Published in final edited form as:

Mol Microbiol. 2020 February ; 113(2): 399–417. doi:10.1111/mmi.14427.

Characterization of 6S RNA in the Lyme disease spirochete

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Summary

6S RNA binds to RNA polymerase and regulates gene expression, contributing to bacterial adaptation to environmental stresses. In this study, we examined the role of 6S RNA in murine infectivity and tick persistence of the Lyme disease spirochete *Borrelia* (*Borreliella*) *burgdorferi*. *B. burgdorferi* 6S RNA (Bb6S RNA) binds to RNA polymerase, is expressed independent of growth phase or nutrient stress in culture, and is processed by RNase Y. We found that *rny* (*bb0504*), the gene encoding RNase Y, is essential for *B. burgdorferi* growth, while *ssrS*, the gene encoding 6S RNA, is not essential, indicating a broader role for RNase Y activity in the spirochete. Bb6S RNA regulates expression of the *ospC* and *dbpA* genes encoding outer surface protein C and decorin binding protein A, respectively, which are lipoproteins important for host infection. The highest levels of Bb6S RNA are found when the spirochete resides in unfed nymphs. *ssrS* mutants lacking Bb6S RNA were compromised for infectivity by needle inoculation, but injected mice seroconverted, indicating an ability to activate the adaptive immune response. *ssrS* mutants were successfully acquired by larval ticks and persisted through fed nymphs. Bb6S RNA is one of the first regulatory RNAs identified in *B. burgdorferi* that controls the expression of lipoproteins involved in host infectivity.

Graphical Abstract

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Author contributions

DD, AJB, KMW, and DSS conceived and designed the study; DD, LSH, AJB, JRC, and KMW collected the data; DD, LSH, AJB, KMW and DSS analyzed the data; DD and DSS wrote the manuscript; and all authors approved the manuscript.

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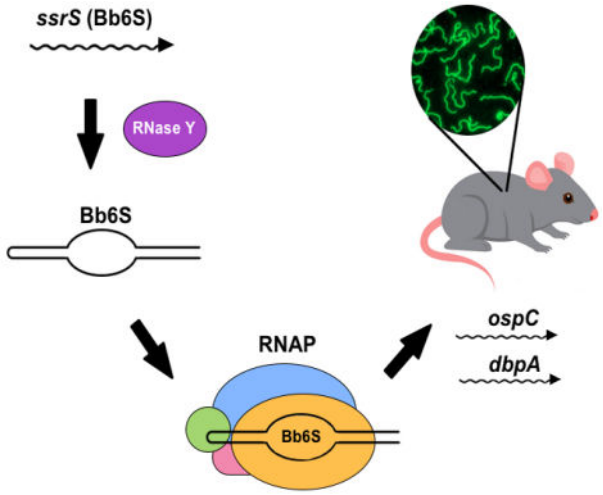
Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest Statement

The authors declare no conflict of interest.



Borrelia burgdorferi, the causative agent of Lyme disease, is maintained in nature in an enzootic cycle involving a tick and a vertebrate. Bb6S RNA, a small regulatory RNA in *B. burgdorferi*, is processed by ribonuclease Y, binds RNA polymerase, regulates transcription, and is involved in infection of the vertebrate host and persistence in the tick vector.

Keywords

Borrelia burgdorferi; Lyme disease; 6S RNA; gene expression regulation, bacterial; RNA, small untranslated; Spirochaetales

Introduction

The Lyme disease agent *Borrelia* (*Borreliella*) *burgdorferi* navigates disparate and challenging environments as it traverses through its enzootic cycle, which alternates between *Ixodes* ticks and vertebrates (Radolf *et al.*, 2012; Caimano *et al.*, 2016). *B. burgdorferi* is acquired when *Ixodes* larvae feed on an infected animal, and the spirochete then persists in the tick midgut as the blood meal is consumed by the larvae before it molts into a nymph. The spirochete can be transmitted to and infect a new host as the nymph takes a blood meal, completing an enzootic cycle. *B. burgdorferi* adapts to and thrives in the different environments of the vector and host via the global regulation of gene expression (Iyer and Schwartz, 2016; Samuels and Samuels, 2016) mediated by the alternative sigma factor RpoS (σ^S) (Fisher *et al.*, 2005; Caimano *et al.*, 2007; Ouyang *et al.*, 2008), the stringent response via guanosine tetraphosphate and pentaphosphate [(p)ppGpp] (Bugrysheva *et al.*, 2015; Drecktrah *et al.*, 2015), and cyclic dimeric GMP (c-di-GMP) (Rogers *et al.*, 2009; He *et al.*, 2011; Caimano *et al.*, 2015). While studies of these signaling systems have begun to uncover the gene products important for infectivity and persistence in the tick, our understanding of global transcriptional regulators remains incomplete.

6S RNA is an abundant small RNA that regulates transcription in *Escherichia coli*, and other bacteria, by direct interaction with the σ^{70}-containing form of RNA polymerase (RNAP), referred to as the σ^{70}-holoenzyme ($E\sigma^{70}$) (Wassarman and Storz, 2000; Cavanagh and

Wassarman, 2014; Steuten *et al.*, 2014a; Steuten *et al.*, 2014b; Burenina *et al.*, 2015; Wassarman, 2018). Therefore, 6S RNA functions as a global regulator, in contrast to other sRNAs that typically bind to target RNAs to affect expression of a more limited set of genes (Waters and Storz, 2009; Storz *et al.*, 2011). The defining characteristic of 6S RNAs is not their nucleotide sequence but their secondary structure, which forms a central bubble of single-stranded RNA flanked by double-stranded RNA stems and is required for 6S RNA interaction with RNAP (Wassarman and Storz, 2000; Barrick *et al.*, 2005; Trotochaud and Wassarman, 2005; Chen *et al.*, 2017). This structure mimics the open promoter DNA complex of transcriptional initiation sites; binding of 6S RNA to σ^{70}-RNAP directly competes with σ^{70}-dependent promoter binding (Wassarman and Storz, 2000). 6S RNA binding to σ^{70}-RNAP leads to decreased expression from many σ^{70}-dependent promoters in *E. coli* (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2004; Trotochaud and Wassarman, 2006; Cavanagh *et al.*, 2008; Cavanagh *et al.*, 2010; Neusser *et al.*, 2010). At the same time, 6S RNA binding to σ^{70}-RNAP leads to increased expression from many σ^{S}-dependent (RpoS-dependent) promoters (Trotochaud and Wassarman, 2004; Cavanagh *et al.*, 2008; Neusser *et al.*, 2010). Thus, increased 6S RNA levels generally shift expression from σ^{70}-dependent to σ^{S}-dependent promoters, although not all promoters are affected in the same manner and the assembly of sequence elements influences the response, at least for σ^{70}-dependent promoters (Cavanagh *et al.*, 2008). These molecular mechanisms are one example of the cellular reprogramming of gene expression to respond to environmental stresses.

In *E. coli*, 6S RNA increases throughout growth in log phase and early stationary phase, accumulating to high levels in late stationary phase (Wassarman and Storz, 2000). The increase in 6S RNA levels and regulated changes in gene expression are thought to contribute to adaptation to the environmental stresses associated with limited nutrients and high cell density. The effects of 6S RNA are amplified by targeting global regulators, including the transcription factors PspF (Trotochaud and Wassarman, 2006; Joly *et al.*, 2010), Crp (Cavanagh *et al.*, 2008; Neusser *et al.*, 2010) and RelA (Cavanagh *et al.*, 2010). Cells lacking 6S RNA are not compromised for growth in exponential phase but have a survival defect during long-term nutrient stress and competitive survival (Lee *et al.*, 1985; Trotochaud and Wassarman, 2004). 6S RNA levels in some bacteria are regulated not by growth phase but by other signals, such as oxidative stress for *Burkholderia cenocepacia* (Peeters *et al.*, 2010), host cell environment for *Coxiella burnetii* (Warrier *et al.*, 2014) and *Yersinia pestis* (Yan *et al.*, 2013), and the cell-cycle and light for cyanobacteria (Axmann *et al.*, 2007). In addition, both *Bacillus subtilis* and *Legionella pneumophila* have two versions of 6S RNA, Bs6S-1 and Bs6S-2 and Lp6S and Lp6S-2, respectively, which are differentially expressed and regulate distinct adaptations to disparate stresses (Ando *et al.*, 2002; Suzuma *et al.*, 2002; Barrick *et al.*, 2005; Trotochaud and Wassarman, 2005; Faucher *et al.*, 2010; Weissenmayer *et al.*, 2011; Cavanagh *et al.*, 2012; Cavanagh and Wassarman, 2013; Burenina *et al.*, 2014). Bs6S-1 RNA levels increase as cells enter stationary phase while Bs6S-2 RNA levels remain relatively unchanged (Ando *et al.*, 2002; Suzuma *et al.*, 2002; Trotochaud and Wassarman, 2005; Beckmann *et al.*, 2011). Mutagenesis experiments showed that Bs6S-1 RNA regulates the timing of sporulation while Bs6S-2 does not (Cavanagh and Wassarman, 2013). Thus, the 6S RNAs clearly function to alter gene

expression to adapt to environmental stresses but the regulatory signals, cellular responses and physiological importance of these sRNAs are varied.

More recently, 6S RNA has been suggested to play a role in virulence of bacteria, including *L. pneumophila* (Faucher *et al.*, 2010), *Y. pestis* (Yan *et al.*, 2013), *C. burnetii* (Warrier *et al.*, 2014), and *Salmonella enterica* serovar Typhimurium (Ren *et al.*, 2017), specifically where replication and stress resistance is tied to pathogenesis. However, *Y. pestis* is the only bacterial pathogen where 6S RNA function has been examined using an animal model (Yan *et al.*, 2013). Expression and function of 6S RNA has not been studied in *B. burgdorferi*, or any other spirochete, to our knowledge. Here, we report the first study of the function of an sRNA in *B. burgdorferi* in the tick-mouse model of Lyme disease. We show that Bb6S RNA binds RNAP in the spirochete, is processed by RNase Y, and regulates expression of lipoproteins important for host infection.

Results

The *B. burgdorferi ssrS* gene (encoding Bb6S RNA), first identified by Barrick *et al.* (2005), is in the intergenic region between *bb0187* and *bb0188* on the chromosome (Fig. 1A). We experimentally determined that Bb6S RNA is 204 nucleotides by 3′ and 5′ RACE and modeled the secondary structure using mfold (Zuker, 2003) (Fig. 1B). Bb6S RNA, while having only about 35% identity with *E. coli* 6S RNA, is predicted to form the stem-bulge-stem structure common to all 6S RNAs that likely mimics the DNA open promoter complex during transcriptional initiation (Wassarman and Storz, 2000; Barrick *et al.*, 2005; Trotochaud and Wassarman, 2005; Chen *et al.*, 2017).

6S RNA regulates transcription in bacteria by binding to σ^{70}-RNAP and sequestering it from binding to σ^{70}-dependent promoters. We assayed the binding of Bb6S RNA to RNAP in *B. burgdorferi* by co-immunoprecipitation. Antibodies specific to *B. burgdorferi* RNAP (*Bb*RNAP) are not available, but we found that antibodies specific to *E. coli* RNAP (*Ec*RNAP) sufficiently cross-reacted with *Bb*RNAP (Fig. 2A, cell lysate (1/2)). Spirochete extracts immunoprecipitated with preimmune serum or serum specific to *Ec*RNAP antibodies were separated by SDS-PAGE, transferred to a membrane and immunoblotted with the same *Ec*RNAP-specific serum used for immunoprecipitation. The RNAP subunits are visible in the one-half equivalent cell extract and in the anti-RNAP core immunoprecipitation (IP), but not in the preimmune serum immunoprecipitated treatment (Fig. 2A). RNA from the other portion of the cell extracts and immunoprecipitated samples were separated on a urea gel and analyzed by Northern blot to examine relative Bb6S RNA levels compared to 5S rRNA levels as a control. Bb6S RNA co-immunoprecipitated with serum specific for *Ec*RNAP, but not the preimmune serum, indicating either a direct or an indirect interaction between Bb6S RNA and *Bb*RNAP (Fig. 2B). 5S rRNA did not co-immunoprecipitate with either serum (Fig. 2B), supporting the specificity of the Bb6S RNA-RNAP interaction in *B. burgdorferi* cells. Bb6S RNA was also able to bind *E. coli* RNAP (σ^{70} holoenzyme) *in vitro* as demonstrated by a gel shift assay using *in vitro* transcribed ^{32}P-labeled Bb6S RNA (Fig S1), suggesting a direct interaction between Bb6S RNA and RNAP. This is only the second identification of an sRNA-protein interaction in *B. burgdorferi* (Lybecker *et al.*, 2010; Lybecker and Samuels, 2017).

6S RNA is processed from a larger transcript in *E. coli* by mechanisms involving RNase BN or the endoribonucleases RNase G and RNase E (Kim and Lee, 2004; Chen *et al.*, 2016) and further trimmed by exoribonucleases RNase T and RNase PH (Li *et al.*, 1998). *B. burgdorferi* has a limited repertoire of ribonucleases, compared to *E. coli* and *B. subtilis*, and lacks genes encoding RNase G and RNase E; the only predicted endoribonuclease homologs are RNase III, RNase M5, RNase P, RNase Y, RNase Z, YbeY, and RNase HII (Fraser *et al.*, 1997; Archambault *et al.*, 2013; Anacker *et al.*, 2018). The size of Bb6S RNA was assayed by Northern blot in individual RNase mutant strains to investigate Bb6S RNA processing. First, using our extant *rnc* mutant (Anacker *et al.*, 2018), Bb6S RNA size and levels were largely unchanged, with only a small portion of Bb6S RNA remaining incompletely processed (Fig S2), which indicates that RNase III has a limited role in processing. Next, we focused on RNase Y since it has been postulated to functionally replace RNase E (Shahbabian *et al.*, 2009). Multiple efforts to generate an *rny* deletion mutant lacking RNase Y were unsuccessful, which suggests that RNase Y is essential in *B. burgdorferi*. Therefore, we constructed a conditional mutant with an IPTG-inducible *flacp-rny* fusion (Gilbert *et al.*, 2007). To assay the regulation of *rny* expression in the *flacp-rny* strain, cells were grown to 10^7 cells ml^{-1} in the presence of IPTG before removing the inducer. Cells were then resuspended with or without IPTG and grown for 48 h. *rny* expression, measured by qRT-PCR, in the *flacp-rny* strain in the presence of 0.5 mM IPTG is slightly less than that seen in wild type (Fig. 3A). Levels of *rny* mRNA decreased as the IPTG concentration was reduced in *flacp-rny* mutants, demonstrating IPTG-dependent *rny* expression (Fig. 3A). To examine the effect of *rny* on growth, spirochetes were treated with or without IPTG and cells were enumerated for eight days. Conditional *flacp-rny* mutants grown in the presence of 1.0 mM IPTG had similar growth kinetics as wild-type *B. burgdorferi* (Fig. 3B, gray squares and black circles). There was a slight decrease in growth rate and final cell density of *flacp-rny* mutants in the presence of 0.05 mM IPTG (Fig. 3B, gray circles). The *flacp-rny* strain failed to replicate in the absence of IPTG (Fig. 3B, open circles). The lack of growth without IPTG supports our hypothesis that the *rny* gene is essential in *B. burgdorferi*. The influence of reduced *rny* expression on Bb6S RNA processing was examined by Northern blot analyses. Cells were treated with or without IPTG and analyzed by Northern blot, using transfer-messenger RNA (tmRNA) as a control. In *flacp-rny* mutants with reduced levels of *rny* mRNA (Fig. 3A, 0 mM IPTG), the mature form of Bb6S RNA was dramatically reduced (Fig. 3C, filled arrow) while tmRNA levels were unchanged (Fig. 3C). To further investigate the role of RNase Y in Bb6S maturation, we used SYBR Green primers spanning the mature 5′ and 3′ ends for qRT-PCR analyses. The levels of Bb6S trended lower as IPTG was removed from the *flacp-rny* strain, but the differences were not significant (Fig. 3D, $P > 0.05$, one-way ANOVA with a Tukey's *post-hoc* test). The levels of the 5′ and 3′ unprocessed ends both increased when RNase Y was depleted by removing IPTG (Fig. 3E and F, 0 mM IPTG). In the *flacp-rny* strain, the ratio of 5′ end to Bb6S increased sixfold (Fig. 3E) and the ratio of 3′ end to Bb6S increased about sevenfold (Fig. 3F) when IPTG was removed compared to cells with 0.5 mM IPTG. Therefore, our data suggest a role for RNase Y in the biogenesis of Bb6S RNA by processing both the 5′ and 3′ ends.

Accumulation of 6S RNA in stationary phase is a hallmark of adaptation in *E. coli* (Wassarman and Storz, 2000; Cavanagh and Wassarman, 2014; Steuten *et al.*, 2014b). To

examine if *B. burgdorferi* follows suit, Bb6S RNA levels were assayed during growth from early log phase through late stationary phase by Northern blot analyses and qRT-PCR. Bb6S RNA levels did not significantly increase, compared to the *flaA* control, as spirochete cultures reached stationary phase (Fig. 4A and B). Nutrient starvation in culture has been used to simulate *B. burgdorferi* persistence in the tick midgut and has been shown to be an important signal for transcriptional regulation (Concepcion and Nelson, 2003; Drecktrah *et al.*, 2015). To assess the effect of nutrient stress on Bb6S RNA levels, cells were grown to stationary phase and starved in RPMI medium for two or six hours before RNA was analyzed by Northern blot and qRT-PCR. Again, Bb6S RNA levels were not significantly affected by starvation in cultured spirochetes (Fig. 4C and D).

To assess the role of Bb6S RNA in gene regulation during the enzootic cycle of the Lyme disease spirochete, we replaced the *ssrS* gene encoding Bb6S RNA with a promoterless *aadA* gene, conferring streptomycin resistance, to yield an *ssrS* null mutant (Fig. 5A). The *ssrS* mutant was complemented in cis by genomic reconstitution using the gentamicin resistance gene *aacC1* inserted after *bb0185*, the last gene of a putative operon including *ssrS* (Fig. 5A). The absence of Bb6S RNA in the null mutant and restoration of Bb6S RNA in the complemented strain was confirmed by Northern blot analysis (Fig. 5B). The plasmid profiles of the *ssrS* mutant and complemented strains were assessed by PCR analyses, and both were the same as the parental 297 wild type, except that the *ssrS* complemented strain lost one of the cp32s (Table S1). Additionally, expression of *bb0187* and *bb0188* (*rplT*), genes adjacent to the *ssrS* locus, was unchanged in the *ssrS* mutant and complemented strains compared to the parental strain as determined by qRT-PCR (Fig. S3), suggesting the absence of polar effects from the genetic manipulations.

In *E. coli*, 6S RNA alters the relative levels of σ^{70}-mediated and σ^{S}-mediated transcription in response to changing environmental conditions. To examine Bb6S RNA function, we assayed the RpoS-dependent synthesis of outer surface protein C, encoded by *ospC*, and decorin binding protein A, encoded by *dbpA* (Schwan *et al.*, 1995; Yang *et al.*, 2000; Hübner *et al.*, 2001; Caimano *et al.*, 2004), which are lipoproteins important for establishing infection in the mammalian host (Fischer *et al.*, 2003; Grimm *et al.*, 2004; Pal *et al.*, 2004; Fingerle *et al.*, 2007; Shi *et al.*, 2008; Weening *et al.*, 2008). A temperature shift from 23°C to 35°C has been experimentally used in cultured spirochetes to induce expression of some RpoS-dependent genes and mimic the transmission from ticks to mammals during the blood meal (Schwan *et al.*, 1995; Obonyo *et al.*, 1999; Yang *et al.*, 2000; Ramamoorthy and Scholl-Meeker, 2001; Alverson *et al.*, 2003; Tokarz *et al.*, 2004). Wild-type, *ssrS* mutant and *ssrS* complemented strains were temperature shifted, grown to mid-log phase and total RNA was isolated. Expression of *ospC*, *ospA* (encoding outer surface protein A), *dbpA*, and *flaB* was assayed by Northern blot analyses. *ospC* and *dbpA* mRNA levels increased in wild type but not the *ssrS* mutant following a temperature shift (Fig. 6A). Expression of *ospC* and *dbpA* was restored in the *ssrS* complemented strain (Fig. 6A). The expression of *ospA*, a gene repressed, probably indirectly, by RpoS (Caimano *et al.*, 2005), was not affected by Bb6S RNA (Fig. 6A). In addition, the effect of Bb6S RNA on the synthesis of OspC during a temperature shift was examined by immunoblot using anti-OspC antibodies, with FlaB as a control. OspC protein levels also were Bb6S RNA-dependent, as OspC levels increased in the wild type but not in the *ssrS* mutant (Fig. 6B). OspC induction was restored in the *ssrS*

complemented strain (Fig. 6B). Neither *rpoS* transcript nor RpoS protein levels were affected by Bb6S RNA (Fig. S4). These results suggest that Bb6S RNA regulates RpoS-dependent gene expression in cultured spirochetes and controls at least a subset of the RpoS regulon, likely by affecting the sigma factor selectivity of RNAP.

To test the role of Bb6S RNA in murine infectivity, mice were intradermally injected with increasing doses (10^2 to 10^4) of wild-type, *ssrS* null mutant and *ssrS* complemented strains. Infection was determined by culturing spirochetes from ear tissues collected three weeks post inoculation and from ear tissues, bladders and tibiotarsal joints five weeks post inoculation. The *ssrS* mutant was compromised for infectivity in a dose-dependent manner and this defect was completely restored in the *ssrS* complemented strain (Table 2). Murine infectivity was also followed by seroconversion. Unexpectedly, mice inoculated with 10^3 *ssrS* mutant cells, in which live spirochetes could not be recovered (Table 2), seroconverted when examined by immunoblot using serum from the inoculated mice (Fig. 7). Thus, the *ssrS* null mutant stimulates an adaptive immune response, but does not establish an infection resulting in recoverable live spirochetes from distal sites.

In nature, *B. burgdorferi* infects mammals via the bite of an ixodid tick. We next investigated the role of Bb6S RNA in tick-transmission using the tick-mouse model of Lyme disease (Barthold *et al.*, 2010). Naive *Ixodes* larvae were allowed to feed to repletion on mice infected with 1×10^4 wild-type, *ssrS* null mutant or *ssrS* complemented spirochetes. Larvae from each mouse were tested one week post-feeding to confirm acquisition of each strain (>80% of larvae were infected for all strains). Fed larvae were allowed to molt into nymphs before nymphs infected with each strain were placed on naive mice and allowed to feed to repletion. Mice were screened three and five weeks post-infestation for infection as described above. The *ssrS* null mutant was able to transmit to mice by tick bite when either two or five ticks were allowed to feed per mouse, suggesting that Bb6S RNA is not absolutely required for tick transmission, at least in the tick-mouse model (Table 2).

In other bacteria, 6S RNA plays an important role in adapting to environmental stresses, including nutrient limitation (Sharma and Chatterji, 2010; Cavanagh and Wassarman, 2014; Wassarman, 2018). To gain insight into Bb6S RNA regulation, Bb6S RNA levels were followed in *Ixodes* ticks infected with *B. burgdorferi* using the tick-mouse model of Lyme disease (Barthold *et al.*, 2010). Briefly, mice were infected with 10^4 wild-type spirochetes by intradermal needle inoculation. Successful infection was determined by positive cultures from ear biopsies taken three weeks post inoculation. Naive *Ixodes scapularis* larvae were allowed to feed to repletion on infected mice. Levels of Bb6S RNA were measured by qRT-PCR and normalized to *flaB* transcript levels in infected larvae (at one week and three weeks post feeding), flat nymphs (at six weeks post feeding) and fed nymphs (at one week after feeding to repletion on naive mice) as previously described (Yang *et al.*, 2004). The amount of Bb6S RNA significantly increased after larval feeding (fed larvae, one week) to reach a maximum in unfed nymphs (Fig. 8). These data suggest Bb6S RNA functions in the persistence of *B. burgdorferi* in unfed ticks.

To address our hypothesis that Bb6S RNA functions in adapting to environmental stresses during persistence in the tick, we followed the acquisition and persistence of the *ssrS* null

mutant through the tick life cycle. Naive larvae were fed to repletion on mice infected with wild-type, *ssrS* null mutant or *ssrS* complemented *B. burgdorferi*, and *B. burgdorferi* DNA was quantified by qPCR from fed larvae one week post-detachment, unfed nymphs and fed nymphs to determine spirochete loads (genomic equivalents). We found the absence of *ssrS* had little effect on spirochete loads in the tick; however, there was a slight decrease of *B. burgdorferi* numbers in fed larvae and unfed nymphs, although only the decrease in nymphs was significant ($P < 0.05$) and the spirochete load was restored after the nymphs fed (Fig. 9B). Examination of fed larval midguts by immunofluorescence microscopy showed no obvious morphological differences in ticks infected with the *ssrS* mutant compared to wild-type or *ssrS* complemented strains (Fig. S5). Thus, the *ssrS* null mutant successfully persists throughout the larval and nymph stages of the tick life cycle in the animal model of Lyme disease.

Discussion

An sRNA was recently shown to be expressed in the noncoding sRNA transcriptomes of *B. burgdorferi* (Arnold *et al.*, 2016; Popitsch *et al.*, 2017; Drecktrah *et al.*, 2018) from the region of the genome predicted to encode Bb6S RNA (Barrick *et al.*, 2005). In this study, we present experimental evidence to establish that this sRNA is indeed the canonical 6S RNA: we demonstrate specific binding of Bb6S RNA to RNAP in cells, describe the processing of Bb6S RNA by RNase Y, and examine the role of Bb6S RNA in murine infectivity and persistence in the tick. This is the first study to examine the role and expression of an sRNA in *B. burgdorferi* through the tick-mouse model of Lyme disease. Furthermore, only a few 6S RNAs have been shown to bind RNAP *in vivo*; besides for Bb6S RNA, these include the 6S RNAs from *E. coli* (Wassarman and Storz, 2000), *B. subtilis* (Trotochaud and Wassarman, 2005) and *L. pneumonia* (Faucher *et al.*, 2010). We determined that Bb6S RNA is 204 nucleotides and that the 3′ end matches that predicted by Barrick *et al.* (2005) although the 5′ end differs slightly (Fig. 1B). Our modeling of the Bb6S RNA suggests a secondary structure conserved among bacteria with a single-stranded central bubble flanked by double-stranded stem regions (Barrick *et al.*, 2005; Trotochaud and Wassarman, 2005; Chen *et al.*, 2017), although the upstream stem is shorter than that of *E. coli* 6S RNA (Fig. 1B).

Many bacteria, such as *E. coli*, accumulate 6S RNA to adapt to environmental stresses as cells enter stationary phase (Wassarman and Storz, 2000; Sharma and Chatterji, 2010; Cavanagh and Wassarman, 2014; Wassarman, 2018). Bb6S RNA levels did not significantly change in response to any environmental signals examined in culture, including growth phase, even to late stationary phase, and nutrient stress (Fig. 4), which are known to affect 6S RNA levels in some other bacteria (Wassarman and Storz, 2000; Cavanagh and Wassarman, 2014; Steuten *et al.*, 2014b). Similarly, the RNA-seq studies that have identified expression of *ssrS* (Bb6S) also found no dependence on growth phase or temperature (Arnold *et al.*, 2016; Popitsch *et al.*, 2017). The lack of Bb6S RNA accumulation in stationary phase suggests its regulation may be similar to Bs6S-2 RNA from *B. subtilis*, which also does not accumulate in a growth phase-dependent manner (Suzuma *et al.*, 2002; Trotochaud and Wassarman, 2005). However, Bb6S RNA levels increased in the tick as fed larvae molted into unfed nymphs and then decreased after nymphs fed to repletion (Fig. 8),

which suggests a role in persistence in the tick (Caimano *et al.*, 2016). Furthermore, we have recently shown that Rel_{Bbu} and the stringent response upregulate Bb6S RNA (Drecktrah *et al.*, 2018), implying that the mechanism of Bb6S RNA accumulation in the tick may involve (p)ppGpp and nutrient deprivation. However, we found the absence of *ssrS* had little effect on persistence in the tick throughout the larval and nymph stages (Fig. 9). While there is a slight reduction in genomic equivalents of the *ssrS* null mutant observed in unfed nymphs that is restored in fed nymphs in the tick-mouse model, this phenotype may have more severe consequences for *B. burgdorferi* in competition with other microbes in the complex environment of the tick midgut in nature. A number of studies have found global transcriptomic changes mediated by signaling pathways, in addition to those mediated by Rel_{Bbu} (Drecktrah *et al.*, 2015), to be important for persistence in the tick (Caimano *et al.*, 2016; Samuels and Samuels, 2016), including the alternative sigma factor RpoS (Dunham-Ems *et al.*, 2012) and the Hk1/Rrp1 two-component system regulating c-di-GMP levels (Caimano *et al.*, 2011; He *et al.*, 2011; Kostick *et al.*, 2011; Sultan *et al.*, 2011). In *E. coli*, 6S RNA causes an increase in *relA* transcription and, subsequently, (p)ppGpp levels (Cavanagh *et al.*, 2010), so there may be positive feedback regulation. Whether the RpoS or c-di-GMP regulatory systems influence Bb6S RNA levels is currently unknown.

The biogenesis of 6S RNA in *E. coli* involves transcription from two promoters and processing of a long transcript that extends into the *ygfA* gene downstream of *ssrS*. RNase E and RNase G, along with exoribonucleases, process the pre-6S RNA to the mature form (Kim and Lee, 2004; Chae *et al.*, 2011). In *B. burgdorferi*, *ygfA* is not adjacent to *ssrS* and the genome lacks homologs of RNase E and RNase G (Fraser *et al.*, 1997; Archambault *et al.*, 2013; Anacker *et al.*, 2018). We found that processing of both the 5′ and 3′ ends of Bb6S RNA requires the endoribonuclease RNase Y (Fig. 3E and F). When *rny* expression was repressed in the inducible strain, the overall levels of Bb6S RNA were not significantly changed (Fig. 3D), but the mature Bb6S RNA was largely absent while a considerably longer transcript (>1000 nt) containing the Bb6S RNA sequence was visualized (Fig. 3C, open arrow). These results suggest that Bb6S RNA is also processed from a pre-Bb6S RNA transcript. RNase Y degrades mRNA transcripts (Durand *et al.*, 2012; Lehnik-Habrink *et al.*, 2011; Chen *et al.*, 2013) and has not been previously implicated in 6S RNA processing in any other bacteria. However, RNase Y is thought to functionally replace RNase E in Gram-positive bacteria (Shahbabian *et al.*, 2009). Our data further support the hypothesis that RNase Y is the counterpart of RNase E, at least for Bb6S RNA processing. Additionally, we found *rny* to be an essential gene in *B. burgdorferi*. Our multiple attempts to generate an *rny* null mutant were unsuccessful, so, to circumvent this obstacle, we replaced the native *rny* promoter with the IPTG-inducible promoter *flacp* (Gilbert *et al.*, 2007). The *flacp-rny* cells failed to grow once IPTG was removed, but cell numbers and tmRNA levels remained constant for four and two days, respectively, suggesting that a lack of RNase Y is not immediately lethal (Fig. 3B and C). These results add *rny* to a short list of genes, including *bamA* (Lenhart and Akins, 2010), *dedA* (Liang *et al.*, 2010), *rrp2* (Groshong *et al.*, 2012), *resT* (Bandy *et al.*, 2014), *bb0028* (Dunn *et al.*, 2015), *ftsH* (Chu *et al.*, 2016), and *oppDF* (Groshong *et al.*, 2017), that have been shown to be essential in *B. burgdorferi* using artificially regulated gene expression. *ssrS* null mutants are viable, which suggests that

RNase Y has a crucial role(s) in *B. burgdorferi* other than Bb6S RNA maturation, possibly in mRNA turnover.

Bb6S RNA regulates transcript levels of genes with RpoS-dependent promoters that encode proteins known to be essential for host infection (*ospC*) and dissemination (*dbpA*), at least *in vitro* (Fig. 6). This regulation is reminiscent of the canonical 6S RNA function observed in *E. coli*, although RpoS and its regulon have a different function in *B. burgdorferi*. In most bacteria, RpoS serves to regulate gene expression to adapt to environmental stresses, such as entering stationary growth phase, while RpoS in *B. burgdorferi* controls the expression of genes that encode proteins crucial for transmission to and infection of the vertebrate host (Hübner *et al.*, 2001; Caimano *et al.*, 2004; Fisher *et al.*, 2005; Caimano *et al.*, 2007; Ouyang *et al.*, 2008) and, thus, RpoS has been termed the “gatekeeper” of the enzootic cycle (Caimano *et al.*, 2007). Regulation of the RpoS pathway is complex, and includes transcriptional and post-transcriptional mechanisms (Samuels, 2011), and our data suggest that Bb6S regulates levels of at least some RpoS-dependent transcripts (*ospC* and *dbpA*), but not RpoS protein levels (Fig. S4B), illuminating yet another factor influencing the RpoS regulon.

The phenotypes of 6S RNA mutants in other bacteria are often subtle (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2004; Trotochaud and Wassarman, 2006; Cavanagh *et al.*, 2012; Cavanagh and Wassarman, 2013); Lp6S mutants of *L. pneumophila* have an intracellular replication defect only uncovered using a competition assay (Faucher *et al.*, 2010). Needle inoculation with up to 3×10^3 cells of the *ssrS* null mutant was not infectious as determined by reisolation of spirochetes from murine tissues (Table 2), but the inoculated mice seroconverted (Fig. 7). These data imply that the *ssrS* mutant was present long enough to trigger an adaptive immune response, although the extent of survival during the infection is unclear. Some non-infectious *B. burgdorferi* mutants, including *ospC* (Tilly *et al.*, 2007), *guaAB* (Jewett *et al.*, 2009), *plzA* (Kostick-Dunn *et al.*, 2018), and lp36$^-$ strains (Jewett *et al.*, 2007), illicit no adaptive immune response in mice. While only *vlsE* mutants (Bankhead and Chaconas, 2007) and *lmp1* mutants (Yang *et al.*, 2009), which lack surface-exposed lipoproteins involved in evading host immunity, seroconverted in the absence of infection. An *htrA* mutant strain, lacking a virulence-associated protease, elicited a weak immune response without being able to establish infection (Ye *et al.*, 2016). The murine immune system reacted to considerably fewer antigenic proteins in the *ssrS* null mutant compared to either the wild-type or *ssrS* complemented strains, which could be due to Bb6S RNA regulating expression of proteins targeted by the adaptive immune system and, possibly, involved in host interactions, but these questions await further investigation. The host infectivity phenotype of the *ssrS* null mutant can be overcome by increasing the number of inoculated spirochetes to 10^4 or by transmission via infected nymph bite. These results suggest that Bb6S RNA is not absolutely required for host infection and dissemination, which is not surprising given the subtle phenotypes of *ssrS* mutants in other bacteria. *B. burgdorferi* adaptation in the tick or tick factors present during feeding may mitigate the infectivity defect during tick-to-mammal transmission, and demonstrates the importance of the route of infection using animal models to study pathogens.

Bb6S RNA joins a growing list of 6S RNAs that bind RNAP to regulate transcription as bacteria adapt to environmental stress, including that encountered during infection of hosts. Here, we provide the first description of 6S RNA in any spirochete and demonstrate a role for RNase Y in Bb6S maturation.

Experimental Procedures

Ethics Statement

All animal experiments were approved by the University of Montana Institutional Animal Care and Use committee and followed the *Guidelines for the Care and Use of Laboratory Animals* from the National Institutes of Health.

B. burgdorferi *strains and growth conditions*

Low-passage *B. burgdorferi* strains 297 (BbAH130) (Hübner *et al.*, 2001) and B31–5A4 (Purser and Norris, 2000), and genetically manipulated derivatives were grown and maintained in Barbour-Stoenner-Kelly II (BSK) liquid medium, pH 7.6, containing 6% rabbit serum (Pel-Freez Biologicals) (Barbour, 1984) without gelatin unless otherwise noted. Cultures were inoculated at 1×10^3 and grown at 35°C to mid log phase ($1 – 3 \times 10^7$ cells ml^{-1}), late log phase ($4 – 9 \times 10^7$ cells ml^{-1}), stationary phase ($1 – 2 \times 10^8$ cells ml^{-1}) or late stationary phase ($\geq 3 \times 10^8$ cells ml^{-1}) before RNA and whole-cell protein lysates were collected. *B. burgdorferi* was starved in RPMI 1640 without L-glutamine and without serum as previously described (Drecktrah *et al.*, 2015). Cell density was determined by enumeration using a Petroff-Hausser cell counting chamber (Samuels *et al.*, 2018). *E. coli* TOP10F′ grown in lysogeny broth (Bertani, 1951) were used for cloning.

Construction of mutant strains

To generate an *ssrS* null strain, the gene encoding the *B. burgdorferi* 6S RNA (Barrick *et al.*, 2005) was replaced with the streptomycin resistance gene *aadA* (Frank *et al.*, 2003), without a promoter (Sultan *et al.*, 2010), by homologous recombination (Samuels *et al.*, 2018). Genomic regions upstream and downstream of *ssrS* (encoding Bb6S RNA) were amplified using KOD polymerase (Novagen) with the primers 6S U971F and 6S 5R+AatIIAgeI and primers 6S 109F+AatII and 6S 1325R+AgeI, respectively. PCR products were separated on an agarose gel, extracted using a Qiagen kit, poly(A)-tailed, cloned into pCR2.1-TOPO (Invitrogen), and verified by sequencing. Both constructs were digested with AatII and AgeI, and ligated together. A promoterless streptomycin resistance gene (*aadA*) flanked by AatII sites was ligated into the synthetic AatII site. This plasmid was linearized with AhdI and electroporated into competent *B. burgdorferi* 297 as previously described (Drecktrah *et al.*, 2013; Samuels *et al.*, 2018). Transformants were cloned in liquid BSK containing 50 μg ml^{-1} streptomycin in 96-well plates (Yang *et al.*, 2004). Mutants were confirmed by PCR analysis. To complement the *ssrS* null strain in *cis* by genetic reconstitution, two segments of DNA were amplified by PCR using KOD polymerase: a region containing *ssrS* and upstream sequence using primers 6S U441F and 6S D1564R+AatIIAgeI and a slightly overlapping downstream region with primers 6S D1463F+AatII and 6S D2498R+AgeI. PCR products were prepared as described above, cloned into pCR-XL-TOPO and pCR2.1-TOPO, respectively, and verified by sequencing. The plasmids were digested with AatII and AgeI,

separated on an agarose gel, and ligated together. A gentamicin resistance cassette with the *flgB* promoter from *B. burgdorferi* (Elias *et al.*, 2002) and the *trpL* terminator from *B. subtilis* (Babitzke *et al.*, 1996) (*flgBp-aacC1-trpLt*) was inserted into the synthetic AatII site (Drecktrah *et al.*, 2015; Samuels *et al.*, 2018). This *ssrS*-containing plasmid was linearized and transformed into competent *ssrS* mutant cells as described above.

The *B. burgdorferi flacp-rny* mutant strain was constructed by replacing the native *rny* promoter with the inducible promoter *flacp* (Gilbert *et al.*, 2007). Briefly, primers rny U921F and rny U4R+AatII+AgeI were used to amplify the genomic region upstream of the *rny* gene and primers rny 1F+NdeI+AatII and rny 1000R+AgeI were used to amplify a portion of the *rny* gene (*bb0504*). The two PCR products were cloned into pCR2.1-TOPO. The plasmids were purified, digested with AatII and AgeI, and ligated together. The inducible promoter *flacp* was fused to the *rny* gene by digesting with NdeI and AatII, and ligating the promoter into the *rny* plasmid. The gentamicin resistance cassette *flgBp-aacC1* was inserted into the synthetic AatII site. *B. burgdorferi* strain 297 carrying the *lacI* gene inserted into *bbe02* on the endogenous plasmid lp28–1 (Gilbert *et al.*, 2007) was transformed with linearized DNA containing the *flacp-rny* construct. Transformed cells were plated in 40 μg ml^{-1} gentamicin as described above. The promoter fusion was confirmed by PCR analysis.

Immunoprecipitation of RNAP from *B. burgdorferi*

Co-immunoprecipitation of RNAP and Bb6S RNA was by modification of a previously described protocol (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2005). *B. burgdorferi* strain B31–5A4 was grown in 100-ml cultures to early stationary phase ($1 – 2 \times 10^8$ cells ml^{-1}) and collected by centrifugation at 3000 × *g*, 10 min at 4°C. Cell pellets were washed in BSA-free BSK, resuspended in 200 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM KCl, 1 mM $MgCl_2$, 1 mM DTT) and placed in a flat-bottom tube containing 200 μl of 100 μm glass beads. Cells were lysed by 10 cycles of vortexing for 30 s and incubating on ice for 15 s; 400 μl of lysis buffer was added to the lysed cells and centrifuged at 20,800 × *g* for 10 min at 4°C. Antibody-protein A Sepharose (PAS) complexes were prepared by mixing 2 mg of pre-swollen PAS and 10 μl of serum generated against *E. coli* core RNAP (WI-151) or 10 μl of preimmune serum in Net2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-100). The antibody-PAS mixture was then nutated for 2 h at 23°C and the beads were washed three times at 20,800 × *g* for 30 s at 4°C in Net2 buffer before addition of 100 μl of *B. burgdorferi* cell extract supernatant. The mixture was nutated for 2 h at 4°C to allow antibody binding. The antibody-PAS complexes were centrifuged at 20,800 × *g* for 30 s at 4°C and washed five times with Net 2 Buffer before final resuspension in 500 μl of Net 2 buffer. One hundred μl of the antibody-PAS complexes was removed for immunoblot analyses using antiserum against *E. coli* core RNAP (WI-151), as described below, and RNA was isolated from the remaining 400 μl to be analyzed by Northern blot. To remove bound protein from the PAS beads, the sample was first centrifuged at 20,800 × *g* for 30 s at 4°C and 20 μl of 2× SDS-PAGE loading buffer was added to the pelleted beads. The supernatant was used for immunoblot analyses. RNA was isolated by adding 50 μl of 3M NaOAc (pH 5.5), 5 μl of 10% SDS and 600 μl of phenol:chloroform:isoamyl alcohol (25:24:1), pH 8, to the sample, vortexing, and centrifuging at 20,800 × *g* for 5 min at 23°C, followed by ethanol precipitation.

Growth of the *flacp-rny* conditional mutant

flacp-rny was cultured at 35°C in the presence of 0.5 mM IPTG to 10^7 cells ml^{-1}. Two-thirds of the cells were divided into two aliquots, washed twice with BSK, and grown for 48 h at 35°C in 0 or 0.05 mM IPTG. The remaining one-third of the *flacp-rny* cells were allowed to grow in the presence of 0.5 mM IPTG for 48 h. The parental strain was grown in the presence of 0.5 mM IPTG until late log phase and RNA was isolated using Trizol for qRT-PCR as described below. Additionally, RNA was harvested using hot phenol for Northern blot analyses as previously described (Popitsch *et al.*, 2017).

Immunoblot analyses

Equivalent amounts of total cell lysates collected from *B. burgdorferi* cultures ($1 – 3 \times 10^7$ cells ml^{-1}) were analyzed by SDS-PAGE using pre-cast Novex 4–20% Tris-Glycine polyacrylamide gels (Invitrogen) and transferred to PVDF Immobilon membranes (Millipore) as previously described (Drecktrah *et al.*, 2013). Protein levels were analyzed by incubating membranes with rabbit antibodies against OspC (1:1000) or mouse antibodies against FlaB (1:50) followed by goat anti-rabbit or goat anti-mouse HRP-linked antibodies (Bio-Rad Laboratories) (1:5000). Blots were developed by chemiluminescence (Amersham ECL Prime, GE Healthcare) and visualized using an LAS-3000 Intelligent Dark Box (Fujifilm Medical Systems USA).

Seroconversion

To determine if mice seroconverted after intradermal inoculation of wild-type, *ssrS* null or *ssrS* complemented strains, blood was collected five weeks post injection. Serum was prepared by allowing blood to clot at 23°C for 45 min, followed by centrifugation at 1500 × *g* at 4°C for 15 min and collection of the supernatant. Equivalent amounts of cell lysates were analyzed by an immunoblot, as described above, using serum (1:200 dilution) instead of the primary antibodies. *E. coli* strain DH5α cell lysate was a negative control for immunoreactivity to mouse serum.

qRT-PCR

Total RNA was isolated from *B. burgdorferi* cultures using TRIzol™; 2 μg of RNA was treated with TURBO DNase as previously described (Lybecker *et al.*, 2010; Drecktrah *et al.*, 2015). The absence of contaminating DNA was determined by PCR analysis using the primers flaB 423F and flaB 542R. One μg of total RNA from each sample was converted to cDNA using the SuperScript III kit (Invitrogen). TaqMan or SYBR Green quantitative PCR (Table 1) was performed with gene transcript copy numbers normalized to *flaB* or *flaA* copy number as previously described (Drecktrah *et al.*, 2013; Drecktrah *et al.*, 2015). Values represent the mean ± SEM from three independent experiments.

Northern hybridization

Northern blots were probed with either single-stranded RNA as previously described (Lybecker *et al.*, 2010) or oligonucleotides as previously described (Popitsch *et al.*, 2017; Drecktrah *et al.*, 2018). Briefly, Bb6S, *flaA*, 5S RNA, and tmRNA probes were constructed by PCR amplification of *B. burgdorferi* genomic DNA using primers 6S 19F and 6S 180R

+T7, primers flaA 64F and flaA 284R+T7, primers rrf 7F and rrf 110+T7, and primers ssrA 53F and ssrA 231R+T7, respectively, followed by *in vitro* transcription with the MEGAScript T7 kit (Ambion) using biotin-16-UTP (Roche) according to the manufacturer's instructions. DNA was removed from reactions with TURBO DNase (Invitrogen). RNA was precipitated twice with ammonium acetate and washed with 70% ethanol after each precipitation. Probes were separated on a precast 6% TBE urea gel (Invitrogen) and stained with acridine orange. Bands were extracted by overnight incubation in gel extraction buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS) at 37°C. Liquid containing the extracted probes was transferred to a fresh tube and precipitated by the addition of three volumes of cold 100% ethanol and incubation at −20°C overnight. RNA was pelleted by centrifugation, washed in 70% ethanol, dried, resuspended in nuclease-free water and stored at −80°C. Three μg of total RNA was separated on a 6% TBE urea gel and transferred to a BrightStar Plus membrane (Ambion). Membranes were hybridized with biotinylated RNA probes overnight at 68°C and washed; the probe was detected with streptavidin-alkaline phosphatase (Invitrogen) diluted 1:10,000. Blots were developed by incubation with CDP-*Star* chemiluminescent substrate (Applied Biosystems). Images were collected on a Fujifilm LAS-3000.

Oligonucleotide probes to *ospC*, *ospA*, *dbpA* and *flaB* (Table 1) were ^{32}P-end-labeled using T4 PNK (New England Biolabs) and $\gamma-^{32}$P-ATP (Perkin-Elmer). Total RNA was isolated from *B. burgdorferi* cultures by the hot phenol method (Popitsch *et al.*, 2017). RNA was treated with DNase to remove DNA before 5 μg per sample was loaded and run on an 0.8% agarose-formaldehyde gel in 1× MOPS buffer at 70 V for 3 h. The gel was soaked in nuclease-free water for 10 min to remove the formaldehyde and RNA was transferred from the gel to Hybond XL membranes (Amersham) by capillary action in 20× SSC buffer overnight. The membrane was then UV-crosslinked, blocked in ULTRAhyb Oligo Hybridization Buffer (Invitrogen) for 1 h at 40°C and ^{32}P-labeled probe allowed to hybridize overnight at 40°C. The blot was washed twice with 2× SSC + 0.5% SDS and covered in plastic wrap. Images were obtained with a Fujifilm FLA-3000G phosphorimager.

Identification of 5′ and 3′ ends of Bb6S RNA

The ends of Bb6S RNA were determined by 5′ and 3′ rapid amplification of cDNA ends (RACE) as previously described (Anacker *et al.*, 2018). RNA was isolated from *B. burgdorferi* cultures grown to late log phase as described above, except that samples were not treated with DNase. The 5′ and 3′ RACE PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, gel-extracted (Qiagen), and cloned into pCR2.1-TOPO. DNA was isolated with a Qiagen mini-prep kit from positive clones and sequenced.

Quantification of *B. burgdorferi* in tick

Spirochete loads were quantified in fed larvae (one week and three weeks post feeding, groups of 5), unfed nymphs and fed nymphs (one week post feeding) as previously described (Drecktrah *et al.*, 2015) using the DNeasy Blood/Tissue kit (Qiagen) and TaqMan qPCR with primers and probe to the *flaB* gene (Table 1).

Immunofluorescence microscopy of infected ticks

B. burgdorferi in infected ticks were visualized by indirect immunofluorescence microscopy as previously described (Hoon-Hanks *et al.*, 2012; Drecktrah *et al.*, 2015). Briefly, dissected tick midguts were fixed in acetone and *B. burgdorferi* was visualized using mouse anti-*Borrelia* antibodies (a gift from T. Schwan) followed by goat anti-mouse Alexa Fluor 488 (Invitrogen). Tick cells were stained with wheat germ agglutinin (WGA)-Alexa Fluor 594 (Invitrogen).

Mouse infectivity and tick persistence

The role of Bb6S RNA in murine infectivity was examined as previously described (Barthold *et al.*, 2010) by intradermal injection of female C3H-HeJ mice with 1×10^2 to 1×10^4 cells of wild-type (297 clone AH130), *ssrS* null mutant or *ssrS* complemented strains. Infection was determined by culturing mouse ear biopsies in BSK containing 50 μg ml^{-1} rifampicin, 20 μg ml^{-1} phosphomycin and 2.5 μg ml^{-1} amphotericin B and examining cultures by dark-field microscopy for the presence of spirochetes. Five weeks post-infection, mice were sacrificed and ear, ankle and bladder tissues were collected, cultured and examined for spirochetes. Uninfected *Ixodes scapularis* larvae (National Tick Research and Education Resource, Oklahoma State University) were maintained in a 98% humidified chamber. To allow ticks to acquire *B. burgdorferi*, approximately 100 larvae per mouse were allowed to feed to repletion. To examine tick acquisition of *B. burgdorferi* from infected mice, larvae were dissected and processed for immunofluorescence microscopy, as described above. Spirochete loads per tick were quantified by qPCR as described above. After infected larvae molted into nymphs (about 8 weeks), three mice were infested with two or five nymphs each and allowed to feed to repletion. Murine transmission was monitored by culturing ear, ankle and bladder tissues as described above. Persistence of *B. burgdorferi* in ticks was followed by immunofluorescence microscopy and qPCR as described above.

RNA isolation from infected ticks

Five to ten ticks were placed in a nuclease-free tube containing 100 μl TE, pH 8.0 and crushed with a nuclease-free pestle. Two hundred μg lysozyme (2 μl of 100 mg ml^{-1}) was added and tubes were incubated at 37°C for 10 min. RNA was isolated using a Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was processed for qRT-PCR as described above. Bb6S RNA levels were quantified by TaqMan qRT-PCR using the primers and probe listed in Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are grateful to Ron Breaker and Zasha Weinberg for sequence data, Jessica Wexler for assistance with mouse blood collection, Meghan Lybecker for critical reading of the manuscript, Tom Schwan for the anti-*Borrelia* and anti-FlaB antibodies, George Chaconas, Mike Norgard, and Frank Yang for sharing *B. burgdorferi* strains, Paul Babitzke for providing the *B. subtilis trpL* terminator, and Melissa Anacker and Meghan Lybecker for useful discussions.

Funding

This work was supported by Public Health Service grant R01 AI051486 to DSS.

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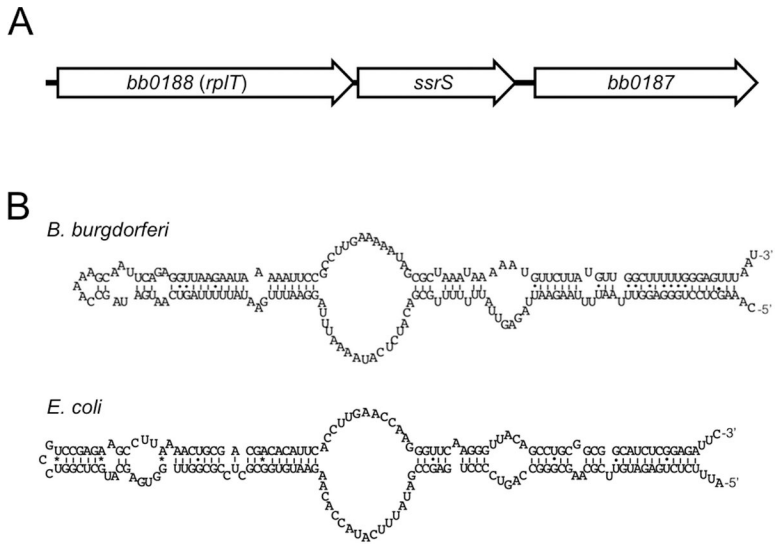

Figure 1.

Bb6S RNA in *B. burgdorferi*. (A) Genomic locus of *ssrS* encoding the *B. burgdorferi* homolog of 6S RNA (Bb6S RNA). (B) The predicted secondary structure of 6S RNA from *B. burgdorferi* and, for comparison, *E. coli* (Wassarman, 2018). The 5′ and 3′ ends of Bb6S RNA were experimentally determined by RACE. The majority of 5′ ends (5/6) mapped to a C five nucleotides downstream from the end of the *bb0188* (*rplT*) ORF. The majority of 3′ ends (11/14) mapped to a U 24 nucleotides upstream from the start of the *bb0187* ORF.

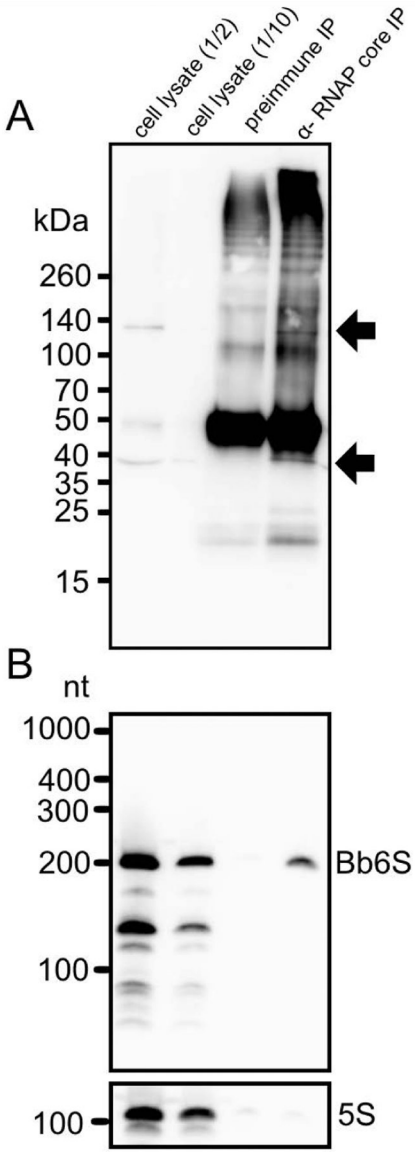

Figure 2. Bb6S RNA binds to *B. burgdorferi* RNAP.

(A) Immunoblot of *B. burgdorferi* cell lysate one-half (1/2) or one-tenth (1/10) the equivalent used for immunoprecipitation (IP) of extracts with preimmune serum or anti-*E. coli* RNAP antiserum (clone WI 151). The blot was probed with the anti-*E. coli* RNAP antiserum (clone WI 151) to visualize *B. burgdorferi* RNAP core. Arrows denote proteins present in the cell extract and enriched in IP with anti-*E. coli* RNAP antiserum but not preimmune serum. (B) Northern blot of a *B. burgdorferi* total cell lysate and extracts immunoprecipitated with preimmune serum (preimmune IP) or anti-*E. coli* RNAP antiserum WI-151 (α-RNAP IP) separated on a urea gel and hybridized with a biotinylated RNA probe to Bb6S RNA (upper panel) or *B. burgdorferi* 5S rRNA (lower panel). The amount of cell lysate was 10% of the equivalent used for the immunoprecipitation.

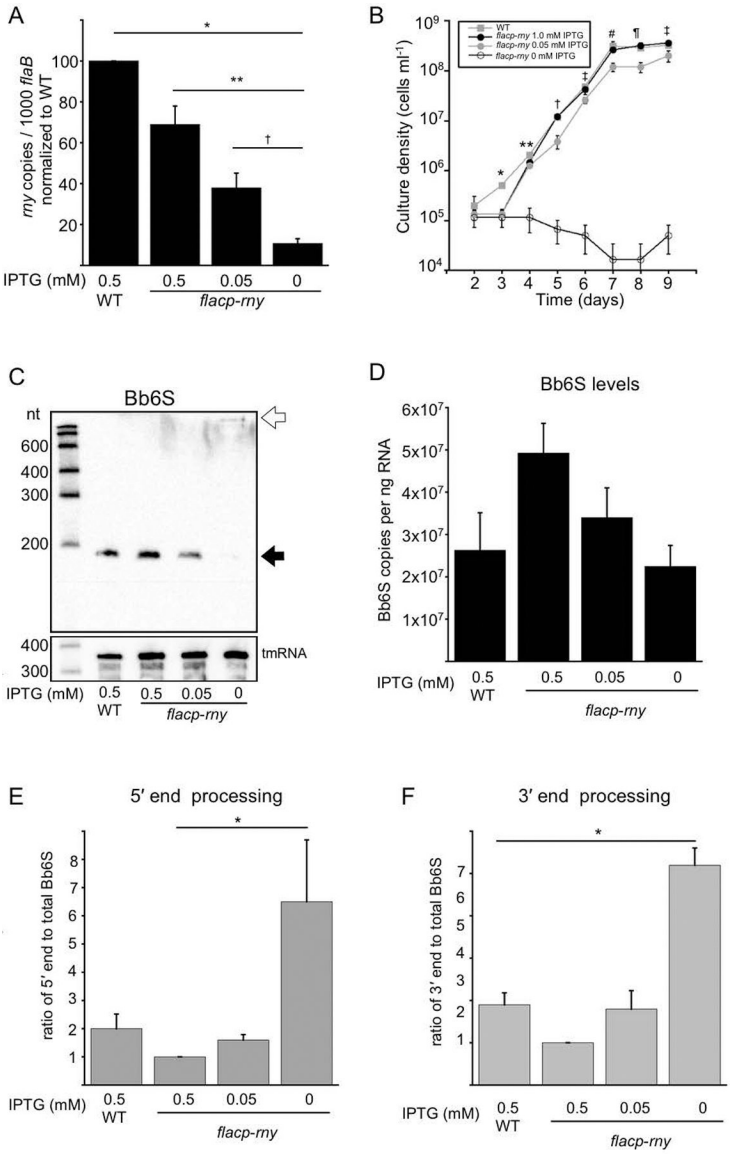

Figure 3. Bb6S RNA is processed by RNase Y.

RNase Y levels were depleted by fusing the artificially inducible promoter *flacp* to the *rny* gene, encoding RNase Y, and inserted into the *rny* locus on the *B. burgdorferi* chromosome. (A) *flacp-rny* spirochetes were grown in 0.5 mM IPTG to 10^7 cells ml^{-1}. IPTG was removed from two-thirds of the cells, which were then placed in growth medium containing either 0 or 0.05 mM IPTG for 48 h. One-third of the *flacp-rny* cells remained in 0.5 mM IPTG for 48 h. Wild-type (WT) spirochetes were grown in the presence of 0.5 mM IPTG until late log phase. Levels of *rny* mRNA were quantified by TaqMan qRT-PCR and normalized to *flaB* mRNA levels relative to wild type. Values are the mean of four independent biological replicates and error bars represent the SE. * denotes a significant difference between WT and *flacp-rny* strains in all levels of IPTG, ** denotes a significant difference between *flacp-rny* in 0.5 mM IPTG and both 0.05 and 0 mM IPTG and † denotes a significant difference between 0.05 mM and 0 mM IPTG ($P < 0.05$ determined by one-way ANOVA with Tukey's

post-hoc test). (B) WT and *flacp-rny* spirochetes were grown in 0.5 mM IPTG for two days before IPTG was removed and cells were resuspended in growth medium containing 0, 0.05 or 1.0 mM IPTG. Cells were enumerated each day for the next eight days (days 2 to 9). Values are the means of three independent biological replicates and error bars represent the SE. Significance ($P < 0.05$) was determined by one-way ANOVA with Tukey's *post-hoc* test. * denotes WT significantly different from 1.0, 0.05, and 0 mM IPTG-treated *flacp-rny* strains, ** denotes 0 mM IPTG-treated *flacp-rny* significantly different from all others and WT significantly different from 1.0 and 0.05 mM IPTG-treated *flacp-rny* strains, † denotes 0 mM IPTG-treated *flacp-rny* significantly different from all others and 0.05 mM IPTG-treated *flacp-rny* significantly different from WT and 1.0 mM IPTG *flacp-rny* strains, ‡ denotes 0 mM IPTG-treated *flacp-rny* significantly different from all others, # denotes 0 mM IPTG *flacp-rny* significantly different from WT and 1.0 mM IPTG *flacp-rny* and 0.05 mM IPTG *flacp-rny* significantly different from WT, ¶ denotes 0 mM IPTG-treated *flacp-rny* significantly different from WT and 0.05 mM IPTG-treated *flacp-rny* significantly different WT and 1.0 mM IPTG-treated *flacp-rny*. (C) Northern blot analyses of Bb6S RNA levels from the RNA isolated in (A) using a biotinylated RNA probe to Bb6S RNA and tmRNA as a control. The filled arrow marks the size of the mature Bb6S RNA and the open arrow indicates a large (>1000 nt) species present in the sample lacking mature Bb6S RNA, potentially representing an unprocessed Bb6S RNA transcript. (D) qRT-PCR analyses of RNA isolated from the same conditions in (A) using SYBR Green primers 6S 5F and 6S 114R (Table 1) to quantify Bb6S levels. (E) qRT-PCR analyses of the 5′ end of Bb6S using SYBR Green primers 6S U26F and 6S 24R (Table 1) expressed as a ratio of 5′ end to total Bb6S and normalized to the ratio in *flacp-rny* cells in 0.5 mM IPTG. * denotes significant difference between the 5′ end to Bb6S ratio in the *flacp-rny* strain in 0.5 mM and 0 mM IPTG ($P < 0.05$ by one-way ANOVA with Tukey's *post-hoc* test). (F) qRT-PCR analyses of the 3′ end of Bb6S using SYBR Green primers 6S 95F and 6S D224R (Table 1) expressed as a ratio of 3′ end to total Bb6S and normalized to the ratio in *flacp-rny* cells in 0.5 mM IPTG. * denotes significant difference between the 3′ end to Bb6S ratio in the *flacp-rny* strain in 0 mM IPTG and all other values ($P < 0.05$ by one-way ANOVA with Tukey's *post-hoc* test). Values are the mean of three independent biological replicates and error bars represent the SE in panels D, E and F.

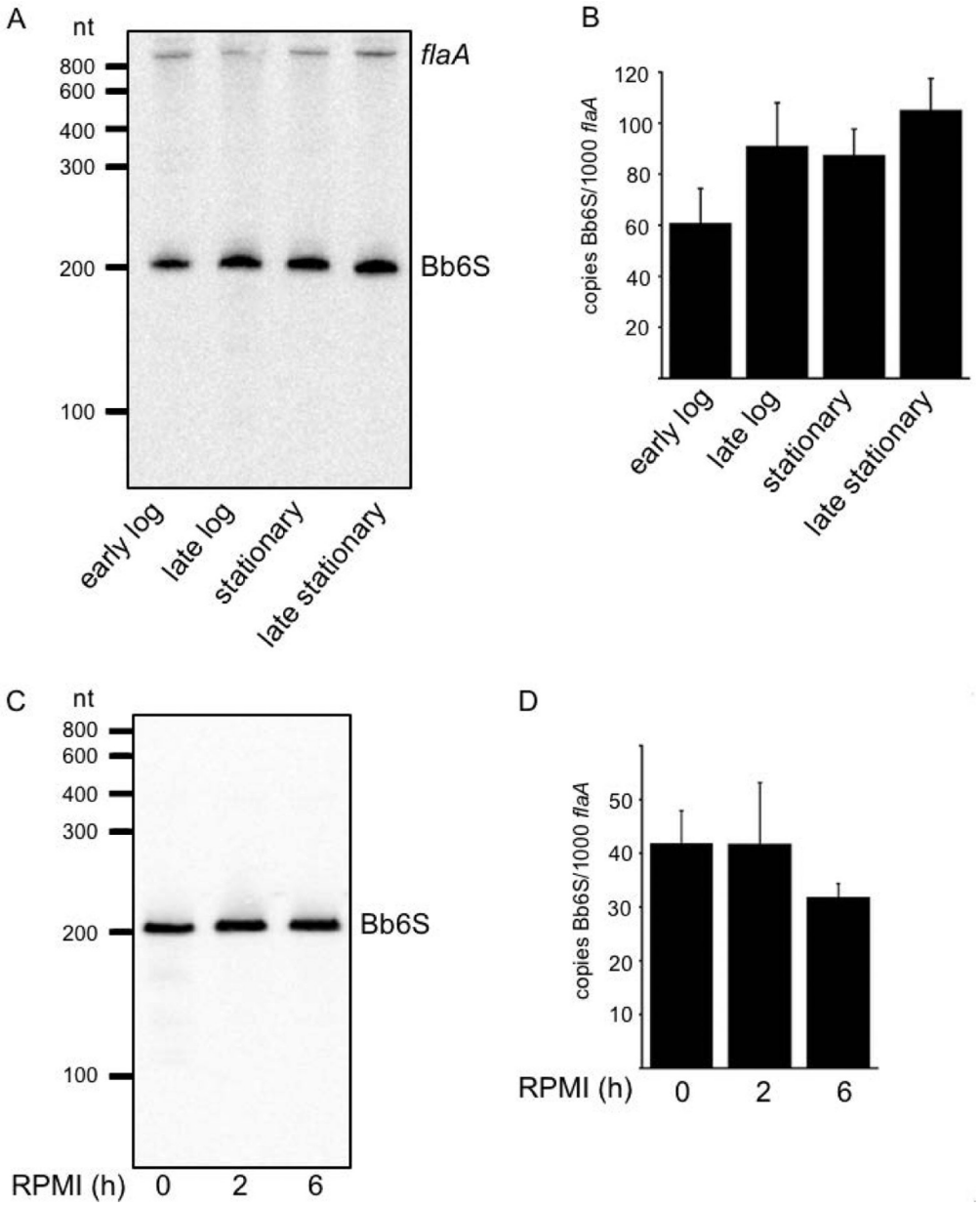

Figure 4. Bb6S RNA expression *in vitro*.

(A) Northern blot and (B) qRT-PCR analyses of Bb6S RNA and *flaA* mRNA levels from total RNA isolated from wild-type cultures grown at 35°C to different cell densities. (C) Northern blot and (D) qRT-PCR analyses of Bb6S RNA levels from wild-type cells grown to stationary phase (RPMI 0 h) and starved in RPMI for 2 h or 6 h. Values are the mean of three independent biological replicates and error bars represent the SE. $P > 0.05$ by one-way ANOVA with Tukey's *post-hoc* test for comparison of Bb6S levels in both panels B and D.

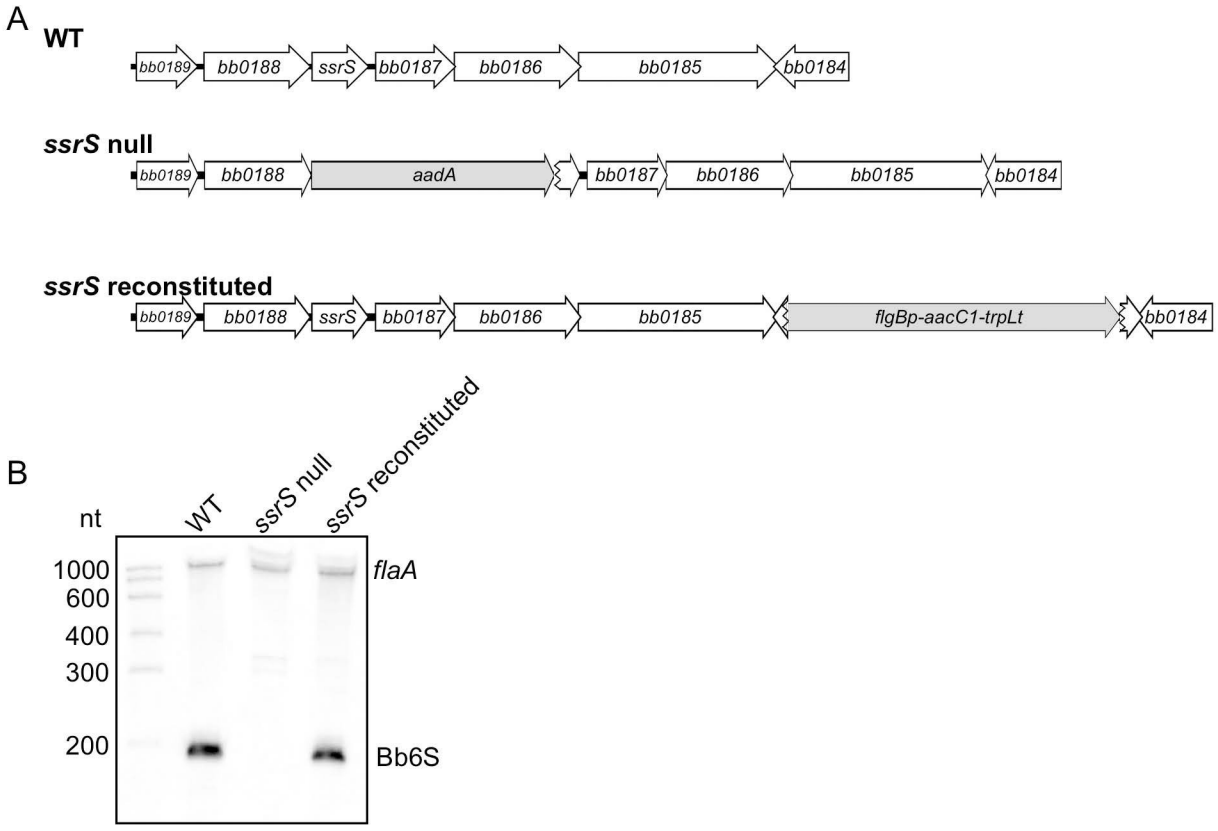

Figure 5. Mutation and complementation of *ssrS* encoding Bb6S RNA in *B. burgdorferi*.

(A) The *ssrS* mutant was constructed by replacing the *ssrS* gene encoding Bb6S RNA with a promoterless streptomycin resistance gene (*aadA*). The *ssrS* mutant was complemented in *cis*, using a gentamicin resistance gene (*aacC1*) fused to a *B. burgdorferi* promoter (*flgBp*) and a *B. subtilis* terminator (*trpLt*), to generate the *ssrS* complemented strain (*ssrS* reconstituted). (B) Bb6S RNA expression in the wild-type (WT), *ssrS* null mutant and *ssrS* complemented strains analyzed by Northern blot of total RNA. Samples were separated on a 6% TBE urea gel, blotted to membrane and hybridized with biotinylated *flaA* and Bb6S RNA single-stranded RNA probes.

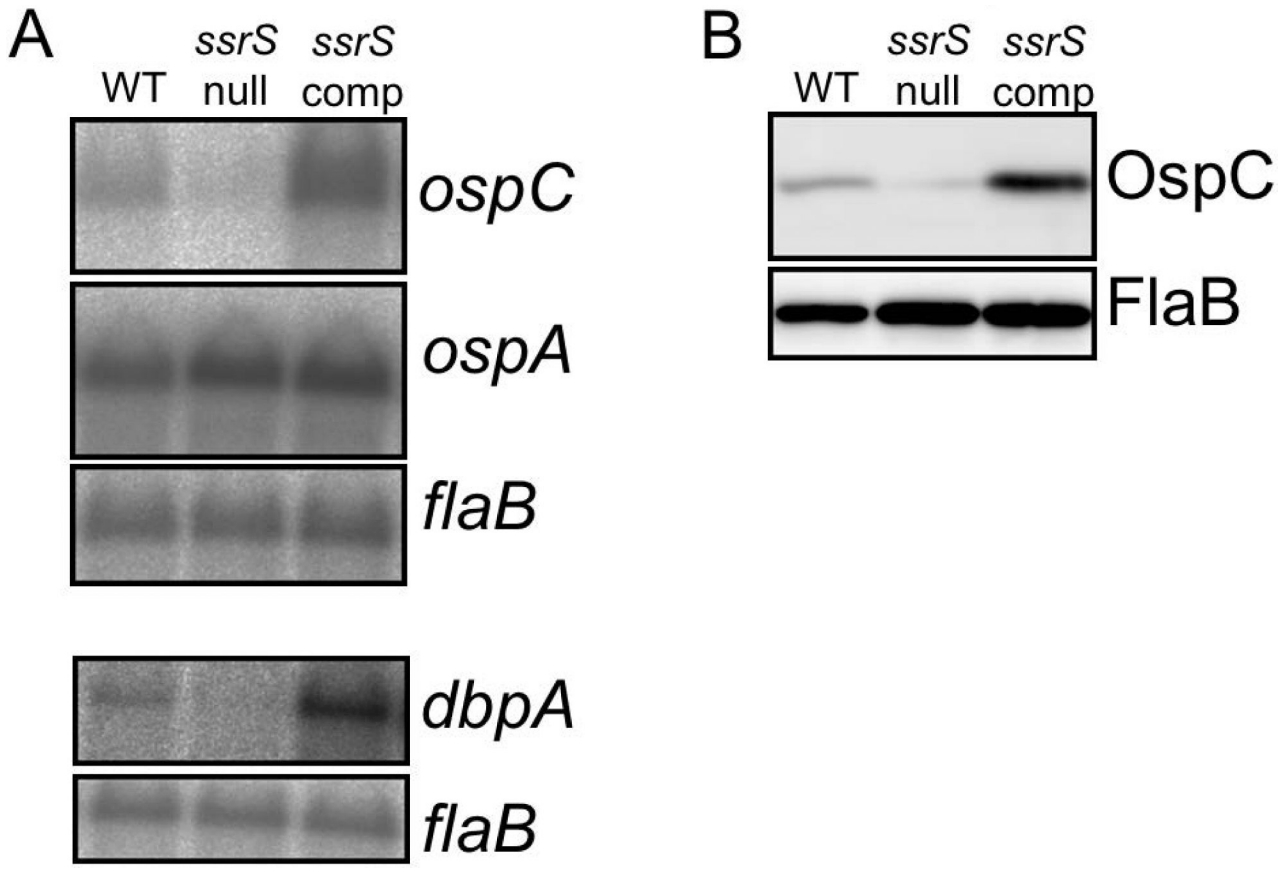

Figure 6. Bb6S RNA affects *ospC* and *dbpA* gene expression.

(A) Northern blot analyses of total RNA isolated from wild-type (WT), *ssrS* mutant (*ssrS* null) and *ssrS* complemented (*ssrS* comp) strains temperature-shifted from 23°C and grown at 35°C until mid-log phase. RNA was separated on an 0.8% agarose gel, transferred to membranes and hybridized with ^{32}P-labeled probes to *ospC*, *ospA*, *dbpA* and *flaB* mRNA (Table 1). (B) Total cell lysates from strains and conditions listed in (A) were separated by SDS-PAGE, transferred to PVDF membranes and analyzed by immunoblot using antibodies against OspC and FlaB.

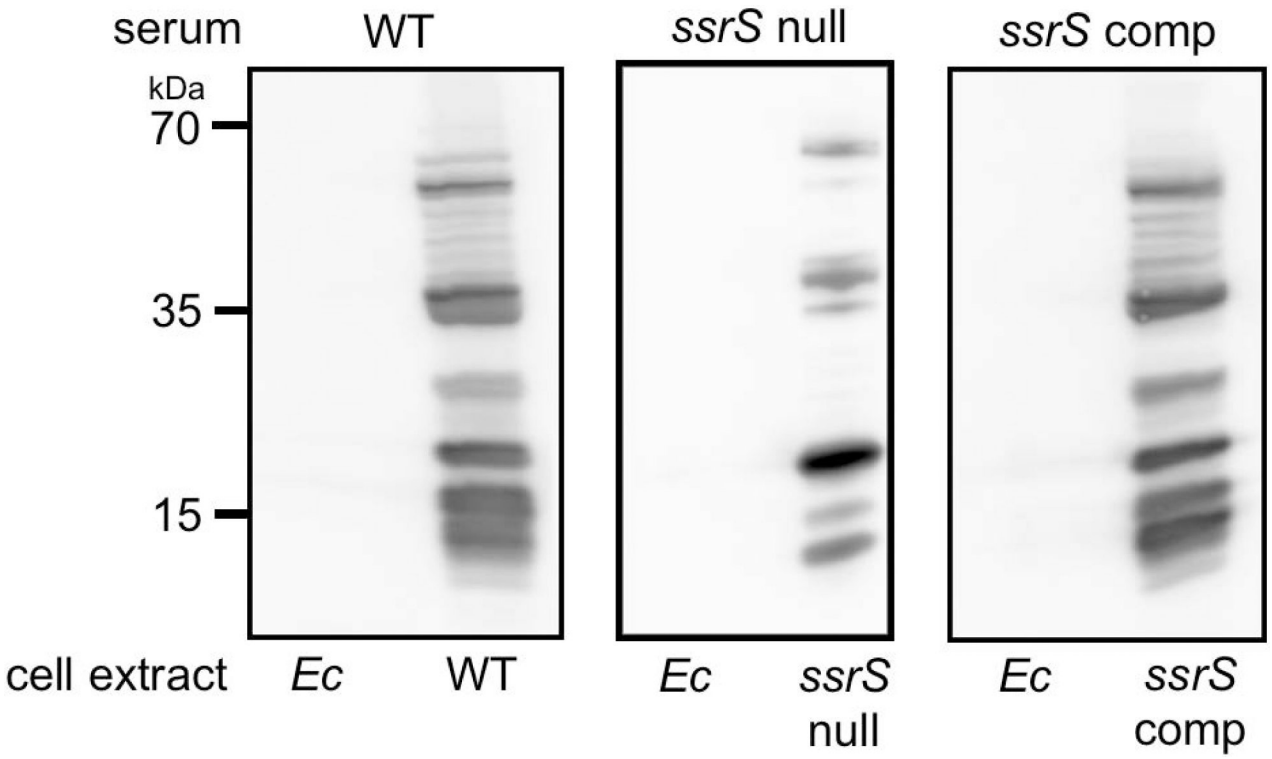

Figure 7. Serological response of mice injected with the *ssrS* null mutant.

Whole cell lysates from wild-type (WT), *ssrS* mutant (*ssrS* null) and *ssrS* complemented (*ssrS* comp) *B. burgdorferi*, as well as *E. coli* (*Ec*) as a negative control, were separated by SDS-PAGE, transferred to membranes and incubated with mouse serum collected five weeks post-injection of 1×10^3 cells of the corresponding strains.

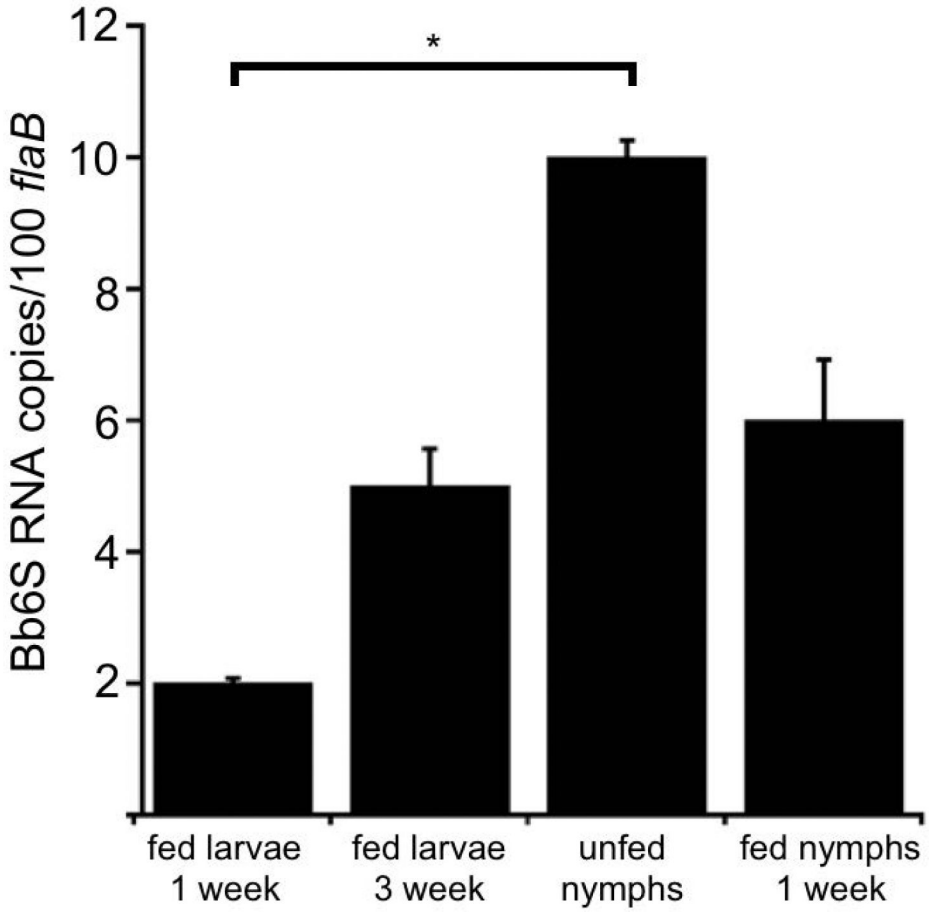

Figure 8. Bb6S RNA expression *in vivo* in ticks.

Bb6S RNA levels in wild type-infected ticks as measured by qRT-PCR from RNA isolated from ticks: naive larvae one week or three weeks post-feeding to repletion on an infected mouse, unfed nymphs one month after molting into nymphs, and fed nymphs one week after feeding to repletion on a naive mouse. Values are the means of at least two independent groups of ticks and error bars represent the SE. * denotes $P < 0.05$ as determined by one-way ANOVA with a Tukey's *post hoc* test.

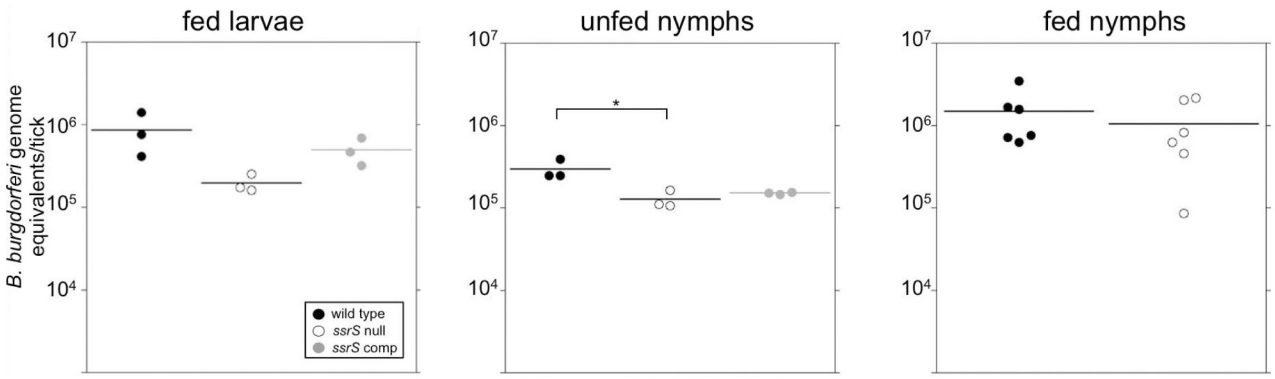

Figure 9. Persistence of the *ssrS* null mutant in ticks.

Quantification of spirochetes in ticks that had fed on mice infected with wild-type (black circles), *ssrS* null mutant (white circles) or *ssrS* complemented (gray circles) strains. Total DNA was isolated from larvae that had fed to repletion (fed larvae) or after larvae had molted to nymphs (unfed nymphs) or one week after nymphs had fed to repletion on uninfected mice (fed nymphs). The number of *B. burgdorferi* genome equivalents per tick was determined by qPCR using TaqMan primers/probe to *flaB*. Data were analyzed using one-way ANOVA with a Tukey's *post hoc* test where * indicates $P < 0.05$.

Table 1.

Oligonucleotides used in this study.[a]

Name	Sequence (5′ to 3′)
6S U971F	TTGAGGTTTCTCCTAATGTA
6S 5R+AatIIAgeI	ACCGGTAGCGACGTCTTTTCATCTTCTTATTTCTAAAA
6S 109F+AatII	GACGTCAGCAATTCAGAGGTTAAGA
6S 1325R+AgeI	ACCGGTGGATCAATCTGATTCAAATA
6S U441F	GAGAAATTTAAGGAAATCGG
6S D1564R+AatIIAgeI	ACCGGTCACGACGTCAGCAAAATTAAAAGCCTTTT
6S D1463F+AatII	GACGTCAAGGTGATGATATTTTGTCA
6S D2498R+AgeI	ACCGGTAAGTAGGTCTATTTTCGATG
rpoC 3285F	TGCATCTTATGTATTACCAG
rpsL U149F+AatII	GACGTCTGGACATTTAATTCCTACTG
rpsG 385R+AgeI	ACCGGTATGCATTTAAAAGTTCGTTT
rny 467F	AAAGAGATGCTCAAGTTATT
rny 1455F+AatII	GACGTCAGAAGCTGAAATGAGATATC
rny D2344R+AgeI	ACCGGTCTGGATACCTTTTACAAAG
Northern blot probes	
6S 19F	GGGAGGTTTAATTTTAAGAA
6S 180R+T7	**TAATACGACTCACTATAGGC**CAACATAAGAACATTTT
flaA 64F	GCTCAAGAGACTGATGGATTAGC
flaA 284R+T7	**TAATACGACTCACTATAGGC**GCAGAAGGAGTAAGTAAAACGCTC
rrf 7F	TGGTTAAAGAAAAGAGGAAA
rrf 110+T7	**TAATACGACTCACTATAGG**CTGGCAATAACCTACTCT
ssrA 53F	GGGAATCTCTTAAAACTTCT
ssrA 231R+T7	**TAATACGACTCACTATAGG**AAGTCCCTAAAAATCAACTT
ospC 118R	GCCCTTTAACAGACTCATCAGCAGAATTTGCAGATGTATTCCCATCTTTCCC
dbpA 254R	GCCCTTTAACAGACTCATCAGCAGAATTTGCAGATGTATTCCCATCTTTCCC
ospA 105R	AGGCAAATCTACTGAAACGCTGTTTTTCTCGTCAAGGCTGCTAACATTTTGC
flaB 698R	CCGCCTTGAGAAGGTGCTGTAGCAGGTGCTGGCTGTTGAGC
RACE	
6S 131F	AAATTCCGCCTTGAAAAATAGCGCTAAA
6S 155R	AGCGCTATTTTTCAAGGCGGAATTTTTA
qRT-PCR (TaqMan)	
6S 96F	CCAAAAGCAATTCAGAGGTT
6S 183R	ACTCCCAAAAAGCCAACATA
6S probe	6-FAM-AAGAATAAAAATTCCGCCTTGAAAAATAGCGCT-TAM
rny 1147F	TGCGGAGAGAGCGAAATTG
rny 1247R	ATGGCATCTGCTATTTGAACCA
rny probe	6-FAM-CGCATCACAATGAGGTGAAACCCG-TAM
flaB 423F	TTCTCAAAATGTAAGAACAGCTGAAGA

Name	Sequence (5′ to 3′)
flaB 542R	TGGTTTGTCCAACATGAACTC
flaB probe	6-FAM-TCACTTTCAGGGTCTCAAGCGTCTTGGAC-TAM
flaA 90F	GGGTTCTAAAAGGCCAGAGC
flaA 213R	ACCAGAAGCGCCCGAATATA
flaA probe	6-FAM-TGCCGAGCTTGCAAGAGATCCAAGTTCA-TAM
qRT-PCR (SYBR Green)	
6S U62F	TTAGCAATTGAGGATGTTGAAGCTTT
6S 24R	AAATTAAACCTCCCAGGAGCTTTGA
6S 5F	GCTCCTGGGAGGTTTAATTTTAAGAATT
6S 114R	ACCTCTGAATTGCTTTTGGCTATC
6S 95F	GCCAAAAGCAATTCAGAGGTTAAGA
6S D224R	GAGAAATAAGCTGCACAATAATAATCCT

[a] Restriction sites are underlined and T7 promoter sequences are in bold.

Table 2.

Mouse infectivity of the *ssrS* null mutant strain.

Route	Number of cells injected	Strain	3 weeks		5 weeks	
			Ear	Ear	Ankle	Bladder
Needle[a]	1×10^2	WT	1/3	1/3	1/3	1/3
		ssrS mutant	0/3	0/3	0/3	0/3
		ssrS complement	1/3	1/3	1/3	1/3
	1×10^3	WT	3/3	2/2	2/2	2/2
		ssrS mutant	0/3	0/2	0/2	0/2
		ssrS complement	2/3	2/3	2/3	2/3
	3×10^3	WT	3/3	3/3	3/3	3/3
		ssrS mutant	0/3	0/3	0/3	0/3
		ssrS complement	3/3	3/3	3/3	3/3
	1×10^4	WT	6/6	6/6	6/6	6/6
		ssrS mutant	4/6	4/6	4/6	4/6
		ssrS complement	3/3	3/3	3/3	3/3
Nymph bite[b]		WT	3/3	3/3	3/3	3/3
		ssrS mutant	3/3	3/3	3/3	3/3
		ssrS complement	2/2	2/2	2/2	2/2
Nymph bite[c]		WT	3/3	3/3	3/3	3/3
		ssrS mutant	3/3	3/3	3/3	3/3
		ssrS complement	3/3	3/3	3/3	3/3

[a] Intradermal injection.

[b] Mice were infested with five nymphs each.

[c] Mice were infested with two nymphs each.