.

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Table 2. Relative expression levels of rpoS and ospC as determined by qRT-PCR

In Experiment I, spirochaetes were inoculated at an initial density of 250 spirochaetes ml\(^{-1}\) and cultured at 37 °C for 6 days (late-exponential phase) in BSK-II medium (pH 6.8) containing 6 % rabbit serum. In Experiment II, spirochaetes were inoculated at an initial density of 143 spirochaetes ml\(^{-1}\) and cultured at 37 °C for 7 days (early stationary phase) in BSK-II medium (pH 6.8) containing 6 % rabbit serum.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Experiment I</th>
<th>Relative expression level*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT A3</td>
<td>bosR mutant L9</td>
</tr>
<tr>
<td>bb0771 (rpoS)</td>
<td>1.000</td>
<td>0.490±0.005</td>
</tr>
<tr>
<td>bb0771L (rpoS-L)</td>
<td>1.000</td>
<td>2.103±0.147</td>
</tr>
<tr>
<td>bbb19 (ospC)</td>
<td>1.000</td>
<td>0.008±0.001</td>
</tr>
<tr>
<td>bb0647 (bosR)</td>
<td>1.000</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>bb0646</td>
<td>1.000</td>
<td>0.132±0.001</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bb0771 (rpoS)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>bb0771L (rpoS-L)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>bbb19 (ospC)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>bb0647 (bosR)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>bb0646</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

*Mean ± SEM of two determinations, each with four replicates. Statistical significance was evaluated by Student’s t-test for the following comparisons. Total rpoS transcript: L9 vs A3 (P=0.01), L9 vs LN101 (P<0.01), LN101 vs LN1 (P=0.02), LN202 vs LN1 (P=0.07) and LN101 vs LN202 (P<0.01). Long rpoS transcript: L9 vs A3 (P=0.04), L9 vs LN101 (P=0.02), LN101 vs LN1 (P=0.01), LN202 vs LN1 (P=0.08) and LN101 vs LN202 (P=0.23).
†ND, Not done.

(Fig. 3a, b). These data do not indicate if BosR acted directly or indirectly; however, two additional studies gave similar results (Tables S4 and S5). Taken together, the combined data (Fig. 4a, b) showed that the increased expression of total rpoS transcript by the bosR mutant was highly statistically significant (P<0.01) while the decreased expression of the long rpoS transcript by the bosR mutant was not quite statistically significant (P=0.11).

Ouyang et al. (2009) previously reported that BosR activated rpoS transcription; however, their qRT-PCR analysis determined the level of total rpoS transcript and did not distinguish between the long and short transcripts. Here, the qRT-PCR analysis showed that BosR activated short rpoS transcription (Fig. 4a), but not long rpoS transcription (Fig. 4b). All three studies showed, however, that the WT strain and bosR mutant strain differed in their expression of bb0646 (Tables 2, S4 and S5). Thus, the rpoS result is valid only if it was unaffected by the varied expression of bb0646. To address this concern, additional studies were carried using the complemented strains. Experiment I showed that the bosR-complemented strain mirrored the behaviour of the WT strain, yet expressed neither BosR nor BosRR39K and thus functioned as the bosR mutant (Table 2, Fig. 3a, b). Thus, adopting this strategy of comparing the complemented strains could circumvent the issues with bb0646.

Experiment II employed three complemented strains: the bosR complement LN101 which expressed only BosR, the bosRR39K complement LN202 which expressed only BosRR39K, and the mock complement LN1 which expressed neither BosR nor BosRR39K and thus functioned as the bosR mutant. Each strain was cultured at 37 °C, pH 6.8 to ensure high-level expression of ospC and upregulation of rpoS. Spirochaetes were harvested during early stationary phase and the RNA was isolated.
for qRT-PCR analysis. The results of the qRT-PCR analysis (Table 2) showed that the \( \text{bosR} \) complement produced 333-fold more \( \text{ospC} \) transcript than the mock complement, confirming that the culture conditions did allow for high-level expression of \( \text{ospC} \). The results also showed that, as expected, all three complemented strains expressed similar levels of \( \text{bb0646} \) transcript. Finally, the results showed that the \( \text{bosR} \) complement produced eightfold more total \( \text{rpoS} \) transcript than the mock complement and 1.5-fold less long \( \text{rpoS} \) transcript than the mock complement (Table 2, Fig. 3, d). Therefore, BosR appears to have both activated total \( \text{rpoS} \) transcription and repressed long \( \text{rpoS} \) transcription. A second study gave similar results (Table S6). Student’s \( t \)-test was used to evaluate the data from the three studies which compared the \( \text{bosR} \) complement (LN101) with a \( \text{bosR} \) mutant (L9 or LN1).

### Footnotes

3.0
2.0
1.0
0.0
1.5
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0.0
2.0
1.6
1.2
0.8
0.4
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Day 6
Day 7
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Day 8
Day 6
Day 7
Day 8

**Fig. 3.** Regulation of \( \text{rpoS} \) transcription by BosR versus BosRR39K. The plots show the relative expression levels for total \( \text{rpoS} \) transcript (a, c and e) and long \( \text{rpoS} \) transcript (b, d and f) as determined by qRT-PCR. (a, b) Experiment I: WT strain B31-A3 was compared with \( \text{bosR} \) mutant L9 and \( \text{bosR} \) complement LN101. (c, d) Experiment II: \( \text{bosR} \) complement LN101 was compared with \( \text{bosRR39K} \) complement LN202 and mock complement LN1. (e, f) Experiment III: \( \text{bosR} \) complement LN101 (black bars) was compared with \( \text{bosRR39K} \) complement LN202 (white bars) using spirochaetes harvested during mid-exponential phase (day 6), late-exponential phase (day 7) and stationary phase (day 8). Error bars show the SEM (\( n=2 \)).
The next question was: what effect did BosRR39K have on rpoS transcription? The results of Experiment II showed that the bosRR39K complement produced about the same level of ospC transcript as the mock complement, indicating that, unlike BosR, BosRR39K did not upregulate ospC (Table 2). These results also showed that the bosRR39K complement produced 1.7-fold less total rpoS transcript than the mock complement, and therefore while BosR activated total rpoS transcription, BosRR39K did not (Table 2, Fig. 3c). A second study gave similar results (Table S6). Taken together, the combined data from the two studies gave a P value of 0.53, according to Student’s t-test.

These data indicate that, unlike BosR, BosRR39K did not activate total rpoS transcription.

The results for the long rpoS transcript were less clear. Experiment II showed that the bosRR39K complement produced less long rpoS transcript than the mock complement (Table 2). However, the second study showed that the bosRR39K complement produced a similar level of long rpoS transcript as the mock complement (Table S6). In neither study was the difference between LN202 and LN1 statistically significant (Tables 2 and S6). Taken together, the combined data gave a P value of 0.40, according to Student’s t-test. These data suggest that BosRR39K did not repress long rpoS transcription; however, further studies are needed to clarify this point.
Experiment III was designed to gain a better picture of how BosR regulates rpoS transcription. Strains LN101 and LN202 were grown in parallel under conditions conducive to upregulation of rpoS. Spirochaetes were harvested during mid-exponential phase (6 day cultures), late-exponential phase (7 day cultures) and stationary phase (8 day cultures) and the RNA was isolated and analysed by qRT-PCR. Table S7 shows the results of this analysis. Plots of the rpoS data also appear in Fig. 3(e, f).

As expected, strains LN101 and LN202 expressed similar levels of bb0646 transcript and similar levels of bosR or bosRR39K transcript (Table S7). Also as expected, strain LN101 expressed high levels of ospC transcript while strain LN202 expressed trace levels of ospC transcript (Table S7). Compared with strain LN202, strain LN101 expressed 4.7-fold more total rpoS transcript on day 6, 5.2-fold more on day 7 and 5.8-fold more on day 8 (Table S7). And, compared with strain LN202, strain LN101 expressed 1.1-fold less long rpoS transcript on day 6, 1.3-fold less on day 7 and 1.6-fold less on day 8 (Table S7). Thus, while strains LN101 and LN202 differed greatly in their production of total rpoS transcript, they differed less in their production of long rpoS transcript.

Strains LN101 and LN202 were also compared in Experiment II (see Tables 2 and S6). The results in Table 2 show that, compared with strain LN202, strain LN101 produced 12.7-fold more total rpoS transcript and 1.3-fold more long rpoS transcript. The results in Table S6 show that, compared with strain LN202, strain LN101 produced 4.2-fold more total rpoS transcript and 1.3-fold less long rpoS transcript. Taken together, the combined data from all three studies showed that strains LN101 and LN202 differed statistically in their expression of total rpoS transcript (Fig. 4e, P < 0.01), but not in their expression of long rpoS transcript (Fig. 4f, P = 0.23). Of course, BosR and BosRR39K may both regulate long rpoS transcription, but by different means.

Fig. 3(e, f) shows the same rpoS data as Table S7, but expressed in different form. In the plotted data, all of the results are expressed relative to the day 6 – LN202 data point (which is arbitrarily assigned a value of 1). It is clear from the plotted data that the BosR-expressing spirochaetes increased their expression of total rpoS transcript during the transition from mid-exponential phase to stationary phase (Fig. 3e), yet maintained a fairly constant level of expression of long rpoS transcript (Fig. 3f). Because the level of long rpoS transcript remained essentially constant, it is clear that the increase in total rpoS transcript was owing to short rpoS transcript. These data provide direct evidence that BosR activates short rpoS transcription while BosRR39K does not.

**BosR, but not BosRR39K, binds the DR sequence in vitro**

Ouyang et al. (2011) reported that recombinant BosR bound the DR sequence TAAATTAAT within the rpoS promoter from strain 297. The rpoS promoter in strain B31 differs slightly from that in strain 297 (Fig. S5); however, it still contains all four DR sequences identified in the 297 promoter (Ouyang et al., 2011). To test binding to the B31 promoter, recombinant BosR and BosRR39K were prepared in parallel. Both proteins ran with an estimated mass of 20.5 kDa on SDS-PAGE and appeared free of major contamination (Fig. S6). The results of gel shift assays showed that although both proteins bound the B31 promoter, BosR showed a different pattern of binding than BosRR39K (Fig. 5a). Because competitor DNA was not added to the assay, both proteins may have bound the DNA non-specifically. However, at low concentration (8 nM), BosR generated three shifted bands while BosRR39K generated a single shifted band (Fig. 5a). The
same results were obtained when the two proteins were assayed together (Fig. S7).

The putative BosR binding site located immediately upstream of the −24/−12 RpoN site contains a perfect DR sequence in both strains (Fig. S5). This site (Fig. 5b) was inserted into the EcoRV cloning site of pST-Blue1 and the insert plus flanking vector sequence was PCR-amplified to generate BbDR target DNA for testing (Fig. S8). The vector sequence with no insert was also PCR amplified to provide control DNA (Fig. S8). The BbDR target DNA was 257 bp. The control DNA was 229 bp. Thus, the insert (28 bp) accounted for ~10 % of the entire length. The results of the gel shift assay showed that BosRR39K and BosR both bound the BbDR target DNA (Fig. 5c) and both also bound the vector control DNA (Fig. 5d). However, while BosRR39K showed the same pattern of binding for both targets, BosR showed a different pattern of binding. Thus, at 10 nM, BosR generated a major shifted band with the BbDR target DNA (Fig. 5c, arrow) that was not present in the assay with the vector control (Fig. 5d). These data provide preliminary evidence that BosR bound the BbDR insert while BosRR39K did not.

**DISCUSSION**

This study shows that a single nucleotide change in the bosR gene to generate bosRR39K had the effect of rendering *B. burgdorferi* non-infectious in mice. Others have shown that eosR is required for *B. burgdorferi* to activate expression of the alternative sigma factor RpoS (Hyde et al., 2009; Ouyang et al., 2009). Because RpoS controls the expression of virulence factors needed for mammalian infection (Radolf et al., 2012), failure to upregulate rpoS could account for why the spirochaetes expressing BosRR39K were non-infectious. Assays carried out to determine the level of rpoS transcript present in the spirochaetes grown under conditions conducive to rpoS expression found that while the BosR-expressing spirochaetes did transcribe rpoS, the BosRR39K-expressing spirochaetes did not. Furthermore, the BosR-expressing spirochaetes produced high levels of ospC transcript and OspC protein while the BosRR39K-expressing spirochaetes did not. Transcription of ospC is known to require RpoS (Radolf et al., 2012). In vivo, OspC is expressed on the surface of the spirochaete at the point when the spirochaete moves from the gut of the tick into the haemolymph just prior to invading a mammalian host (Grimm et al., 2004; Pal et al., 2004; Tilly et al., 2006). Therefore, although genes other than rpoS may have had a role in making the BosRR39K-expressing spirochaetes non-infectious, failure to upregulate rpoS appears to have been a major cause.

Studies published in 2009–2010 established that BosR controls expression of rpoS in *B. burgdorferi* (Samuels & Radolf, 2009). Ouyang et al. (2011) identified sites within the promoter of strain 297 rpoS that bound BosR in vitro. They observed that these sites contained near matches to the DR sequence TAAATTAAT and suggested that BosR activated rpoS transcription by binding one or more of these sites in vivo. Later, Ouyang et al. (2014, 2015) redefined this putative BosR binding site as a palindromic 6-1-6 inverted repeat consisting of the sequence ATTAA-TTTAAA. They termed this sequence the BosR box. If BosR does bind this BosR box in vivo, it is still unclear how this binding activates rpoS transcription.

The preliminary results of the gel shift assays suggested that only BosR bound the BbDR insert. However, because the BbDR insert contained both a perfect DR sequence (tTTTAAATTAAAAT) and an imperfect BosR box (tTTTAAATTAAT), it is not possible to distinguish which of these sequences BosR recognized.

The protein families database (Pfam) lists BosR as belonging to the FUR family of DNA-binding proteins (Finn et al., 2004). Pfam alignments of FUR proteins (PF01475) show that BosR’s Arg39 aligns with a highly conserved arginine present in virtually all FUR proteins. X-ray crystallography studies show that FUR proteins consist of two domains: an N-terminal DNA-binding domain and a C-terminal dimerization domain (An et al., 2009; Butcher et al., 2012; Dian et al., 2011; Gilston et al., 2014; Jacquamet et al., 2009; Lin et al., 2014; Lucarelli et al., 2007; Makthal et al., 2013; Pohl et al., 2003; Sheikh & Taylor, 2009; Shin et al., 2011; Traoré et al., 2006, 2009). The DNA-binding domain contains a winged helix–turn–helix (wHTH) motif that provides the protein with the means to bind DNA (Pohl et al., 2003). The wHTH motif contains a three-helix bundle: the conserved arginine that aligns with BosR’s Arg39 is located near the N-terminal end of α-helix 1 (Pohl et al., 2003). Typically, residues within the recognition helix (α-helix 3) determine the binding specificity of wHTH proteins and therefore Arg39 would not be expected to be involved in defining the binding specificity of BosR (Aravind et al., 2005; Huffman & Brennan, 2002). However, others have suggested that positively charged residues located within the N-terminal region of FUR proteins are likely to form electrostatic interactions with their target DNAs (Butcher et al., 2012; Lucarelli et al., 2007; Pecqueur et al., 2006). Recently, *Streptococcus pyogenes* PerR was found to require Arg21, Arg26 and Arg31, as well as other positively charged residues, to bind DNA in vitro (Lin et al., 2014). Of interest here, Arg31 is the conserved arginine in PerR that aligns with BosR’s Arg39.

Models of BosR and BosRR39K (Fig. S9) were built using the *S. pyogenes* PerR as template (Lin et al., 2014). In these models, Arg39 and Lys39 were each located at the base of a pocket in a position adjacent to α-helix 3 (Fig. S9a). The electrostatic surface potential was determined for both proteins and showed that the surfaces appeared virtually identical (Fig. S9b).

In 2014, Gilston and coworkers published the crystal structure of *E. coli* Zur bound to DNA (Gilston et al., 2014). Included in their data was the observation that Zur’s Arg28 was located sufficiently close to the DNA to allow an electrostatic interaction to form between the arginine
BosR requires Arg39 to direct spirochaete infection

Fig. 6. Model of BosR regulation of rpoS transcription. RpoD-dependent transcription from an upstream promoter produces a long transcript in the absence of BosR (top). RpoN-dependent transcription from a downstream promoter produces a short transcript in the presence of BosR (bottom). BosR is pictured binding to all four DR sequences within the rpoS promoter; however, binding to all four sequences may not be required to allow transcription to proceed from the downstream promoter. The extent of long versus short transcription is indicated by the line thickness.

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references


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