



Global Repression of Host-Associated Genes of the Lyme Disease Spirochete through Post-Transcriptional Modulation of the Alternative Sigma Factor RpoS

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Abstract

Borrelia burgdorferi, the agent of Lyme disease, is a vector-borne pathogen that transits between *Ixodes* ticks and vertebrate hosts. During the natural infectious cycle, spirochetes must globally adjust their transcriptome to survive in these dissimilar environments. One way *B. burgdorferi* accomplishes this is through the use of alternative sigma factors to direct transcription of specific genes. RpoS, one of only three sigma factors in *B. burgdorferi*, controls expression of genes required during tick-transmission and infection of the mammalian host. How spirochetes switch between different sigma factors during the infectious cycle has remained elusive. Here we establish a role for a novel protein, BBD18, in the regulation of the virulence-associated sigma factor RpoS. Constitutive expression of BBD18 repressed transcription of RpoS-dependent genes to levels equivalent to those observed in an *rpoS* mutant. Consistent with the global loss of RpoS-dependent transcripts, we were unable to detect RpoS protein. However, constitutive expression of BBD18 did not diminish the amount of *rpoS* transcript, indicating post-transcriptional regulation of RpoS by BBD18. Interestingly, BBD18-mediated repression of RpoS is independent of both the *rpoS* promoter and the 5' untranslated region, suggesting a mechanism of protein destabilization rather than translational control. We propose that BBD18 is a novel regulator of RpoS and its activity likely represents a first step in the transition from an RpoS-ON to an RpoS-OFF state, when spirochetes transition from the host to the tick vector.

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Introduction

Many vector-borne pathogens are maintained in a natural infectious cycle in which they transition between specific vectors and susceptible hosts. During this vector->host->vector cycle, pathogens are exposed to disparate environments, to which they must quickly adapt through immediate changes in gene expression to ensure successful transmission and acquisition. One such vector-borne pathogen, and the causative agent of Lyme disease, is the spirochete *Borrelia burgdorferi* [1–3]. *B. burgdorferi* is transmitted by the bite of infected *Ixodes* ticks and maintained in an enzootic cycle between ticks and small mammalian hosts [4]. Larval *I. scapularis* ticks acquire the pathogen by feeding on an infected host. Spirochetes survive through the molt from larvae to nymph, and are subsequently transmitted to a new host by tick feeding. The spirochetes establish a persistent infection in the host, completing the infectious cycle and making them available for acquisition by feeding ticks [5–7].

Throughout the infectious cycle, spirochetes are exposed to difficult environmental conditions, including acquired and innate immune pressures, oxidative and nitrosative stress, and nutrient limitation [8–14]. To survive within and transit between these environments, *B. burgdorferi* must quickly and effectively adjust its transcriptome. Characteristic examples of changes in gene expression during the *B. burgdorferi* infectious cycle include the

timely and critical expression of *ospC*, *dbpA*, and *bba66* early in mammalian infection [15–21], as well as the expression of *ospA* and *glpD* in the tick vector [20,22–25]. While the specific functions of some of these factors are unknown, they have demonstrated roles in the in vivo fitness of *B. burgdorferi* [19–21,25–31]. Inappropriately timed or unregulated expression [12,32–34] of some key virulence-associated factors can lead to spirochete clearance from the host, or the inability to survive in the vector. The ability to adapt to environmental changes is requisite for successful transmission of the spirochete; precise control and proper timing of gene expression is critical for survival of the spirochete throughout its infectious cycle.

Control of gene expression in bacteria is complex and occurs through many different molecular mechanisms. One of those mechanisms is the coordinated control of sigma factor-directed transcription. In response to environmental conditions, growth phase or cellular stresses, specific sigma factors become available, bind to the RNA polymerase holoenzyme (RNAP), and direct the transcription of genes required to adapt to particular environments. When the stress is removed, bacteria switch sigma factors, thereby remodeling the transcriptome, to adapt to changing conditions (For review see-[35]). Reprogramming of gene expression through sigma factor-directed transcription, and the use of alternative sigma factors are vital to the success of many pathogens

[36,37]. *B. burgdorferi* accomplishes this during the infectious cycle using only three sigma factors, *rpoD* ($\sigma 70$), *rpoN* ($\sigma 54$), and *rpoS* ($\sigma 38$) [38,39]. Both RpoN and RpoS play critical roles in the *B. burgdorferi* infectious cycle and, interestingly, RpoN controls the majority of *rpoS* transcription [40–44].

In *E. coli* RpoS controls the stress response [45], but in *B. burgdorferi*, as in many other pathogenic bacteria, RpoS controls the transcription of several virulence factors, including *ospC* and *bba66* [44,46]. *OspC* and BBA66 are critical for establishing infection when spirochetes are transmitted from a tick to a mammalian host [21,28,47], and *rpoS* appears to be maximally expressed during this transmission stage [48,49]. RpoS, or an RpoS-dependent factor, also plays a central role in the repression of genes that have important roles in the arthropod vector, including *ospAB*, *bba62* and *bba74* [48,50,51]. Therefore, proper expression and repression of *rpoS* is crucial throughout the infectious cycle; inappropriately timed expression of *OspC* or repression of *OspA* would be detrimental to the survival of *B. burgdorferi*. Consequently, *B. burgdorferi* exerts tight control over *rpoS*, using transcriptional and translational activators and transcriptional repressors [43,52–61]. The regulation of RpoS in *B. burgdorferi* is complex [60]. RpoS is requisite for expression of critical virulence factors, is fundamental to establishing an infection in a mammalian host, and must be repressed to allow expression of genes required when spirochetes transition from the mammalian host into the tick.

Linear plasmid 17 (p17) of *B. burgdorferi* encodes a protein, BBD18, that can repress expression of *ospC* [62]. However, expression of *ospC* is RpoS-dependent, and induction of RpoS-dependent gene transcription requires the activation of a multistep signaling cascade [42,43,60,63,64]. Additional control of *ospC* expression is also exerted through inverted repeat (IR) sequences located upstream of the *ospC* promoter [33,65–67]. BBD18 is a small (25.7kDa), basic protein that contains sequence determinants suggestive of a role in nucleic acid binding, but where BBD18 is exerting its regulatory effect leading to *ospC* repression was previously undetermined. Here we report that BBD18-mediated repression is not limited to *ospC* and that BBD18 is in fact a novel regulator of RpoS, exerting its effect at a post-transcriptional level, and therefore repressing the entire RpoS regulon. We demonstrate that repression of RpoS is not a result of inhibition of translation initiation, or mediated through the *rpoS* ribosome binding site or the *rpoS* 5' untranslated region (UTR). Our data suggest that BBD18 plays a role in destabilizing RpoS and signifies a "first step" in transitioning from an RpoS-ON state to an RpoS-OFF state. BBD18-mediated post-transcriptional repression of RpoS adds yet another layer of complexity to the sophisticated mechanisms used by *B. burgdorferi* to regulate this critical sigma factor.

Results

BBD18 represses RpoS-dependent virulence factors in wild-type *B. burgdorferi*

OspC is an RpoS-dependent virulence factor and the level of RpoS is typically tightly regulated in *B. burgdorferi*, as well as in many other pathogenic bacteria [60,68]. BBD18 can repress expression of *ospC* in attenuated *B. burgdorferi* strains that demonstrate high *ospC* expression levels under normal in vitro conditions [62]. These strains were developed in vitro by serial passage and selective pressure, resulting in the displacement of most of the *B. burgdorferi* plasmids [69]. The mechanism leading to high level *ospC* expression in these strains, and their precise genetic makeup, was undetermined. Therefore, BBD18-mediated repression of *ospC* was difficult to comprehensively characterize in these strains. To better describe the effect of BBD18 on *ospC* expression,

we used a genetically defined wild-type *B. burgdorferi* strain, and analyzed the effect of BBD18 on the production of *OspC*. We first used allelic exchange to generate an isogenic *rpoS* mutant in our wild-type strain B31-S9, as previously described for the B31-A3 strain [70]. We confirmed the disruption of the *rpoS* locus (B31-S9 Δ *rpoS*) and that the plasmid content of the mutant was identical to the parental strain by PCR (data not shown).

During normal in vitro growth of *B. burgdorferi*, RpoS levels and RpoS-dependent gene transcription are low [41,48]. Consistent with these observations [63], we detected low levels of *ospC* transcript or protein under normal in vitro growth conditions (BSKII medium, pH7.6/35°C/5%CO₂), making it difficult to study the effect of BBD18 on *OspC* under these conditions (data not shown). However, several in vitro culture conditions have been developed that induce expression of *rpoS*, leading to increased RpoS-dependent gene transcription. These conditions include growing spirochetes at a reduced pH [71], subjecting spirochetes to a temperature shift [15], or growth in increased levels of acetate [61], and are thought to partially mimic the tick-to-mouse transition, the point in the infectious cycle where *rpoS* appears to be maximally expressed in vivo [48,49]. To induce expression of *rpoS*, and consequently *ospC*, we subjected *B. burgdorferi* strains B31-S9 (wild type), B31-S9 Δ *rpoS* (Δ *rpoS*) or B31-S9 containing a shuttle vector constitutively expressing *bdd18* (wild type/*flaBp-bdd18*), to growth conditions at a reduced pH (pH6.8). Consistent with previous reports [71], we found that *OspC* was readily produced by the wild-type strain and that synthesis was RpoS-dependent (Fig. 1, panels A-B) [41,65]. *OspC* was undetectable in both the Δ *rpoS* and wild-type/*flaBp-bdd18* strains grown under identical conditions (Fig. 1A-B). The absence of *OspC* in the presence of BBD18 (Fig. 1C) demonstrates that BBD18 expression prevents the production of *OspC* in *B. burgdorferi* wild-type strains.

A palindromic sequence upstream of the *ospC* promoter plays a role in both the expression and repression of *ospC*, although a specific repressor protein that binds to the putative operator sequence has not been identified [33,66,67]. One possible mechanism of BBD18-mediated repression of *ospC* is through the interaction of BBD18 with the *ospC* promoter, either with the inverted repeats (IR) upstream of the *ospC* promoter, or within the region proximal to the transcriptional start site. However, when this potential BBD18-*ospC* promoter interaction was analyzed biochemically using electrophoretic mobility shift assays (EMSA), we found no evidence for a BBD18-*ospC* promoter interaction (Fig. S1). Taken together, these results suggest that BBD18-mediated repression of *ospC* does not involve an interaction of BBD18 with the *ospC* promoter.

In addition to *OspC*, *B. burgdorferi* RpoS-dependent virulence factors BBA66 and DbpA are synthesized early during mammalian infection. To determine if BBD18 production alters any other specific RpoS-dependent virulence factors, we analyzed the production of RpoS-dependent proteins BBA66 and DbpA by immunoblot. While both BBA66 and DbpA were detected in wild type, we were unable to detect these proteins in the Δ *rpoS* strain or when BBD18 was constitutively expressed (Fig. 1, panels D-E). The correlation between the presence of BBD18 and absence of *OspC*, BBA66, or DbpA suggests that BBD18 expression prevents the production of additional RpoS-dependent virulence factors.

BBD18 alters the *B. burgdorferi* Antigenic-Protein Profile

Since our analysis was consistent with BBD18 being a global regulator of RpoS-dependent expression, we took advantage of the natural induction of RpoS-dependent proteins that occurs during the infectious cycle [15,48,72]. We anticipated that sera collected from mice infected by *B. burgdorferi* could be used to detect global

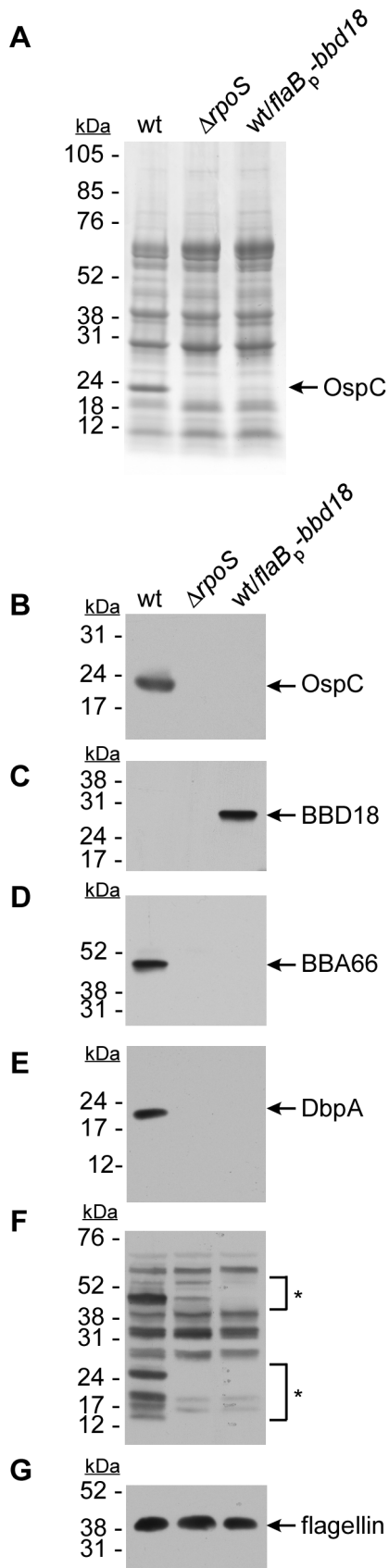


Figure 1. Effect of BBD18 on proteins in the RpoS regulon. *B. burgdorferi* strains B31-S9 (wt), B31-S9 $\Delta rpoS$ ($\Delta rpoS$) and B31-S9/pBSV2*-flaBp-bbd18 (wt/flaBp-bbd18) were grown under *rpoS*-inducing conditions (BSKII medium, pH 6.8). Cell lysates were subjected to SDS-PAGE, Coomassie blue staining (A), and immunoblot analysis (B-G). Membranes were probed for the presence of OspC (B), BBD18 (C), BBA66 (D), or DbpA (E), using protein-specific antibodies or antisera. Pooled sera from mice infected with *B. burgdorferi* by tick bite was used to detect changes in the antigenic protein profile (F). A mouse monoclonal α -flagellin antibody (H9724) was used to detect flagellin as a protein loading control (G). Positions of molecular mass standards are shown on the left in kiloDaltons (kDa).
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changes in RpoS-dependent protein production and provide a broader assessment of the effect of BBD18 on the *B. burgdorferi* proteome.

We performed Western blots using pooled sera collected from mice infected with *B. burgdorferi* by tick bite, and analyzed cell lysates from wild type, $\Delta rpoS$, and wild type/flaBp-bbd18 strains that had been grown under *rpoS*-inducing conditions. In the wild-type strain, we detected several prominent protein bands that were recognized by these sera, demonstrating that proteins normally recognized by the acquired immune response were being produced (Fig. 1F). However, in the $\Delta rpoS$ and wild type/flaBp-bbd18 strains, many of the immuno-reactive bands were absent or markedly reduced (Fig. 1F, see brackets and asterisks). These proteins were more prominent in wild type, suggesting that their genes are RpoS-dependent. Interestingly, some protein bands appear reduced, although not absent, and likely are not strictly RpoS-dependent, consistent with previous reports demonstrating that some genes in the RpoS regulon are not strictly RpoS-dependent [42,48]. We do not know the identity of all the proteins recognized by these sera, but they range in molecular mass from ~12–65 kDa. These data demonstrate that the effect of BBD18 on proteins in the RpoS regulon is not limited to OspC, BBA66, and DbpA. Therefore, BBD18 represses the expression of numerous RpoS-dependent genes, indicating a global regulatory effect imparted by BBD18 on many, if not all, RpoS-dependent proteins.

BBD18 specifically represses RpoS-dependent gene transcription

Having established that BBD18 represses the synthesis or presence of proteins that are RpoS-dependent and normally produced under *rpoS*-inducing conditions, we next sought to determine if BBD18-mediated repression was at the level of transcription. To do so, we determined the transcript level of core genes within the RpoS regulon under *rpoS*-inducing conditions, using quantitative reverse transcriptase PCR (qRT-PCR). We analyzed the transcription of *ospC*, *bba66*, *bba72*, *bbg01*, *bbj24* and *bba34*, and found that all were readily expressed under *rpoS*-inducing conditions in our wild-type strain (Fig. 2A). Consistent with the RpoS-dependent nature of these genes, transcript levels were all substantially reduced in the $\Delta rpoS$ strain (Fig. 2A). Under the same *rpoS*-inducing conditions, the wild-type strain constitutively producing BBD18 was unable to induce expression of these genes, and expression levels were similar to those in a $\Delta rpoS$ strain (Fig. 2A). To determine if BBD18 were acting specifically on RpoS-dependent transcripts or acting as a global repressor of transcription, we analyzed the expression of *rpoS*-independent genes *bbj41*, *bba62* (lp6.6) and *bba15* (*osp4*). The transcript levels of *bbj41*, *bba62*, and *bba15* are increased in the arthropod vector or during growth conditions mimicking the arthropod vector [73–75]. These genes are also subject to RpoS-dependent repression in dialysis membrane chambers in vivo [48,50]. Our data

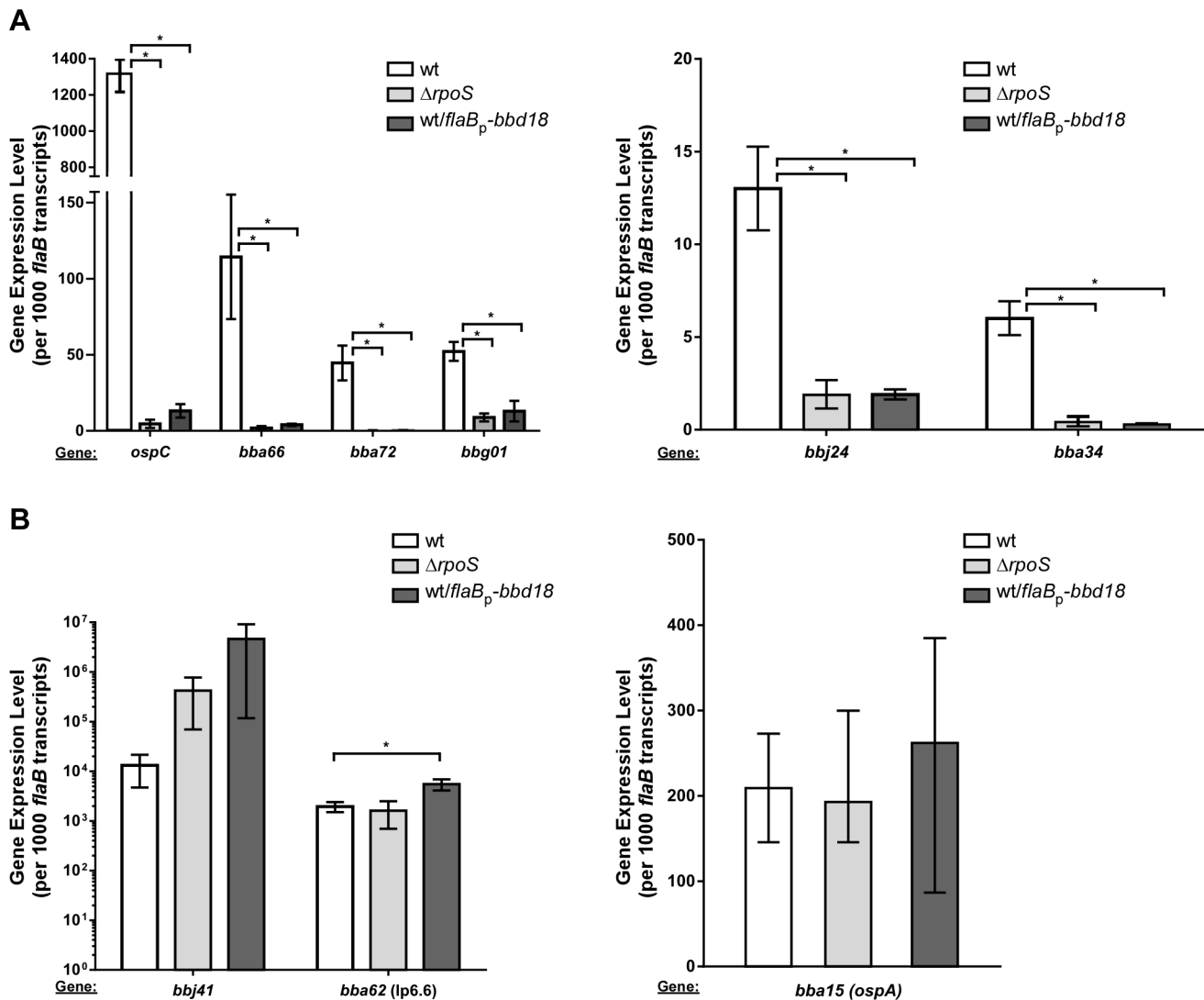


Figure 2. Analysis of RpoS-dependent and RpoS-independent gene transcription. Quantitative reverse transcriptase-PCR (qRT-PCR) analysis of gene expression in strains B31-S9 (wt), B31-S9 $\Delta rpoS$ ($\Delta rpoS$) and B31-S9/pBSV2*-*flaBp-bbd18* (wt/*flaBp-bbd18*), grown under *rpoS*-inducing conditions (BSKII medium, pH6.8). The transcript level of RpoS-dependent genes *ospC*, *bba66*, *bba72*, *bbg01*, *bbj24*, and *bba34* (A) and RpoS-independent genes *bbj41*, *bba62* (lp6.6) and *bba15* (*ospA*), (B) are shown as relative units, and normalized to the constitutively expressed *flaB* transcript. Data were analyzed using Student's unpaired t-test and brackets marked with asterisks represent a statistically significant difference ($p < .05$).

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demonstrate that BBD18 does not repress the transcription of *bbj41*, *bba62*, or *bba15* (Fig. 2B). These data demonstrate that BBD18-mediated repression is specific for RpoS-dependent transcripts and is not reflective of a global repression of transcription. In fact, *bba62* and *bbj41* show an increase in gene expression in the $\Delta rpoS$ and wild type/*flaBp-bbd18* strains (Fig. 2B). These data indicate that BBD18 represses RpoS-dependent transcripts at the level of transcription, and is specific to RpoS-dependent transcripts.

BBD18-mediated repression of RpoS is post-transcriptional

The gene expression cascade leading to induction of the RpoS regulon is a multistep process [60]. The response regulator protein-2 (Rrp2) is activated and promotes the transcription and subsequent translation of the alternative sigma factor RpoN.

RpoN transcribes *rpoS*, and following translation of the *rpoS* transcript, RpoS-dependent gene transcription occurs (Rrp2 \rightarrow RpoN \rightarrow RpoS \rightarrow RpoS-dependent gene transcription)[40,42–44,51]. Our data establish a role for BBD18 in the specific repression of RpoS-dependent transcripts. However, BBD18 could be exerting its effect on any of the upstream components in the cascade, resulting in the repression of RpoS-dependent gene transcription. To determine where in this regulatory cascade BBD18 exerts its effect, we used qRT-PCR to analyze the transcript levels of the alternative sigma factors *rpoN* and *rpoS*. Since transcription of these genes is low during normal in vitro growth conditions, we analyzed transcripts from *B. burgdorferi* cultured at pH6.8 to induce *rpoS* expression. We found that both *rpoN* and *rpoS* were expressed at similar levels in both wild type and wild type/*flaBp-bbd18* strains (Fig. 3). Having detected robust levels of RpoS-dependent transcripts (*ospC*, *bba66*, *bba72*, *bbg01*,

bbj24, *bba34*) in the wild-type strain and low-to-undetectable levels of RpoS-dependent gene transcripts in the wild type/*flaBp-bbd18* strain (compare Fig. 2A and Fig. 3), we found these results surprising. To address this apparent inconsistency, we analyzed cell lysates of spirochetes grown under RpoS-inducing conditions using RpoS-specific antiserum. We found that RpoS was readily detected in the wild-type strain, but not in the $\Delta rpoS$ or wild type/*flaBp-bbd18* strains (Fig. 4). Comparing wild-type to the wild type/*flaBp-bbd18* strain, cultured under identical conditions, we found equivalent *rpoS* transcript levels, yet dissimilar protein levels. Taken together, these data demonstrate that BBD18 exerts its regulatory effect on RpoS at a post-transcriptional level.

BBD18 does not inhibit translation initiation of RpoS

A common mechanism of post-transcriptional regulation in bacteria is through inhibition of translation initiation, which can result from the binding of a regulatory factor, or formation of an inhibitory secondary structure that occludes the ribosome binding site and prevents translation [58,76–78]. Because BBD18 exhibits characteristics of a nucleic acid binding protein [62], we hypothesized that BBD18 might interact with the 5' untranslated region (UTR) of the *rpoS* mRNA and inhibit translation. To address this possibility, we constructed a shuttle vector containing a transcriptional fusion of the *rpoS* promoter to the *lacZ_{Bb}* reporter gene [79] (Fig. 5A). The transcriptional fusion contains a 141bp region 5' of the *rpoS* open reading frame (ORF), and includes the transcriptional start site [58,63,80] and a Shine-Dalgarno sequence (RBS) [81] (Fig. 5A). The transcriptional fusion between the *rpoS* 5'UTR and the *lacZ_{Bb}* gene allows detection of β -galactosidase (β -gal) activity as a measure of translation efficiency from the *rpoS* 5'UTR, and can be used to determine if BBD18-mediated repression of RpoS requires the *rpoS* 5' UTR.

We first confirmed the activation of the *rpoN*->*rpoS* regulatory cascade in both a wild-type strain and wild type/*flaBp-bbd18* harboring the transcriptional fusion (*rpoSp₁₄₁-lacZ_{Bb}*). Strains were

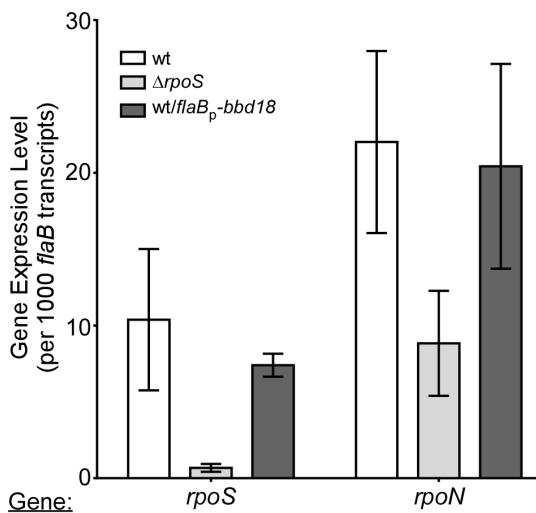


Figure 3. Analysis of the transcript level of alternative sigma factors *rpoN* and *rpoS*. qRT-PCR data displaying the transcript levels of *rpoS* and *rpoN* in strains B31-S9 (wt), B31-S9 $\Delta rpoS$ ($\Delta rpoS$) and B31-S9/pBSV2*-*flaBp-bbd18* (wt/*flaBp-bbd18*) grown under *rpoS*-inducing conditions. Levels of *rpoS* and *rpoN* transcripts are displayed in relative units per 1000 copies of *flaB* transcript. Transcript levels were analyzed using Student's unpaired t-test and no statistically significant difference was detected ($p > .05$).
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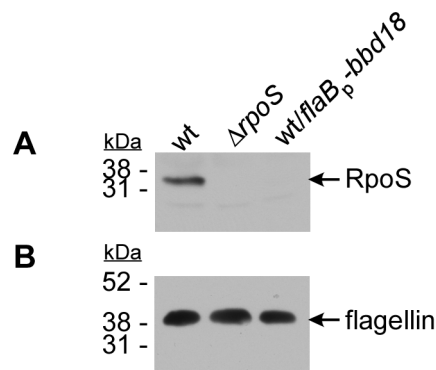


Figure 4. Synthesis of RpoS by wild type but not *bbd18*-expressing *B. burgdorferi* Immunoblot analysis of cell lysates from strains B31-S9 (wt), B31-S9 $\Delta rpoS$ ($\Delta rpoS$) and B31-S9/pBSV2*-*flaBp-bbd18* (wt/*flaBp-bbd18*) grown under *rpoS*-inducing conditions. Cell lysates were analyzed using RpoS antiserum (A) or a mouse monoclonal antibody to flagellin (B) to assess flagellin levels as a protein loading control. Cell lysates and immunoblot for flagellin were the same as those used in Fig. 1. Positions of molecular mass standards are shown on the left in kiloDaltons (kDa).
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grown under RpoS-inducing conditions (BSKII pH6.8) and cell lysates analyzed for the presence of OspC by Western blot. As expected, we detected OspC in the wild-type strain and the wild type/*rpoSp₁₄₁-lacZ_{Bb}* strain, but not in the wild type/*rpoSp₁₄₁-lacZ_{Bb}/flaBp-bbd18* strain (Fig. 5B). Having established that the RpoS regulon was being transcribed under these conditions, we next measured β -gal activity in these cell lysates. We did not detect any β -gal activity in the wild-type strain lacking the reporter construct (Fig. 5D), whereas we detected significant and equivalent levels of β -gal activity in both strains containing the *rpoSp₁₄₁-lacZ_{Bb}* transcriptional fusion (Fig. 5D). These data demonstrate that the RpoS regulon is activated under these conditions and, consistent with the qRT-PCR data, suggest that transcription from the *rpoS* promoter is equivalent in both wild type and wild type/*flaBp-bbd18* strains. These results suggest that in the wild-type/*flaBp-bbd18*/*rpoSp₁₄₁-lacZ_{Bb}* strain, BBD18 is mediating repression of RpoS and the RpoS regulon, however, it is unable to repress expression of, or translation of, a transcriptional fusion containing the *rpoS* promoter and 5' UTR (Compare 4B and 4D). Equivalent levels of β -gal activity suggest that BBD18-mediated repression of RpoS is not through inhibition of translation initiation but is likely specific to the RpoS protein. It is important to note that under similar growth conditions, where robust β -gal activity was detected, we were unable to detect RpoS or RpoS-dependent transcripts (Fig 2B and Fig 4). Taken together, these data suggest that BBD18-mediated repression of RpoS is post-transcriptional, specific to the RpoS protein and independent of the *rpoS* promoter and 5'UTR.

Discussion

To be maintained in nature, *B. burgdorferi* must be transmitted from an infected tick to a susceptible host, establish a persistent infection, and subsequently be re-acquired by feeding ticks. During this alternating tick->mouse->tick infectious cycle, the Lyme disease spirochete remodels its transcriptome in response to specific environmental cues in order to adapt to and survive in these environments. This is accomplished through the use of multiple alternative sigma factors [60]. Exchanging the sigma factor bound to the RNAP holoenzyme allows quick and efficient

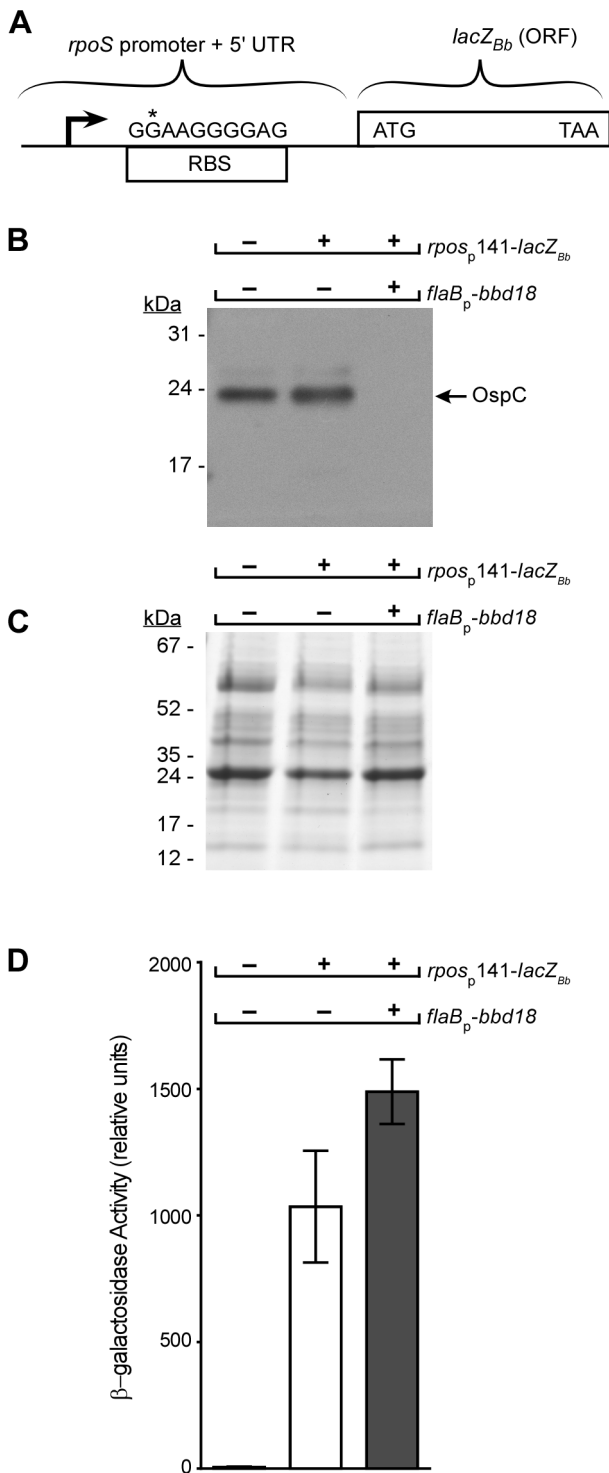


Figure 5. Analysis of BBD18 repression of an *rpoS* promoter *lacZ_{Bb}* transcriptional fusion. (A) A schematic diagram of the transcriptional fusion of the *rpoS* promoter and 5' untranslated region (UTR) fused directly to the *lacZ_{Bb}* open reading frame (ORF). The position of the *rpoS* transcriptional start site [58,63] is indicated by a filled arrowhead. The Shine-Dalgarno sequence (RBS), and the translational start site of β-galactosidase (*lacZ_{Bb}*), indicated by the ATG, are also shown. Regions corresponding to the *rpoS* promoter and 5' UTR (141bp) or the *lacZ_{Bb}* ORF are indicated with brackets. The "G" in the RBS marked with an asterisk is identified because the reporter construct harbored an A->G mutation relative to the published sequence at that position. Cell lysates from strains B31-S9 (wt), B31-

S9/pBSV2G-*rpoSp₁₄₁-lacZ_{Bb}* (wt/*rpoSp-lacZ_{Bb}*), and B31-S9/pBSV2G-*rpoSp₁₄₁-lacZ_{Bb}/pBSV28-flaBp-bbd18* (wt/*rpoSp-lacZ_{Bb}/flaBp-bbd18*) were grown under *rpoS*-inducing conditions and analyzed with OspC antisera (B) or stained with Coomassie blue (C) to demonstrate equivalent protein loads in each lane. The positions of molecular mass standards are shown on the left in kiloDaltons (kDa). (D) β-galactosidase activity in cell lysates from strains B31-S9 (wt), B31-S9/pBSV2G-*rpoSp₁₄₁-lacZ_{Bb}* (wt/*rpoSp-lacZ_{Bb}*), and B31-S9/pBSV2G-*rpoSp₁₄₁-lacZ_{Bb}/pBSV28-flaBp-bbd18* (wt/*rpoSp-lacZ_{Bb}/flaBp-bbd18*) grown under *rpoS*-inducing conditions.

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remodeling of the transcriptome by promoting the transcription of sigma factor-specific genes. Of particular focus in *B. burgdorferi* has been the Rrp2->RpoN->RpoS regulatory cascade, leading to RpoN- and RpoS-dependent gene transcription. This regulatory cascade is turned on early during nymphal tick feeding and controls the expression of virulence factors required for the establishment of mammalian infection [40,41,44,48,49,51,60]. Several environmental cues responsible for turning on this pathway have been identified, and the critical role played by RpoS in virulence factor gene expression in this process has been well established [15,40,44,71,82]. However, the molecular mechanisms involved in the transition from an RpoS-ON state during nymph feeding (and presumably within a mammalian host) to an RpoS-OFF state in fed larvae and unfed nymphs remains less than well defined. We have now identified a role for a novel factor, BBD18, which likely represents a "first step" in the transition from an RpoS-ON to an RpoS-OFF state in *B. burgdorferi*.

BBD18 was identified originally as a repressor of *ospC* in high passage *B. burgdorferi* strains [62], and initially we focused on the role of BBD18 in the repression of the requisite virulence factor OspC. We have now demonstrated that BBD18 prevents the transcription of RpoS-dependent genes, including *ospC*, in wild-type *B. burgdorferi* indirectly. BBD18 prevents RpoS-dependent gene transcription through its regulatory effect on the alternative sigma factor RpoS, not direct transcriptional repression of genes in the RpoS regulon. Under conditions mimicking the tick->mammalian transition, where RpoS-dependent gene transcripts are expressed, *bbd18* expression represses transcription of RpoS-dependent genes to levels equivalent to those in an *rpoS* mutant (Fig. 2A). We also detected an increase in the expression of RpoD-dependent transcripts *baa62* and *bbj41* (Fig. 2B) [51]. Since all of the RpoS-dependent transcripts we analyzed were repressed in the presence of constitutive BBD18, we analyzed the transcription level of *rpoS*. Direct analysis by qRT-PCR, and analysis of an *rpoS* transcriptional fusion, demonstrated that the *rpoS* transcript levels, and expression from the *rpoS* promoter, were equivalent to wild type levels when *bbd18* was being constitutively expressed (Fig. 3 and Fig. 5D). However, under similar RpoS-inducing conditions, we were unable to detect RpoS protein when *bbd18* was constitutively expressed (Fig. 4). Moreover, when *rpoS* was induced by a temperature shift from 25°C -> 35°C, we detected equivalent levels of *rpoS* transcript in wild type and wild type/*flaBp-bbd18* strains by qRT-PCR, but were unable to detect RpoS protein in strains where *bbd18* was constitutively expressed (data not shown). Additionally, the *rpoS* promoter-*lacZ_{Bb}* transcriptional fusion data (Fig. 5) demonstrate that BBD18-mediated post-transcriptional repression of RpoS is not facilitated through the *rpoS* promoter or 5' UTR, either through direct BBD18 interaction with the RBS, or through sequestration of a translation-promoting factor that interacts with the 5'UTR. Cumulatively, these data suggest that BBD18-mediated repression of RpoS occurs post-transcriptionally, is specific for the RpoS protein, and is likely facilitating, either directly or indirectly, destabilization of RpoS.

In *B. burgdorferi*, RpoS regulation is complex [60]. Protein factors BosR, CsrA, BadR, and HrpA play roles in the regulation of *rpoS* transcription [52–54,56,83,84]. Previously, only the small RNA DsrA and RNA-binding protein Hfq were clearly shown to play roles in the post-transcriptional regulation of RpoS in *B. burgdorferi* [58,59]. DsrA plays a similar role to DsrA in *E. coli*, [85,86], binding to the 5' UTR of the *rpoS* mRNA, relieving an inhibitory secondary structure, and promoting translation of *rpoS* under certain conditions. One possible mechanism of BBD18-mediated repression of RpoS would be through the sequestration of the *rpoS*-translation-promoting factor DsrA. However, BBD18 was unable to repress translation of β -galactosidase from the transcriptional fusion (*rpoS*_{141P}-*lacZ*_{BB}) containing a DsrA binding site (Fig. 5D), making this action through DsrA unlikely. Additionally, following a temperature shift, where DsrA is not active, BBD18-mediated repression of RpoS-dependent transcripts still occurred (data not shown). These results suggest that BBD18-mediated repression is independent of DsrA and the *rpoS* promoter, and specific for the RpoS protein.

A likely mechanism of the RpoS repression described here is through BBD18-mediated targeted degradation of RpoS. Targeted degradation of specific protein factors is characterized in both eukaryotes and prokaryotes as a mechanism to directly reorganize the proteome, as well as modify the transcriptome. Degradation of specific transcriptional activators, repressors, or sigma factors can redirect transcription of specific sets of genes and has been hypothesized as a mechanism for regulating the level of RpoS in *B. burgdorferi* [59,87–89]. In *E. coli*, targeted degradation of RpoS has clearly been demonstrated as a robust regulatory mechanism for controlling this alternative sigma factor [45,90]. This mechanism involves the use of an adaptor protein RssB, which binds to and delivers RpoS to the ClpXP proteasome-like complex, where RpoS is quickly degraded [91]. *B. burgdorferi* harbors homologs of the Clp protease complex, but lacks an RssB homolog. If BBD18 were fulfilling the role of RssB, it may be doing so in a way that is unique to *B. burgdorferi*, as the primary amino acid sequence of BBD18 shares little identity with this factor. However, testing the effect of BBD18 on RpoS repression in a *B. burgdorferi* Clp protease mutant has not been possible. There are no available Clp mutants in the transposon mutagenesis library [92], and previous attempts to disrupt these alleles through direct allelic exchange have been unsuccessful (J.A.Carroll, unpublished observations). The Clp protease complex may be essential for cell homeostasis if the loss of Clp proteolytic components leads to unregulated RpoS levels, which are toxic to *B. burgdorferi* [93]. Alternatively, BBD18 might be acting directly on RpoS as a protease, although BBD18 is not homologous to any known protease or contain any proteolytic domains. Preliminary experiments to assess whether the addition of exogenous BBD18 results in enhanced RpoS turnover were inconclusive (data not shown). Whether BBD18 is acting directly as a protease, indirectly as an adaptor, or through some alternative mechanism to destabilize RpoS, remains to be elucidated. Defining the components involved in RpoS destabilization should help determine the molecular mechanisms involved in BBD18-mediated repression of RpoS.

Our data demonstrate that BBD18-mediated repression of the *rpoS* regulon is through regulation of RpoS protein, not at the level of *rpoS* transcription. These data suggest that the role of BBD18 is likely at the point where *B. burgdorferi* needs to transition from having RpoS available for transcription (RpoS-ON), to one where a different alternative sigma factor (presumably σ -70) is required (RpoS-OFF). During the infectious cycle, RpoS must be shut off when spirochetes transition from an infected host to a feeding vector (as well as in unfed nymphs), to allow expression of genes

required in the arthropod vector. Targeted degradation of RpoS would allow quick and efficient repression of RpoS activity. RpoS degradation would result in changing the alternative sigma factor bound to RNAP by altering the relative concentration of sigma factors available for RNAP binding. This change would allow *B. burgdorferi* to transition quickly from transcription of mammalian-specific genes like *bba66*, *bba72*, *bbj24*, *bbg01*, to arthropod-specific genes, like *ospA*, *bbj41* and *bba62*. In fact, we see hints of that possible scenario in this study. Expression of BBD18 led to a decrease in *rpoS*-specific transcripts and a reproducible increase in *rpoD*-dependent transcripts (Fig. 2 A-B). One possible explanation for these data is that a decrease in the level of RpoS makes additional RNAP available for RpoD binding, and thus leads to an increase in RpoD-dependent transcription. Our focus here was on the mode of action of BBD18, and a more comprehensive analysis of the effect of BBD18 would be required to determine if this observation is reproducible and is transcriptome-wide.

Our hypothesis, that BBD18 is required for the host-to-tick transition to repress RpoS-dependent gene transcription, would suggest that *bbd18* is expressed at the beginning of the arthropod phase of the infectious cycle. Analysis of the available microarray and gene expression data for *B. burgdorferi* suggest that this is likely the case. Expression of *bbd18* mimics the expression patterns of genes that are specifically expressed under arthropod-like conditions [73,74]. Consistent with an in vivo role for BBD18 in the regulation of RpoS at the RpoS ON-> RpoS OFF transition, Tokarz et al. detected *bbd18* transcript in unfed nymphs but not fed nymphs. Also, many of the arthropod-induced genes, including *bbd18*, are transcriptionally repressed in an RpoS-dependent manner in response to mammalian-like environmental signals [48,50,51]. Since transcription of *bbd18* appears to be subject to RpoS-mediated repression, and, as our data demonstrate, BBD18 represses RpoS, a delicate balance must exist between the level of BBD18 and the level of RpoS. We have presented a model of how the interplay between RpoS and BBD18 might work (Fig. 6). In response to mammalian signals, *rpoS* transcript levels increase, and subsequent RpoS protein production leads to expression of genes required for the mammalian environment and repression of arthropod-induced genes, including *bbd18* (Fig. 6A). In response to some as-yet unidentified cue upon entering a tick, *bbd18* expression increases, leading to production of BBD18, destabilizing RpoS, allowing de-repression of RpoS-mediated repression and the concomitant expression of arthropod-specific genes (Fig. 6B).

If BBD18 plays a role in the transition from RpoS-ON to RpoS-OFF, it must be regulated appropriately. Inappropriately timed expression of *bbd18* inhibits mammalian infection (Hayes et al. Submitted). How *bbd18* expression is regulated within ticks and what signals turn on expression of this gene are still undefined. One possibility is an increase in BBD18 activity in response to the phosphorylation state of the protein, similar to the modulation of RssB activity in *E. coli* [94]. Although we do not have evidence for BBD18 phosphorylation, *B. burgdorferi* encodes two histidine kinases whose roles in controlling gene expression have begun to be elucidated [95,96]. The signals and mechanisms involved in adapting to the arthropod environment and transitioning from mammalian-specific gene expression pattern to the arthropod-specific gene expression pattern are just starting to be examined. Our data demonstrate that expression of *bbd18* likely represents a first step in transitioning from an RpoS-ON to an RpoS-OFF state and as such, its induction might be a harbinger for the induction of arthropod-specific genes.

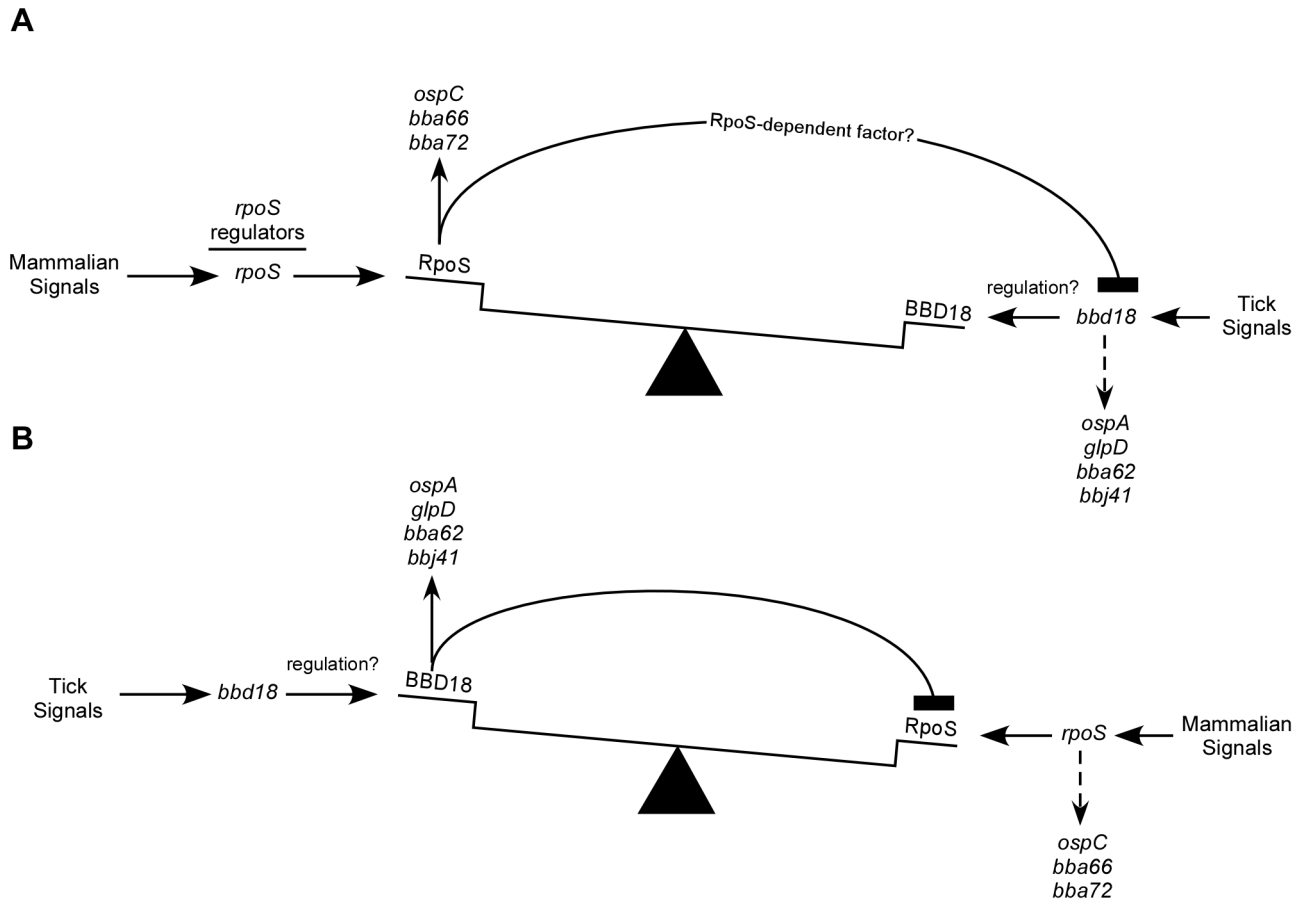


Figure 6. Proposed balance between RpoS and BBD18 in *B. burgdorferi*. (A) In response to mammalian environmental conditions, expression of *rpoS* is induced. Several factors, including BosR, CsrA, Hfq, DsrA and BadR, play roles in regulating *rpoS*. When RpoS protein is available to bind RNAP, it directs the expression of *ospC*, *bba66*, *bba72* and other RpoS-dependent genes. RpoS represses transcription of *bbd18*, *ospA*, *glpD*, *bba62*, *bbj41* and other arthropod-induced genes, possibly through an RpoS-dependent factor[51]. (B) In response to arthropod environmental conditions, *bbd18* is expressed and BBD18 production destabilizes RpoS and represses RpoS-dependent transcription, allowing induction of genes required in the arthropod vector.

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Materials and Methods

Borrelia burgdorferi Strains and Growth Conditions

Typically, *B. burgdorferi* strains were cultured in BSKII medium (pH7.6) as previously described [97,98]. For pH induction of the RpoS regulon, strains were grown in BSKII medium (pH7.6) to late exponential phase (1×10^8 spirochetes/mL) and transferred to pH-adjusted BSKII medium (pH6.8) at a density of 5×10^7 spirochetes/mL and allowed to grow to $\sim 2 \times 10^8$ spirochetes/mL at 35°C. Strain B31-S9, termed wild type in this study, is an infectious derivative of B31-A3 that lacks the known restriction modification systems encoded on linear plasmid 56 and linear plasmid 25 that limit introduction of shuttle vector constructs [99–101]. Strain B31-S9/pBSV2*-*flaBp-bbd18* is a derivative of wild-type B31-S9 strain harboring a shuttle vector constitutively expressing *bbd18* under control of the flagellin (*flaB*) promoter [62]. Strain B31-S9 Δ *rpoS* was constructed as previously described [70] and disruption of the *rpoS* locus and plasmid content verified by PCR (primers 3 & 4, Table S1). Where appropriate, kanamycin, streptomycin and gentamicin were used at 200 ug/mL, 50 ug/mL or 40 ug/mL, respectively.

SDS-PAGE and Immunoblots

For analysis of cell lysates by SDS-PAGE and Western blot, equivalent numbers of spirochetes were harvested by centrifugation based on enumerating spirochetes in a Petroff-Hausser chamber. Spirochetes were washed twice in HN buffer (50 mM HEPES (pH 7.5), 50 mM NaCl), and resuspended in equal volumes of Laemmli gel loading buffer for protein analysis, or mixed with TRIzol reagent (Life Technologies, Carlsbad CA) and stored at -80°C for RNA extraction. Protein samples were resolved by SDS-PAGE on 4–15% gradient gels (Bio-Rad, Hercules CA) and either stained with Coomassie blue or transferred to a nitrocellulose membrane for immunoblotting. For detection of OspC, DbpA, RpoS, FlaB, BBD18, and proteins detected with infected-mouse sera, membranes were blocked for 1 hr in a 4% nonfat milk solution (Lab Scientific, Livingston, NJ) prepared in Tris-buffered saline containing 0.1% Tween 20 (TBST-20), with rocking. Membranes were then transferred to a 1% solution of nonfat milk in TBST-20 containing one of the following: rabbit α -OspC polyclonal antiserum (1:1000) [32], rabbit α -RpoS polyclonal antiserum (UGA-17, 1:500), α -DbpA purified antibody (Rockland Immunochemicals, Gilbertsville, PA, 401-B98, 1:500), mouse monoclonal α -FlaB antibody (H9724, 1:500) [102], rabbit α -BBD18 polyclonal antiserum (1:500), generated, as described

[103], using purified recombinant BBD18 protein (see below), or pooled infected-mouse sera (pooled from several mice infected with wild-type B31-A3 spirochetes by tick transmission, 1:200). Immunoblots were then incubated in TBST-20 with the appropriate peroxidase-conjugated secondary antibody for 30 min and developed using SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific, Rockford, IL) and X-ray film (Lab Scientific Inc., Livingston, NJ). Detection of BBA66 was performed as previously described [46].

Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was isolated from *B. burgdorferi* strains using TRIzol (Life Technologies, Carlsbad, CA) reagent following the manufacturer's instructions. Subsequently, 5 µg of RNA from each strain was treated with DNase for 1 hr at 37°C using TurboDNase (Life Technologies) and purified following the manufacturer's protocol. Following purification, 1 µg of RNA was used to generate cDNA using High Capacity cDNA Reverse Transcriptase kit (Life Technologies), following the manufacturer's protocol. Quantitative PCR (qPCR) reactions were performed using TaqMan Universal PCR Mastermix (Life Technologies) with gene specific primer and probe sets (primer 2 nM each, probe 5 nM) (Integrated DNA Technologies, Coralville, IA) (Table S1). Experiments were performed in biological and technical triplicate and analyzed on an ABI 7900 using Sequence Detection Software (SDS 2.4), or a Viia7 using the Viia 7 software package (Life Technologies). The mean and standard deviations were determined using PRISM software (PRISM). qPCR reactions performed on cDNA samples generated in the absence of reverse transcriptase were similar to no-template control reactions.

β-Galactosidase Activity Assays

A 141bp fragment upstream of *rpoS*, containing the *rpoS* promoter sequence, was amplified by PCR using primers 7 & 8 (Table S1) and cloned into *Sall* and *BspHI* sites immediately upstream of the *lacZ_{Bb}* gene on pBH*lacZ_{Bb}** [62], creating pBSV2G-*rpoSp₁₄₁*-*lacZ_{Bb}*. Construction of pBSV28-*flaBp*-*bbd18* shuttle vector, containing the flagellin (*flaB*) promoter driving expression of *bbd18* on pBSV28, is described elsewhere (Hayes et al. Submitted). The relevant plasmid sequences were verified by Sanger sequencing and the pBSV2G-*rpoSp₁₄₁*-*lacZ_{Bb}* plasmid contained an A->G mutation in the RBS relative to the published sequence. The pBSV2G-*rpoSp₁₄₁*-*lacZ_{Bb}* shuttle vector was transformed into strain B31-S9 and transformants were confirmed by PCR using primers 1 and 2 (Table S1). B31-S9/pBSV2G-*rpoSp₁₄₁*-*lacZ_{Bb}* was then transformed with pBSV28-*flaBp*-*bbd18* generating B31-S9/pBSV28-*flaBp*-*bbd18*/pBSV2G-*rpoSp₁₄₁*-*lacZ_{Bb}*. For analysis of β-galactosidase activity, strains B31-S9, B31-S9/pBSV2G-*rpoSp₁₄₁*-*lacZ_{Bb}*, B31-S9/pBSV28-*flaBp*-*bbd18*/pBSV2G-*rpoSp₁₄₁*-*lacZ_{Bb}* were grown in BSKII medium (pH7.6) to late exponential phase, transferred to BSKII medium (pH6.8) at a density of 5×10^7 spirochetes/mL, and allowed to grow to $\sim 2 \times 10^8$ spirochetes/mL at 35°C. 1 ml cultures containing equivalent numbers of spirochetes were harvested by centrifugation and washed 3 times with HN buffer. β-galactosidase activity was determined using the Galacto-Light Plus Chemiluminescent reporter gene assay system (Life Technologies) following the manufacturer's protocol for microplate detection. Briefly, spirochetes were lysed in Lysis Solution, cell debris was cleared by centrifugation and, after a 20 min incubation with the chemiluminescent substrate, β-galactosidase activity was measured in a microtiter plate using a BioTek Synergy-2 plate reader (BioTek, Winooski, VT). Each sample was analyzed in triplicate following the manufacturer's instructions. To ensure equivalent sample loads

between wells, sample volumes were normalized based on their absorbance reading at 260nm. Luminescence readings were measured in relative units and the mean and standard deviation from three independent experiments were determined using PRISM software. To check induction of the RpoS regulon, a portion of the lysate used in the β-galactosidase assay was analyzed by immunoblot for the presence of OspC, as described above.

Expression and Purification of BBD18

The *bbd18* open reading frame was amplified by PCR from low passage wild-type B31-A3 genomic DNA using primers 5 & 6 (Table S1). The resulting product was digested with NdeI and XhoI (New England Biolabs, Ipswich, MA) and purified using a PCR purification kit (Qiagen, Valencia, CA). The purified product was ligated into similarly-digested pET28 (EMD Millipore, Billerica, Massachusetts) in frame with an N-terminal six-histidine tag, creating pET28-6XHis-*bbd18*. The relevant portion of this plasmid was confirmed by Sanger sequencing and transformed into BL21-CodonPlus (DE3)-RIPL *E. coli* (Agilent Technologies, Santa Clara, CA) for protein expression. Typically, BL21-CodonPlus(DE3)-RIPL/pET28-6XHis-*bbd18* were grown in 1 to 4L of LB at 37°C to an O.D.₆₀₀ of 0.5 with shaking. Isopropyl-β-D-1 thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for induction of expression. Expression continued for 3 hr and cultures were harvested by centrifugation and stored at -80°C. Cell pellets were resuspended in 30 mL of lysis buffer (1 M NH₄Cl, 150 mM KCl, 50 mM Hepes (pH 7.5), 2 mM β-mercaptoethanol, 10 mM imidazole, 5 mM MgCl₂), disrupted by sonication, and cell debris was cleared by centrifugation. The soluble fraction was combined with 1 mL of pre-equilibrated Ni-NTA slurry and allowed to incubate for 1hr at 4°C with rocking. Following incubation, the slurry was applied to a chromatography column and washed with 100 bed volumes of lysis buffer. Proteins were eluted from the column in 5 mL fractions using elution buffer (300 mM KCl, 50 mM Hepes (pH 7.5), 2 mM β-mercaptoethanol, 250 mM Imidazole, 5 mM MgCl₂). Elution fractions were analyzed by SDS-PAGE and Coomassie blue staining and the identity of BBD18 was confirmed by mass spectroscopy. Elution fractions containing BBD18 were combined in an Amicon Ultra centrifugal unit (EMD Millipore, Billerica, MA) and subjected to buffer exchange and protein concentration into a buffer lacking imidazole (300 mM KCl, 50 mM Hepes (pH 7.5), 2 mM β-mercaptoethanol, 5 mM MgCl₂). BBD18 antiserum was generated as previously described [103].

Gel Mobility Shift Assays

Gel mobility shift assays were performed as in [104]. Briefly, pPCR8-*ospCp*-5F containing the *ospC* promoter and upstream inverted repeats was digested with FokI (New England Biolabs) and purified using a PCR purification kit (Qiagen). Restriction digested pPCR8-*ospCp*-5F (400 ng) was incubated with 6 µM to 150 µM of purified BBD18 at 25°C for 15 min in binding buffer (10 mM Hepes pH 7.5, 2 mM β-mercaptoethanol, 1 mM MgCl₂, 1 mg/mL BSA, 0.01% NP40). Samples were resolved on 5% nondenaturing polyacrylamide gels in cold 0.5X TBE and visualized by staining gels with a 1X solution of Gel Red Nucleic Acid Stain (Biotium, Hayward, CA).

Supporting Information

Figure S1 Analysis of a potential BBD18-*ospC* promoter interaction. Recombinant BBD18 was purified by affinity chromatography and analyzed by SDS-PAGE and Coomassie blue staining (A). Electrophoretic mobility shift assay of restriction

digested pPCR8-*ospCp*-5F, containing the *ospC* promoter and upstream inverted repeats, was incubated with purified recombinant BBD18 and resolved on a 5% polyacrylamide gel (B). The arrow indicating unbound DNA is directed at the specific restriction fragment containing the *ospC* promoter and upstream inverted repeats.

(TIF)

Table S1 Oligonucleotide primers and probes used in this study.

(DOCX)

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Author Contributions

Conceived and designed the experiments: DPD BMH PAR. Performed the experiments: DPD. Analyzed the data: DPD BMH PAR. Contributed reagents/materials/analysis tools: DPD BMH PAR. Wrote the paper: DPD.

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