2007

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Meghan Catherine Lybecker
The University of Montana

A SMALL RNA REGULATES RPOS IN THE LYME DISEASE SPIROCHETE *BORRELIA BURGDORFERI*

By

Meghan Catherine Lybecker

B.S. Eastern Oregon University, La Grande, Oregon, 1999

Dissertation

presented in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
Integrative Microbiology and Biochemistry

The University of Montana
Missoula, MT

Summer 2007

Approved by:

Dr. David A. Strobel, Dean
Graduate School

D. Scott Samuels, Chair
Division of Biological Sciences

Mike Minnick
Division of Biological Sciences

Michele McGuirl
Division of Biological Sciences

Jean-Marc Lanchy
Division of Biological Sciences

Kent Sugden
Chemistry

Lybecker, Meghan, Ph.D., Summer 2007 Integrative Microbiology/Biochemistry

A small RNA regulates RpoS in the Lyme disease spirochete *Borrelia burgdorferi*

Chairperson: D. Scott Samuels

We have identified and characterized the first sRNA, $DsrA_{Bb}$, in the Lyme disease spirochete, *Borrelia burgdorferi*; as well as, identified a non-canonical RNA chaperone Hfq. The alternative sigma factor RpoS (σ^{38} or σ^{S}) plays a central role in the reciprocal regulation of the virulence-associated major outer surface proteins OspC and OspA. Temperature is one of the key environmental signals controlling RpoS, but the molecular mechanism by which the signal is transduced remains unknown. $DsrA_{Bb}$ post-transcriptionally regulates the alternative sigma factor RpoS in response to an increase in temperature, associated with the tick to mammal transmission signal. A novel 5′ end of the *rpoS* mRNA was identified and $DsrA_{Bb}$ has the potential of extensively base-pairing with the upstream region of this *rpoS* transcript. We demonstrate that *B. burgdorferi* strains lacking $DsrA_{Bb}$ do not upregulate RpoS and OspC in response to an increase in temperature, but do regulate RpoS and OspC in response to changes in pH and cell density. The 5′ and 3′ ends of $DsrA_{Bb}$ were mapped, demonstrating that at least four species exist with sizes ranging from 213 to 352 nucleotides. We demonstrate and characterize in vitro the interaction between DsrA and upstream region of *rpoS* mRNA. We hypothesize that $DsrA_{Bb}$ binds to the upstream region of the *rpoS* mRNA and stimulates translation by releasing the Shine-Dalgarno sequence and start site from a stable secondary structure. Therefore, we postulate that $DsrA_{Bb}$ is a molecular thermometer regulating RpoS in *Borrelia burgdorferi*.

Acknowledgments

First and foremost, I would like to thank my advisor, Scott Samuels. Not only for his patience, technical expertise and wisdom in my scientific endeavors, but for his excellent example, guidance and understanding in balancing a family and successful scientific career.

I would also like to thank the other members of my graduate committee: Steve Lodmell, Mike Minnick, Michele McGuirl, Kent Sugden and Jean-Marc Lanchy. A special thanks to Steve and Jean-Marc for their expertise in RNA-RNA interactions. I would also like to thank our collaborator, Andrew Feig, for valuable advice and support.

Thanks to the members of the Samuels' laboratory, past and present, including Sharyl Bundle, Beth Todd, Mike Gilbert and all of the others in lab who made the Samuels' lab a great place to work and learn. A special thanks to Sharyl Bundle, who not only taught me the laboratory basics, but shared my love and passion for science.

I would also like to thank the members of the newly formed Borrelia Club including Patti Rosa, Frank Gherardini, Tom Schwan and the members of their labs, for their valuable insight and advice.

Others who have contributed to this work by providing strains, reagents, or valuable advice are: Mike Norgard, Frank Yang, Patti Rosa, Justin Radolf, Christian Eggers, Andrew Feig, Susan Gottesman and Michael Zuker.

Thank you also to my family and friends. Their support, patience and laughter have been invaluable to me while undertaking this endeavor. A special thank you to Axel Lybecker, who, with his unceasing patience, support and sense of humor has kept me sane during my graduate school years. Thank you.

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Chapter 1

Introduction

Lyme Disease and the enzootic life cycle of *Borrelia burgdorferi.*

Lyme disease, the most common arthropod-borne disease in the United States, is primarily caused by the bacterium *Borrelia burgdorferi* (Benach *et al*., 1983; Burgdorfer *et al*., 1982; Steere *et al*., 1983). The Center for Disease Control reported 40,792 cases of Lyme disease for the 2001-2002 year, a 40% increase from the previous period. *B. burgdorferi* belongs to a phylogenetically distinct group of bacteria, the spirochetes, that are characterized by their unique cell structure (Baranton and Old, 1995). Spirochetes are generally long, narrow helical bacteria that are highly motile in both liquid and solid media. *B. burgdorferi* has a complex genome that consists of a linear chromosome and both linear and circular plasmids (Barbour and Garon, 1987; Baril *et al*., 1989; Casjens and Huang, 1993; Casjens *et al*., 1997; Casjens *et al*., 2000; Davidson *et al*., 1992; Ferdows and Barbour, 1989; Fraser *et al*., 1997). The linear DNA molecules are covalently closed by terminal hairpin loops (Barbour and Garon, 1987; Casjens *et al*., 1997; Hinnebusch *et al*., 1990; Hinnebusch and Barbour, 1991).

B. burgdorferi is maintained in nature via an enzootic life cycle alternating between a tick vector (*Ixodes* species) and a mammalian host (mice and deer). The enzootic life cycle of *B. burgdorferi* is perpetuated by uninfected tick larvae becoming infected by feeding on an infected mouse. The spirochetes are maintained in the tick larvae midgut through the molt to the nymphal stage. At the next blood meal the

spirochetes migrate from the nymph midgut to the salivary glands and infect the mammal, thus completing the cycle (Lane *et al*., 1991; Spielman, 1994; Steere *et al*., 2004). The infected nymphs are also the primary route of *B. burgdorferi* transmission to humans, which serve as accidental hosts and interrupt the normal enzootic life cycle. In the United States, *B. burgdorferi* is transmitted to humans through the bite of hard-bodied *Ixodes* ticks (Orloski *et al*., 2000). The first signs of infection include a characteristic "bulls-eye" rash termed erythema migrans and flu-like symptoms, such as fever, fatigue, chills, muscle ache and swollen lymph nodes. Most patients recover fully if treated during the early stages of infection with antibiotics, generally doxycycline or amoxicillin. However, if left untreated after a few weeks of infection, patients present with a variety of symptoms that include loss of muscle tone in the face (Bell's palsy), headaches and neck stiffness, dizziness and joint pain. After a few months, long-term effects of Lyme disease are predominately arthritis, swelling of joints and, in a small percentage of patients, neurological and cardiac symptoms (Steere, 2006).

Outer surface lipoprotein function and regulation

B. burgdorferi must survive in and transition between two vastly different environments, the tick vector and mammalian host (Fikrig and Narasimhan, 2006; Singh and Girschick, 2004). Like many other pathogenic bacteria (Guiney, 1997; Mekalanos, 1992), *B. burgdorferi* senses and responds to environmental cues by regulating the gene expression of proteins necessary for survival (Fikrig and Narasimhan, 2006; Singh and Girschick, 2004). The differential gene expression of outer surface lipoprotein A (OspA) and OspC in *B. burgdorferi* has been well documented, but the underlying molecular mechanisms are still not entirely understood. OspC is required for transmission to and

infection of the mammalian host (Gilbert *et al.*, 2007; Grimm *et al.*, 2004; Pal *et al.*, 2004b; Tilly *et al.*, 2006); it binds the tick salivary protein SALP15 (Ramamoorthi *et al.*, 2005). OspA binds the tick midgut protein TROSPA (Pal *et al.*, 2004a) and is necessary for tick infection and maintenance in the midgut (Yang *et al.*, 2004). OspA is highly expressed, while OspC is almost entirely absent, in the unfed tick midgut. During tick feeding OspA synthesis is down-regulated, while OspC is up-regulated (Akins *et al.*, 1998; de Silva *et al.*, 1996; Leuba-Garcia *et al.*, 1998; Montgomery *et al.*, 1996; Rathinavelu and de Silva, 2001; Schwan *et al.*, 1995; Schwan and Piesman, 2000). An assortment of environmental factors play a role in the reciprocal regulation of OspC and OspA, including pH, temperature and host factors (Alverson *et al.*, 2003; Carroll *et al.*, 1999; Fingerle *et al.*, 2000; Hefty *et al.*, 2001; Hübner *et al.*, 2001; Montgomery *et al.*, 1996; Ramamoorthy and Scholl-Meeker, 2001; Rathinavelu and de Silva, 2001; Schwan *et al.*, 1995; Schwan and Piesman, 2000; Stevenson *et al.*, 1995; Yang *et al.*, 2000). The *in vitro* temperature-induced differential regulation of OspC and OspA was first described by Schwan (Schwan *et al.*, 1995). *B. burgdorferi* were passaged from 24°C to 37°C or from 37°C to 24°C and assayed for OspA and OspC expression. Later, Carroll *et al.* (Carroll *et al.*, 1999) demonstrated the effect of pH on the regulation of OspC. The pH of an unfed tick midgut is 8.0, while the pH of the tick midgut after a blood meal is 6.8: a drop in pH from 8.0 to 6.8 further increased OspC levels *in vitro*. Yang *et al.* (Yang *et al.*, 2000) demonstrated that OspA synthesis is drastically reduced with a drop in pH in combination with an increase in temperature; however, our lab and others (Caimano *et al.*, 2007), have not been able to reproduce these results. Notably, both

OspC and OspA are regulated either directly or indirectly by the alternative sigma factor RpoS (Caimano *et al.*, 2005; Hübner *et al.*, 2001).

Alternative sigma factors

Bacterial gene expression is primarily regulated at the level of transcription, which is catalyzed by RNA polymerase (RNAP). The core RNAP complex consists of five subunits (α_2, β, β' and ω) capable of non-specifically binding DNA and transcription elongation. However, the initiation of transcription, often considered the most important step in gene regulation, is controlled by the sigma factor (σ) subunit of RNAP. The sigma factor is responsible for promoter selectivity, greatly increasing the affinity of RNAP for a specific region of DNA and decreasing its affinity for non-specific DNA binding. The sigma factor is released from the RNAP holoenzyme after transcription of approximately ten nucleotides. Many bacteria synthesize several different sigma factors, with different promoter selectivity, thus directing RNAP to a discrete set of genes, which results in the control of a set of genes needed for a certain response. Sigma factors are classified into two families, σ^{70} and σ^{54}, based on their structure and function (Kazmierczak *et al.*, 2005). Most sigma factors belong to the σ^{70} family. These sigma factors have four conserved regions, which are involved in the recognition of the -35 and –10 sites (upstream from the transcriptional start site) and promoter melting (Paget and Helmann, 2003). Promoter sequences and spacing recognized by the σ^{54} sigma factor differ significantly from those recognized by σ^{70}. σ^{54} promoters have highly conserved short sequences located at positions –24 and –12 upstream from the transcriptional start site. The σ^{54} sequences are nearly invariable at the –24/-12 positions (GG and GC, respectively) and with the spacing between the two (Buck *et al.*, 2000; Merrick, 1993).

In addition, the RNAP holoenzyme formed with the σ^{54} subunit has distinct functional properties compared to the σ^{70} holoenzyme. All σ^{54} promoters require an activator protein along with the RNAP holoenzyme to stimulate initiation of an open complex (Buck *et al.*, 2000). σ^{54}-dependent transcription is generally positively regulated in response to environmental signals or a specific cell cycle phase. The active state of the activator protein is dependent on its state of phosphorylation; the activator protein binds to enhancer-like sequences upstream of the promoter and is responsible for transducing the environmental signal. The σ^{54} RNAP holoenzyme binds DNA specifically, but exists in a closed complex when the activator protein is inactive or absent. The activator protein is necessary for open complex formation and transcription initiation (Austin *et al.*, 1991; Berger *et al.*, 1994; Cannon and Buck, 1992; Hopper and Bock, 1995; Lee *et al.*, 1994; Perez-Martin and de Lorenzo, 1996). Historically, RpoN (σ^{54}) is responsible for transcribing genes necessary for specialized metabolic functions, such as utilization of alternative carbon or nitrogen sources.

The number of sigma factor-encoding genes found in bacterial genomes is highly variable, ranging from 18 in the *B. subtilis* genome (Kunst *et al.*, 1997), to a single sigma factor in the *Lactococcus lactis* genome (Bolotin *et al.*, 1999). The *B. burgdorferi* genome encodes three sigma factor genes, σ^{70} (RpoD), σ^{54} (RpoN), and σ^{38} (RpoS) (Fraser *et al.*, 1997).

The use of alternative sigma factors is a well-known mechanism for regulating bacterial gene expression in response to environmental cues. Complex bacterial responses to environmental signals often use a single global or master regulator to control

many downstream cascades, which ultimately controls the expression of genes needed for the response.

RpoS regulation in *Escherichia coli.*

RpoS (σ^{38} or σ^{s}) in *Escherichia coli* is the stress and stationary phase sigma factor and partially replaces RpoD (σ^{70}), the housekeeping sigma factor, when induced, either by a variety of environmental stress cues or entry into stationary phase. As a result, transcription of many RpoS-dependent genes, the RpoS regulon, is activated (Hengge-Aronis, 2002). RpoS is the global regulator of the general stress response, which is initiated by a variety of environmental signals, including starvation, changes in pH, oxidative stress, near-UV irradiation, heat or cold shock, hyperosmolarity and ethanol (Hengge-Aronis, 2002). In addition, a number of virulence genes have been shown to be under the control of RpoS in enteric bacteria. Caimano *et al*. (2004) demonstrated that RpoS in *B. burgdorferi* is not required for the general stress response, but is necessary for the regulation of virulence factors OspC and OspA.

RpoS regulation has been extensively studied in *E. coli* and, despite its complexity, the basic molecular mechanisms have been elucidated. Surprisingly, most of the regulation occurs post-transcriptionally, either at the level of protein degradation or translation initiation. *rpoS* transcript levels in *E. coli* are high and remain relatively constant regardless of environmental conditions, except in rich medium (Arnqvist *et al.*, 1994; Muffler *et al.*, 1997a). Consequently, there are conditions when the *rpoS* mRNA level is high and the RpoS protein is nearly undetectable. The efficient translation of the *rpoS* transcript is contingent upon the secondary structure of the transcript. Under non-inducing RpoS conditions, the *rpoS* transcript forms a secondary structure in which the

Shine-Dalgarno sequence is base paired and unavailable for access by the ribosome (Hengge-Aronis, 2002). The secondary structure of the *rpoS* transcript has been modeled using the MFOLD computer program. The 340 nucleotides located at the 5′ end of the *rpoS* transcript are predicted to fold into a stable cruciform structure. The translation initiation region (TIR) is located downstream of this structure and is predicted, by MFOLD, to form two different stable structures with similar energetics (Hengge-Aronis, 2002). However, genetic evidence suggests that only one of the structures is biologically significant in the regulation of translation. This structure is predicted to be a hairpin stem loop that has the region around the Shine-Dalgarno sequence partially base-paired to an upstream region of the mRNA (Hengge-Aronis, 2002).

The *rpoS* mRNA secondary structure is altered, either directly or indirectly, under certain environmental conditions, which allows for efficient translation of the transcript. There are three small non-coding RNAs (sRNAs) that affect the translation of *rpoS* mRNA: DsrA, RprA and OxyS (Hengge-Aronis, 2002; Massé *et al.*, 2003; Repoila and Gottesman, 2001; Repoila *et al.*, 2003; Sledjeski and Gottesman, 1995; Sledjeski *et al.*, 1996). sRNAs were originally described as fully complementary, *cis*-encoded antisense RNAs, transcribed from the opposite strand of the mRNA target, with only one mRNA target. These *cis*-encoded sRNAs are predominately found on extra-chromosomal DNA and are involved in plasmid replication control, bacteriophage immunity systems and transposon movement (Eguchi *et al.*, 1991; Wagner and Simons, 1994). For example, the ColE1 and R1 plasmid copy number are regulated by the sRNAs. RNAI binds to the RNA primer required for the ColE1 plasmid replication and prevents the RNA/DNA hybrid required for initiation of replication (Tomizawa *et al.*, 1981). The sRNA CopA

inhibits translation of the mRNA encoding a replication protein for the R1 plasmid (Wagner *et al.*, 1987). Although most sRNAs act post-transcriptionally there are examples of transcription attenuation as well (Okamoto and Freundlich, 1986).

More recently, a new class of *trans*-acting sRNAs emerged that often affect several mRNA targets and are not completely complementary to their targets. Close to 50 *trans*-acting sRNAs have been identified in *E. coli*, although their functions have not all been elucidated. Generally, these *trans*-acting sRNAs affect translation initiation or the target mRNA stability (Gottesman, 2004; Majdalani *et al.*, 2005; Repoila *et al.*, 2003). Many sRNAs regulate their targets via complementary base-pairing to the mRNA, although the extent of base-pairing can vary. However, some sRNA regulators act by binding a target protein and changing its activity: 6S RNA binds the σ^{70} RNAP holoenzyme, by mimicking the open promoter complex and inhibits transcription of σ^{70} promoters in response to changes in growth phase (Wassarman and Storz, 2000). In addition, CsrA and CsrB sRNAs bind and inhibit the CsrA protein, which is a translational regulator that controls carbon metabolism, flagellum biosynthesis and represses biofilm formation in *E. coli* (Romeo, 1998; Suzuki *et al.*, 2002).

RpoS in *E. coli* is the only known case where three sRNAs regulate translation. OxyS inhibits *rpoS* translation in response to oxidative stress by an unknown mechanism, while both DsrA and RprA stimulate *rpoS* translation (Hengge-Aronis, 2002; Massé *et al.*, 2003; Repoila *et al.*, 2003; Sledjeski *et al.*, 1996). Translation of *rpoS* mRNA is induced by DsrA in response to low temperatures, which enhance transcription of *dsrA* and increase stability of DsrA (Repoila and Gottesman, 2001). DsrA is an 85-nucleotide RNA that folds into a stable secondary structure consisting of three stem-loops (Lease

and Belfort, 2000; Sledjeski and Gottesman, 1995). The first stem-loop of DsrA base-pairs with the *rpoS* mRNA interfering with the formation of the stem loop structure that sequesters the Shine-Dalgarno sequence, thus leading to an increased level of translation initiation. The second stem-loop interacts with the *hns* mRNA, which encodes a small architectural DNA-binding protein, and inhibits its translation, while the third stem-loop is thought to be a transcription terminator. RprA increases translation of *rpoS* by the same mechanism as DsrA, but is triggered by the response regulator RscB, which is regulated by cell surface stress (Majdalani *et al*., 2002). Importantly, the RNA-binding protein Hfq is required for the activity of all three RNAs in regulating RpoS synthesis (Brown and Elliott, 1997; Hengge-Aronis, 2002; Massé *et al*., 2003; Repoila *et al*., 2003).

The role of Hfq in small non-coding RNA (sRNA or ncRNA) function

Most characterized sRNAs that regulate their mRNA targets via partial RNA-RNA complementary base-pairing require the RNA chaperone Hfq (Majdalani *et al.*, 2005). Hfq was initially identified as an *E. coli* host factor protein necessary for replication of the RNA genome of the Qβ bacteriophage. Specifically, Hfq binds the 3′ end of the positive strand and is thought to denature secondary structure allowing the Qβ replicase to synthesize negative strands (Franze de Fernandez *et al.*, 1968). In addition to Qβ RNA (de Haseth and Uhlenbeck, 1980), Hfq binds to poly(A) (de Haseth and Uhlenbeck, 1980; Zhang *et al.*, 2002) and poly(U) RNA (Brescia *et al.*, 2003; Senear and Steitz, 1976), DsrA (Sledjeski *et al.*, 2001) and OxyS (Zhang *et al.*, 1998), as well as a number of other RNAs (Moller *et al.*, 2002) .

Hfq contains an Sm conserved motif and is listed under the Sm and Sm-like (LSm) protein family in the Conserved Domain database (Marchler-Bauer *et al.*, 2003).

Sm and LSm proteins are involved in several aspects of RNA metabolism and are characterized by two highly conserved motifs; Sm1 and Sm2 (Cooper *et al.*, 1995; Hermann *et al.*, 1995; Seraphin, 1995). The most well known Sm proteins are the seven human proteins that assemble onto the spliceosomal small nuclear RNAs (snRNAs) to form Sm core ribonucleoproteins. The biogenesis and stability of snRNAs are dependent on the Sm proteins and, therefore, the Sm proteins play a major role in pre-mRNA splicing (Burge *et al.*, 1999; Kramer, 1996; Will and Luhrmann, 1997). LSm proteins play a major role in mRNA decapping (Boeck *et al.*, 1998; Bouveret *et al.*, 2000; Tharun *et al.*, 2000) and telomerase activity (Pillai *et al.*, 2003). Structurally, Sm and LSm proteins form cyclic oligomers (heteroheptamer rings) and therefore have a characteristic ring-shaped structure (Achsel *et al.*, 1999; Achsel *et al.*, 2001; Kambach *et al.*, 1999).

Hfq is the first LSm protein discovered in bacteria (Moller *et al.*, 2002; Zhang *et al.*, 1998) and has since been shown to have multiple functions, including a variety of roles in post-transcriptional regulation. Hfq protein, like Sm and LSm, forms a cyclic oligomer displaying the characteristic torus-shaped structure, but it forms a homohexameric, rather than a heteroheptameric ring (Toro *et al.*, 2001; Toro *et al.*, 2002). Hfq targets several mRNAs for degradation by either interfering with ribosome binding or by increasing polyadenylation (Hajnsdorf and Regnier, 2000; Vytvytska *et al.*, 2000). In contrast, several sRNAs are stabilized by Hfq, which is thought to either fold the sRNA into a nuclease-resistant active form or directly compete with RNase E for the binding site (Majdalani *et al.*, 2005). In addition, Hfq binds sRNAs *in vitro* and stimulates or enhances their pairing to target mRNAs, indicating that Hfq plays another role besides stabilizing the sRNAs. The Hfq binding site on sRNAs was identified as a

single-stranded A-U stretch followed or preceded by a stem (Moll *et al.*, 2003; Moller *et al.*, 2002; Zhang *et al.*, 2002). Two hypotheses have been proposed for the role of Hfq in RNA-RNA interactions. One hypothesis is that Hfq acts as an RNA chaperone by unfolding the sRNA and/or the mRNA target and facilitating interactions. There is evidence that suggests that Hfq can change RNA structure, but it does not do so in all cases. Specifically, the structure of DsrA in *E. coli* does not change in response to Hfq binding, but the structures of the sRNA RhyB and the mRNA *ompA* are altered upon Hfq binding (Brescia *et al.*, 2003; Geissmann and Touati, 2004; Lease and Woodson, 2004; Moll *et al.*, 2003). The second hypothesis is that Hfq facilitates RNA/RNA interactions by co-localizing the sRNA and its target mRNA; several studies suggest that Hfq has two separate RNA binding sites, so it can bind both the sRNA and its target mRNA (Brescia *et al.*, 2003; Mikulecky *et al.*, 2004).

An *E. coli hfq* null mutant has pleiotropic effects that include decreased growth rate, increased cell length, and increased sensitivity to ultraviolet light, mutagens and oxidants (Muffler *et al.*, 1997b; Tsui *et al.*, 1994). Hfq is required for *rpoS* post-transcriptional regulation and, therefore, more than 50 proteins are affected by the null mutation. More than 30 species of bacteria have potential Hfq orthologs (Sun *et al.*, 2002) and several species require Hfq for virulence (Christiansen *et al.*, 2004; Ding *et al.*, 2004; Sharma and Payne, 2006; Sittka *et al.*, 2007; Sonnleitner *et al.*, 2003). Notably, *B. burgdorferi* and many other bacteria, including *Streptococcus pneumoniae* do not have annotated homologs of Hfq.

RpoS regulation in *B. burgdorferi.*

RpoS in *B. burgdorferi* does not appear to be responsible for regulating the general stress response as it does in *E. coli*, but instead controls virulence gene expression (Caimano *et al*., 2004). The molecular mechanisms governing the regulation of RpoS in *B. burgdorferi* remain mostly unknown, despite its important role in the regulation of virulence factors, including OspA and OspC. RpoS also regulates many other genes, either directly or indirectly; the vast majority of these are hypothetical or conserved hypothetical genes (Caimano *et al*., 2007; Fisher *et al*., 2005). Genes encoding factors required for mammalian transmission and infection are presumably up-regulated by RpoS and include: *dbpA*, which encodes a protein adhesin that binds the proteoglycan decorin (Eggers *et al*., 2004; Guo *et al*., 1995; Hübner *et al*., 2001); *ospF*, which encodes an outer surface lipoprotein of unknown function (Caimano *et al*., 2004; Eggers *et al*., 2004, 2006); and *oppA-5*, which encodes an oligopeptide permease peptide binding protein (Lin *et al*., 2001; Medrano *et al*., 2007). Genes encoding factors that are necessary for transmission and survival in the tick are presumably down-regulated by RpoS, including *ospA* and *lp6.6* (Caimano *et al*., 2005). Caimano *et al*. (2007) have recently demonstrated the importance of tick and mammalian host factors in the regulation of RpoS-dependent genes. Previous studies have utilized the temperature up-shift *in vitro* to mimic the transition from the tick vector to the mammal to study genes regulated by RpoS (Fisher *et al*., 2005). Caimano *et al*. (2007) exploited the dialysis membrane chamber (DMC), which cultivates *B. burgdorferi* in a dialysis membrane inside the peritoneum of a rat, to more effectively mimic the host environment. Microarray analysis of wild-type and *rpoS*$^{-}$ strains in the DMC vs. traditional *in vitro* methods underscored the importance of host factors in RpoS-dependent regulation of many genes.

The expression of *rpoS* is regulated by the response regulator Rrp2 and the alternative sigma factor RpoN (Hübner *et al.*, 2001; Yang *et al.*, 2003). *rpoS* transcript levels in cell culture increase in response to an increase in temperature and a decrease in pH, but not in response to changes in growth phase (Caimano *et al.*, 2004). In contrast, RpoS protein levels increase in response to an increase in temperature, a decrease in pH and entry into stationary phase (Elias *et al.*, 2000; Hübner *et al.*, 2001; Yang *et al.*, 2000), although distinguishing between the effects of pH and growth phase can be difficult because the pH of the medium decreases as the cell density of a culture increases. More importantly, the physiological relevance of pH and cell density in the enzootic life cycle of *B. burgdorferi* is unknown.

We hypothesize that RpoS regulation occurs post-transcriptionally, as in *E. coli*, and herein demonstrate that temperature-mediated regulation of RpoS is affected post-transcriptionally by a small RNA that we term $DsrA_{Bb}$. Although we, and others (Caimano *et al.*, 2004), have observed regulation of steady-state *rpoS* mRNA levels by temperature, our data demonstrate that $DsrA_{Bb}$ is required for RpoS synthesis after an increase in temperature. However, regulation of RpoS in response to changes in pH and cell density are not dependent on $DsrA_{Bb}$. The data indicate that the regulation of RpoS in *B. burgdorferi*, like in *E. coli*, is complex, occurs at multiple levels, and integrates a variety of different signals. $DsrA_{Bb}$ is the first sRNA to be characterized in *B. burgdorferi* and likely plays a pivotal role in the transmission of Lyme disease. Our model is that $DsrA_{Bb}$ serves as a molecular thermometer (Narberhaus *et al.*, 2006; Repoila and Gottesman, 2003) regulating RpoS in *B. burgdorferi*. We hypothesize that the activity of $DsrA_{Bb}$ at 23°C vs. 37°C is directly related to the structure of $DsrA_{Bb}$ at these

temperatures. Specifically, we hypothesize that the upstream region of *rpoS* at 23°C is folded into a stable stem-loop structure rendering the Shine-Dalgarno and start codon inaccessible to the ribosome. We propose that the secondary structure of $DsrA_{Bb}$ at 23°C does not allow for base-pairing with the *rpoS* transcript, but, the secondary structure changes after a temperature upshift to allow the two RNAs to bind. The RNA-RNA interaction alters the overall structure of the *rpoS* upstream region, freeing the Shine-Dalgarno sequence and start codon allowing for efficient translation. Using this model of translational regulation as a working hypothesis, we endeavored to demonstrate and characterize the DsrA::*rpoS* interaction *in vitro*. In addition, because of the unequivocal role of Hfq in sRNA activity *in vivo,* we hypothesized that *B. burgdorferi* may have an Hfq analog. In this dissertation, we identify a potential non-canonical Hfq in *B. burgdorferi* by demonstrating its ability to partially restore the wild-type phenotype in an *E. coli hfq* null mutant.

Chapter 2

Materials and Methods

B. burgdorferi bacterial strains

Borrelia burgdorferi low-passage strains 297 (clone BbAH130) (Hübner *et al.*, 2001) and B31–A3 (Elias *et al.*, 2002) were cultivated in Barbour-Stoenner-Kelly H (BSK-H) complete medium (Sigma) or Barbour-Stoenner-Kelly II (BSK-II) complete medium at either 23°C or 37°C. The 297 clone was passaged to 1×10^5 cells ml^{-1} from 23°C to 37°C and grown to either 1-3 $\times 10^7$ cells ml^{-1} (low density), which corresponds to mid-log phase or about 1×10^8 cells ml^{-1} (high density), which corresponds to stationary phase. The B31–A3 strain was grown at 23°C to a cell density of 1×10^5 cells ml^{-1}, moved to 37°C and grown to either high or low cell density. Cell density was determined using a Petroff Hausser counting chamber (Hausser Scientific Partnership) as previously described (Samuels, 1995). *B. burgdorferi* transformants were cloned using either limiting dilution in liquid BSK-II (Yang *et al.*, 2004) or plating in semi-solid BSK medium (Samuels, 1995) containing kanamycin (200 μg ml^{-1}) or streptomycin (50 μg ml^{-1}) at 34°C and a 1.5% CO_2 atmosphere.

Gene disruption and complementation of $dsrA_{Bb}$

The $dsrA_{Bb}$ gene was disrupted by deleting 65 nucleotides of the sequence complementary to the *rpoS* upstream region. Regions flanking the $dsrA_{Bb}$ sequence were amplified by PCR (Table 1 and Fig. 6A). The 3´ end of the upstream flanking sequence and the 5´ end of the downstream flanking sequence were engineered with *Aat*II sites for

insertion of either the kanamycin or streptomycin resistance cassettes (Bono *et al*., 2000; Frank *et al*., 2003). The two flanking regions were cloned into pCR®2.1-TOPO and ligated together to form a 2.4 kb target DNA fragment. The antibiotic-resistance cassettes were cloned into the synthetic *Aat*II site. The plasmid was linearized with *Ahd*I and electroporated into competent *B. burgdorferi* as previously described (Samuels, 1995). Transformants were screened by PCR with oligonucleotide primers that flank the insertion site (Table 1 and Fig. 6A).

Five different plasmids, termed pKFSC, pKFLC, pKFMC, pKFG and pKFLCS were constructed to complement the $dsrA_{Bb}$ mutant strain (Table 2). Four of the complementation plasmids, pKFSC, pKFMC, pKFLC and pKFLCS contain the $dsrA_{Bb}$ sequence complementary to the *rpoS* upstream region, but differ in the length of 5′ upstream sequence included (Fig. 9A). The pKFG plasmid contains the predicted *bb0577* promoter and gene without the entire $dsrA_{Bb}$ sequence. These DNA sequences were amplified by PCR and cloned into pCR®2.1-TOPO. The DNA was then excised using *Kpn*I and *Xho*I and cloned into pKFSS1 (Frank *et al*., 2003) using *Kpn*I and *Sal*I. The pKFLCS plasmid was constructed by site-directed mutagenesis (Stratagene QuikChange® Site-Directed mutagenesis kit) of pKFLC to engineer an early stop codon in the *bb0577* ORF. The recombinant plasmids were electroporated into the kanamycin-resistant $dsrA_{Bb}$ mutants and cloned by limiting dilution in both streptomycin and kanamycin. The resulting transformants were assayed for the $dsrA_{Bb}$ expression plasmid by both PCR and transformation of plasmid DNA from *B. burgdorferi* into *E. coli* (xenodiagnosis).

Northern blot analysis

Total RNA was isolated from 100 ml cultures using TRIzol™ (Gibco BRL) as previously described (Alverson *et al.*, 2003). RNA samples were treated with TurboDNase (Ambion) according to manufacturer's instructions and then fractionated in either a 1% formaldehyde-agarose gel (Ambion) or a Novex® Pre-Cast 6% polyacrylamide 7M urea gel (Invitrogen) following the manufacturer's instructions. The RNA was transferred to a BrightStar-Plus™ membrane by vacuum blotting or electroblotting using the Xcell SureLock™ Mini-Cell (Invitrogen) following the manufacturer's specifications. Northern blot hybridizations were performed using the NorthernMax™ kit (Ambion) according to the manufacturer's instructions. Non-isotopic RNA probes were generated from *in vitro* transcription of PCR-generated DNA templates using the MAXIscript® *in vitro* transcription kit (Ambion) according to the manufacturer's instructions. Briefly, the T7 bacteriophage promoter was added to the 5′ end of one of the PCR primers to be incorporated into PCR products. Biotin-14-CTP (GibcoBRL) was used as a CTP analog in the *in vitro* transcription reaction. Northern blot membranes were developed by chemiluminescence using the BrightStar® Biodetect™ nonisotopic detection kit on a Fujifilm LAS-3000.

Identification of 5′ and 3′ ends of DsrA and *rpoS* mRNA

5′ Rapid Amplification of cDNA Ends (RACE) was carried out on DNase-treated total RNA (described above) using the FirstChoice® RLM-RACE kit (Ambion) according to the manufacturer's directions except that the first two phosphatase reactions were omitted. In addition, to characterize the *rpoS* 5′ ends, an *rpoS*-specific primer was used in the reverse transcription reaction instead of random decamers. Furthermore, an

additional nested PCR reaction was necessary to amplify the *rpoS* 5′ ends, presumably due to the low level of *rpoS* transcript.

Ribonuclease Protection Assay (RPA) was performed on DNase-treated total RNA using the RPA III™ kit (Ambion). Riboprobes were generated as described above using the T7 MAXIscript® kit. The traditional RPA III protocol was followed and the products were resolved in a Novex® Pre-Cast 6% polyacrylamide 7M urea gel (Invitrogen). The RNA was transferred to the BrightStar-Plus™ membrane (Ambion) by electroblotting. The membrane was developed by chemiluminescence as described above.

SDS-PAGE and immunoblotting

B. burgdorferi protein extract preparation was performed as previously described (Alverson and Samuels, 2002). Protein extracts were fractionated in Novex® Pre-Cast 4-20% Tris-Glycine gels (Invitrogen) and transferred to Immobilon-P PVDF membranes (Millipore) by electroblotting as described above. Proteins were detected with either an anti-RpoS antiserum (Yang *et al.*, 2000) or anti-OspC monoclonal antibody 4B8F4 (Padula *et al.*, 1993), which were generous gifts of Frank Yang and Mike Norgard (University of Texas Southwestern Medical Center, Dallas, TX, USA) or Craig Sampson (Centers for Disease Control and Prevention, Fort Collins, CO, USA), respectively. The membranes were developed by chemiluminescence using the ECL Plus Western Blotting detection system (Amersham Biosciences) on a Fujifilm LAS-3000 (Gilbert *et al.*, 2007; Yang *et al.*, 2005).

Template preparation for *in vitro* transcription

B. burgdorferi strain 297 total DNA was used as a template in PCR with the T7 bacteriophage promoter added to the 5′ end of the forward primer. The PCR products were visualized on an agarose gel stained with ethidium bromide. If a single product was visualized, the PCR reaction was precipitated with ammonium acetate and resuspended in DEPC-treated water. If more than one product was visible, the appropriate band was gel-purified with the Qiagen gel purification kit following the manufacturer's protocol and resuspended in DEPC-treated water. The $DsrA^{290}$ PCR required two amplifications to generate a product. An initial PCR product was generated using primers that flank the $DsrA^{290}$ region. This PCR was used (1:100 dilution) as a template for the reaction that included the forward primer with the T7 promoter for this specific RNA. The template preparation for the mutagenic RNAs also consisted of an initial *EcoR*I digestion of the pCR2.1 topoo-vector containing the mutagenic sequence. This was necessary to remove an intrinsic T7 promoter found on the pCR2.1 vector. The digestion was fractionated on an agarose gel and stained with ethidium bromide. The appropriate bands were excised and gel-purified as described above. A 1:50 dilution of the gel-purified product was then used as template for a PCR with the appropriate primers including the T7 promoter.

***In vitro* synthesized RNA**

DsrA and *rpoS* RNA for gel shift assays was synthesized *in vitro* using the T7-Megascript In Vitro Transcription kit (Ambion) according to the protocol suggested by the manufacturer. PCR products (described above) were used as templates for the *in vitro* transcription reactions. *In vitro* transcription reaction time was increased to overnight at 37°C to increase the yield of short products. Following *in vitro*

transcription, the full-length RNA transcripts were purified on a 6% polyacrylamide-7M urea gel and eluted into elution buffer (20 mM Tris-HCL pH 7.5, 0.5 M ammonium acetate, 10 mM EDTA, 0.1% SDS) at 37°C overnight or 65°C for 2 hours. The RNA was purified and concentrated by an ammonium acetate precipitation and resuspended in 60 μL of DEPC-treated water. An OD_{260} reading was used to determine the concentration of each RNA.

Overlap extension PCR mutagenesis

Overlap extension PCR mutagenesis was performed as previously described (Samuels *et al*., 1994) to generate the mutations, dGC, dloop, and St/Sw (Fig. 16) in the *rpoS* mRNA upstream region and DsrA RNA for the *in vitro* binding assays. Briefly, two PCR amplifications were used to introduce the mutations. The first reactions used completely complementary mutagenic primers in two separate PCR reactions with appropriate flanking primers (Table 1) and the proofreading enzyme KOD. The amplification products were purified by an ammonium acetate precipitation and resuspended in 20 ul of water. The two products were then mixed together and cycled 6 times (30 s denaturation, 2.5 min anneal, and 1 min extension) in a PCR with Taq in the absence of primers. The flanking primers were then added and another PCR was used to generate full length mutant products. Taq polymerase was used in the second PCR and products were cloned into pCR®2.1-TOPO after visualization of the correct size amplification product on an agarose gel. The mutations were confirmed by sequencing.

RNA-RNA gel mobility shift assays

Binding assays were performed with 2 μg of DsrA RNA and *rpoS* RNA in 1X dimer buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$) or 1X TE (10 mM Tris-HCL pH 7.5, 1

mM EDTA). Binding assays were performed either using a slow cool or snap cool as described below. In the slow cool protocol, the RNAs were incubated separately at 65°C for 10 min, slow cooled to 23°C (≈ 2 h) and then mixed together and allowed to bind at 23°C or 37°C for 40 min or the specified amount of time. In the snap cool protocol, the RNAs were incubated separately at 92°C for 5 min, put on ice immediately for 2 min, then placed at either 23°C or 37°C for 2 h to allow for proper folding. Next the RNAs were mixed together and allowed to bind at 23°C for 40 min. Samples were then mixed with 2 μL of glycerol loading buffer (40% glycerol, 44 mM Tris-borate pH 8.3, 0.25% bromophenol blue) and analyzed on an 0.8% agarose gel in either 0.5X TB with 0.1 mM $MgCl_2$ or 0.5X TBE run at 4°C for 8 to 10 h at 60 V. The gel was stained with ethidium bromide and visualized with UV light on the Fujifilm LAS-3000. As a positive control the two RNAs were mixed together initially and heated to 65°C or 92°C followed by the same protocols as described above.

Bacteria strains and plasmid construction for *E. coli hfq* complementation

E. coli strain DDS1631 is a derivative of MC4100, with an *hfq* null mutation and a RpoS::LacZ translational fusion (kindly provided by Dr. Andrew Feig) (Sledjeski *et al.*, 2001). The pBADHfq plasmid (pDDS400) was generously provided by Andrew Feig and generated by directionally cloning a PCR fragment of the *hfq* gene into the *Eco*RI and *Pst*I sites of the arabinose-inducible vector pBAD24 (Sledjeski *et al.*, 2001). The BB0268 and BB0260 annotated genes were PCR-amplified with the proofreading enzyme KOD with engineered *Mfe*I and *Pst*I sites at the 5′ and 3′ ends respectively. After poly-A tailing with Taq, the PCR products were cloned into pCR®2.1-TOPO. Positive transformants were PCR screened for the proper sized insert and then sequenced

to confirm accurate and intact sequences. The pBAD260 and pBAD268 plasmids were generated by directionally cloning the *Mfe*I-*Pst*I excised BB268 and BB260 fragments into the *Eco*RI and *Pst*I sites of pBAD24. *Mfe*I, which has compatible ends with *Eco*RI, was used with BB0268 and BB0260 instead of *Eco*RI due to an *Eco*RI site within the BB0268 gene.

β-Galactosidase assays

β-Galactosidase units were assayed as described previously with several modifications. The protocol used was derived from Zhang et al. (Zhang and Bremer, 1995), which greatly simplified the original "Miller" assay. Briefly, cells were grown with shaking at 30°C in Luria-Bertani (LB) medium supplied with the kanamycin and carbomycin in the presence or absence of 150 μM arabinose. The cultures were innoculated 1:100 from overnight cultures and samples were taken at cell densities ranging from 0.4 to 1.0 (approximately log phase). The cell density of each culture was measured (OD_{600}) and 30 μL aliquots were removed and mixed with 70 μL of permeabilization solution (100 mM Na_2HPO_4, 20 mM KCL, 2 mM $MgSO_4$, 0.8 mg/mL CTAB (hexadecyltrimethylammonium), 0.4 mg/mL sodium deoxycholate and 5.4 μL/mL 2-mercaptoethanol) and stored at room temperature. After the final samples were taken they were moved to 30°C and incubated for 30 min. The substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/mL *o*-nitrophenyl-β-D-Galacoside (ONPG) and 2.7 μL/mL 2-mercaptoethanol) was also incubated at 30°C for 30 min. 600 μL of substrate solution was added to each sample and the time was recorded. After sufficient color developed, 700 μL of stop solution (1 M Na_2CO_3) was added to each sample and the time was recorded. The samples were centrifuged at 14,000 rpm for 5 min to remove cell

particulate. The OD_{420} was recorded for each sample (needs to be between 0.05 and 1.0) and the total β-galactosidase units were calculated from the formula:

1000* (OD_{420})/((OD_{600} of culture sampled)*(volume [0.03mL])*(reaction time))

The volumes of permeabilization solution and cell culture varied between experiments and were appropriately changed in the calculation of total β-galactosidase units.

Chapter 3

Identification and characterization of the small RNA $DsrA_{Bb}$

(adapted from Lybecker and Samuels, 2007)

We hypothesized that RpoS in *B. burgdorferi* could be regulated post-transcriptionally by a small non-coding RNA (sRNA). A major class of sRNAs base-pair with a target RNA and affect the translation and/or the stability of the mRNA (Majdalani *et al.*, 2005; Repoila and Gottesman, 2003). Therefore, we searched the genome for a DNA sequence that would generate an RNA that could potentially bind the upstream region of *rpoS* mRNA. Our search revealed a DNA sequence on the chromosome, not linked to the *rpoS* locus (BB0771), that could produce an RNA capable of binding the upstream leader region of the *rpoS* transcript (Fig. 1). We report here the identification of four species of this RNA and a novel 5′ end of the *rpoS* mRNA dependent on cell density and presumably growth phase. In addition, we use the computer program MFOLD to model the secondary structures of *rpoS* mRNA and DsrA.

Identification of $DsrA_{Bb}$ and the 5′ ends of the *rpoS* transcript

The putative sRNA, which we termed $DsrA_{Bb}$, has the potential of base-pairing 31 of 34 nucleotides with the *rpoS* mRNA upstream region; including the flanking regions yields base-pairing of 51 of 68 nucleotides. A short direct repeat occurs within the extended complementary region of the sRNA (Fig. 1A) and sequence analyses reveal that strains B31 and HB19 have five of the repeats, while strain 297 only has four repeats. The

$DsrA_{Bb}$ sequence complementary to *rpoS* (Fig. 1A) shares 29 nucleotides with an upstream hypothetical open reading frame (*bb0577*) (Fig 1B).

Fig. 1

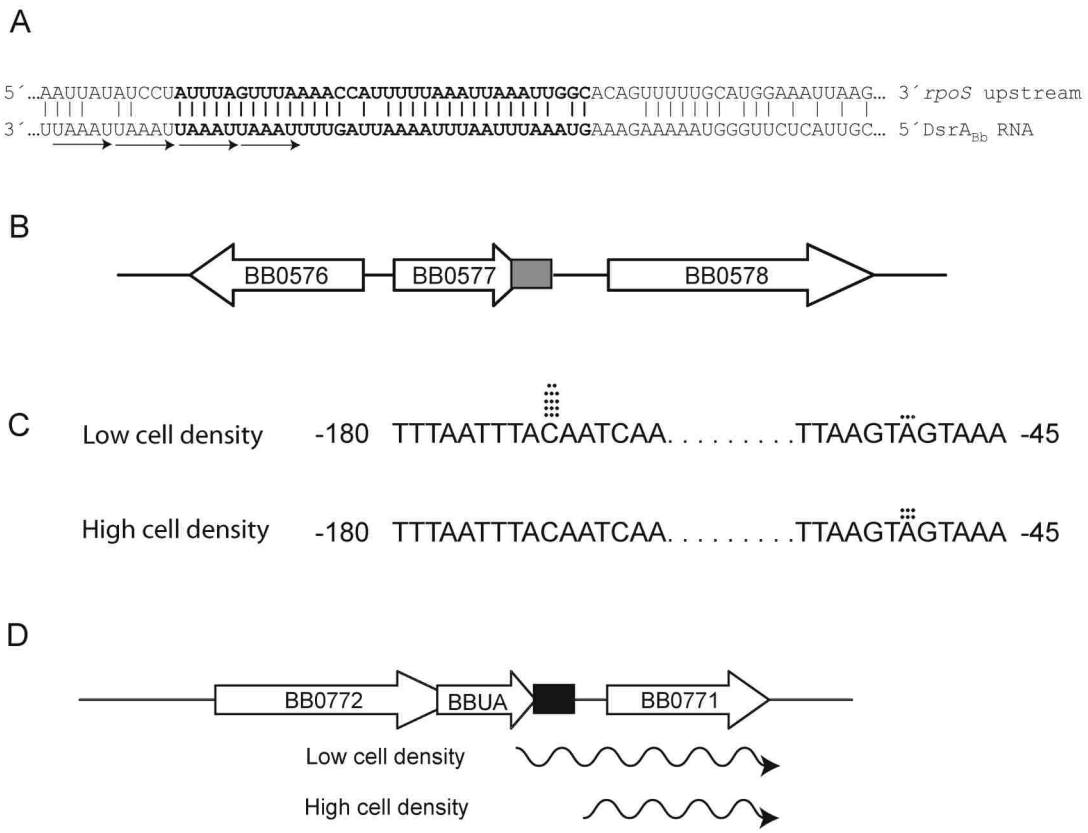

Fig. 1. Complementary binding of a small RNA to the *rpoS* upstream untranslated region. (A) The complementary base-pairing of 51 of 68 nucleotides (31 of 34 nucleotides shown in bold) of $DsrA_{Bb}$ RNA and the upstream region of the *rpoS* transcript. The arrows indicate the short direct repeat (only four shown). (B) A diagram illustrating the chromosomal location of the $DsrA_{Bb}$ sequence displayed in panel A. The $DsrA_{Bb}$ complementary sequence, indicated by the gray box, overlaps with 29 nucleotides of an upstream hypothetical ORF BB0577. (C) The location of the 5′ ends of the *rpoS* transcript characterized by 5′ RACE. Marks (•) denote the number of times the base was sequenced as the 5′ end at either low or high cell density. (D) A diagram illustrating the chromosomal location of the *rpoS* sequence displayed in panel A, indicated by the black box (nucleotides 51 to 120 upstream of the ORF), and the two different transcripts. BBUA is a hypothetical ORF annotated in the *B. garinii* and *B. afzelii* genomes, but not annotated in the *B. burgdorferi* genome.

The *rpoS* sequence that is complementary to $DsrA_{Bb}$ is 51 to 120 nucleotides upstream of the open reading frame (Fig. 1D). We hypothesize that the translation of RpoS is modulated by the complementary base-pairing between $DsrA_{Bb}$ and the *rpoS* transcript. Therefore, the upstream region of the *rpoS* transcript should contain the sequence that is complementary to $DsrA_{Bb}$ to affect regulation by the proposed mechanism. 5′ RACE (rapid amplification of cDNA ends) identified two different 5′ ends of the *rpoS* transcript that appear to be regulated by the cell density or, perhaps, growth phase of the culture (Fig. 1C). *B. burgdorferi* low-passage strain 297 was temperature upshifted to 37°C and grown to low (1-3 × 10^7 cells ml^{-1}) or high (1 × 10^8 cells ml^{-1}) cell density. The predominate 5′ end of the *rpoS* transcript is 171 nucleotides upstream of the start codon (-171) at low cell density, while the predominate 5′ end is 50 nucleotides upstream of the start codon (-50) at high cell density. These data indicate that only the *rpoS* transcript at low cell density contains the sequence with complementarity to $DsrA_{Bb}$.

Northern blot analysis using a single-stranded RNA probe complementary to a region of $DsrA_{Bb}$ shows that the sRNA exists in *B. burgdorferi* and the levels remain constant regardless of temperature or cell density (Fig. 2A). These data were reproduced in a total of six independent RNA isolations from two different strains of *B. burgdorferi*. The probe hybridized to several RNAs as visualized by the multiple bands on the Northern blot (Fig. 2A). The same results were obtained in Northern blots using either a PCR-generated probe or an oligonucleotide probe that were complementary to different regions of the sRNA (data not shown). Furthermore, RT-PCR also confirmed the existence of the RNA (data not shown). We originally hypothesized that the larger bands seen in the $DsrA_{Bb}$ Northern blot were *bb0577* transcripts as the two overlap in sequence

(Fig 1B). However, Northern blot analysis revealed that the major signals of *bb0577* (Fig. 2B), do not correspond with the larger bands in the $DsrA_{Bb}$ Northern blot (Fig. 2A).

Fig. 2

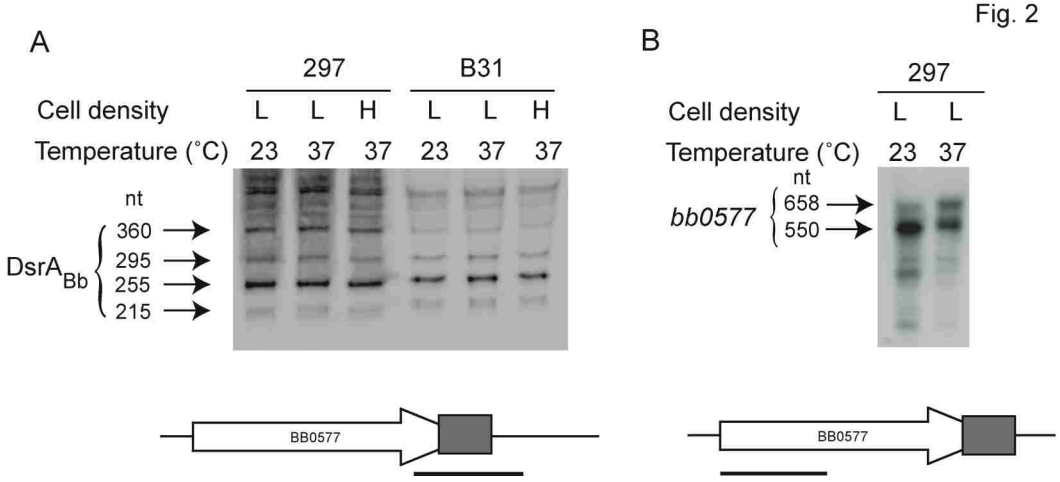

Fig. 2. $DsrA_{Bb}$ levels are not influenced by temperature or cell density. *B. burgdorferi* strains 297 and B31–A3 were grown at 23°C or temperature shifted to 37°C and grown to a low cell density of ~2×10^7 cells ml^{-1} (L) or a high cell density of ~1×10^8 cells ml^{-1} (H). Equal amounts of total RNA (15 μg), confirmed by ethidium bromide staining (data not shown), were fractionated on a 6% TBE-Urea gel, electroblotted to a nylon membrane, and hybridized with either a $dsrA_{Bb}$ or a *bb0577* single-stranded RNA probe (A and B, respectively). The solid bars in the bottom panels illustrate the location of the respective single-stranded RNA probes. Molecular sizes were determined by regression analyses. Three independent experiments with B31–A3 RNA and three with 297 RNA were performed and representative data are shown.

Characterization of $DsrA_{Bb}$ primary structure

The 5′ and 3′ ends of $DsrA_{Bb}$ were characterized by 5′ RACE and RNase protection assays (RPA), respectively (Fig. 3). Two different 5′ ends, separated by 77 nucleotides, were identified (Fig. 3A), and numbered according to the *bb0577* ORF. The RPA identified two 3′ ends of $DsrA_{Bb}$ (Fig. 3B). These data taken together suggest that four size variants of $DsrA_{Bb}$ can exist (Fig. 3C) and the sizes of the four possible species are 213, 275, 290, and 352 nucleotides based on the 5′ RACE and RPA data.

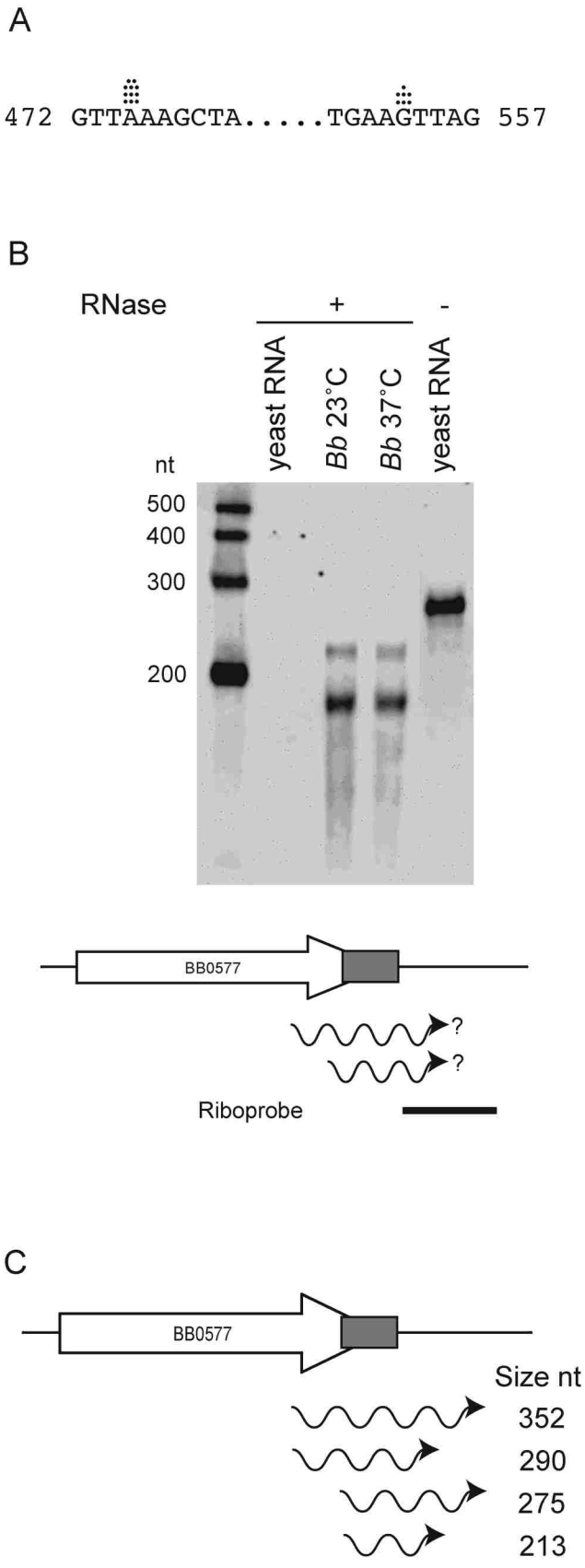

Fig. 3. Characterization of the primary structure of $DsrA_{Bb}$. (A) There are two 5′ ends of $DsrA_{Bb}$ as determined by 5′ RACE. Marks (•) above the base denote the number of times the base was sequenced as the 5′ end. The bases were numbered according to the BB0577 ORF (588 nucleotides). (B) Two 3′ ends were identified by an RNase protection assay (RPA). The products from the RPA were fractionated on a 6% TBE-Urea gel, and electroblotted to a nylon membrane. The bottom panel is a schematic of the RPA strategy. (C) A diagram illustrating the four predicted sizes of $DsrA_{Bb}$ based on the 5′ RACE and RPA data.

These data are consistent with the multiple sizes of $DsrA_{Bb}$ seen in the Northern blot (Fig. 2A). These experiments were performed with both strains B31 and 297; in addition, two different nested primer sets were used in the 5′ RACE and a different riboprobe was used in the RPA: all experiments yielded similar results (data not shown).

Secondary structure modeling of the *rpoS* upstream region and DsrA

MFOLD is a software application available on the internet for the prediction of the secondary structure of single-stranded nucleic acids. Michael Zuker developed the MFOLD software in the late 1980s. However, the MFOLD software was not available on the web until the mid 90s. The 'm' is for 'multiple'. The core algorithm predicts a minimum free energy assuming a nearest neighbor model and using empirical estimates of thermodynamic parameters of neighboring interactions and loop entropies to score structures. Through the years different versions have used different free energy data (Zuker, 2003). Our collaborator, Dr. Andrew Feig, warned of the inaccuracy of MFOLD in predicting secondary structures of RNAs longer than 100 nucleotides (personal communication); however, there is some utility in using it to predict secondary structures. We used MFOLD to model the secondary structure of the *rpoS* mRNA upstream region and DsrA at both 23°C and 37°C. MFOLD predicted a total of 9 different structures for the *rpoS* upstream region at 23°C and 37°C, notably all of the structures have the Shine-Dalgarno (SD) and start codon in the same stem loop structures, and all but one structure has the region of complementarity to DsrA in a stem loop (Fig. 4). In *E. coli,* the Shine-Dalgarno sequence and start site of *rpoS* are base-paired to the upstream sequence that is complementary to DsrA. Therefore, upon DsrA binding to *rpoS,* the SD and start site

become single-stranded and accessible to the ribosome. However, in the upstream region of the *B. burgdorferi rpoS* mRNA secondary structure, the SD and start site are not base-paired with nucleotides that are complementary to DsrA, but are sequestered in stem-loops so they are predicted to be unavailable for ribosome recognition.

Fig. 4

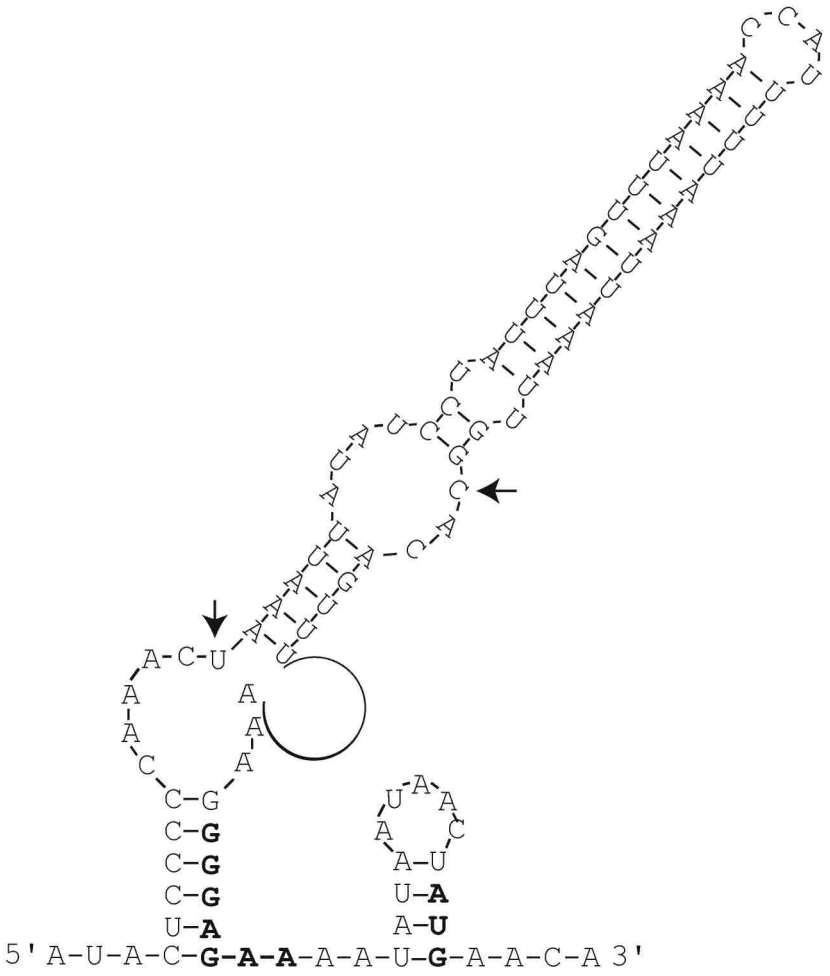

Fig. 4. Predicted secondary structure of the upstream region of *rpoS* mRNA. The arrows indicate the DsrA complementary region (31 of 34 nucleotides). The putative SD sequence and start codon are in bold. The open circle represents a stem-loop structure of 43 nucleotides.

MFOLD predicted a total of 37 different structures for DsrA at 23°C and 37°C; the extensive region of complementarity to the *rpoS* upstream region (31 of 34 nucleotides) is folded into a stem-loop (Fig. 5), except in five of the structures. Notably, the structures that varied from the apparent dominant stem-loop structure still had the *rpoS* complimentary nucleotides base-paired in a stem-loop.

Fig. 5

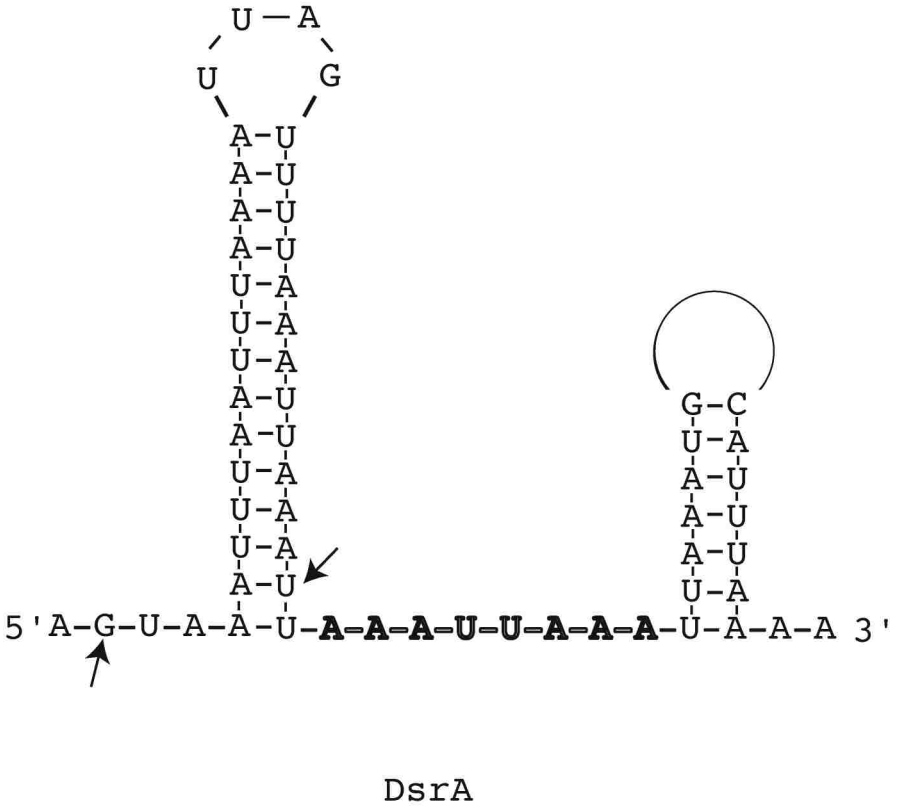

Fig. 5. Predicted secondary structure of DsrA[352]. The arrows indicate the *rpoS* complementary region (31 of 34 nucleotides). The sequence predicted to be the Hfq binding site is in bold. The open circle represents a stem-loop of 32 nucleotides.

Chapter 4

Temperature induced post-transcriptional regulation of RpoS by $DsrA_{Bb}$

(adapted from Lybecker and Samuels, 2007)

Here we demonstrate that $DsrA_{Bb}$ is necessary for the temperature-induced increase in RpoS and OspC at a low cell density, but is not required for the increase in RpoS and OspC in response to a decrease in pH or at a high cell density. In addition, our data indicate that the upstream hypothetical protein BB0577 is required, either directly or indirectly, for DsrA activity.

Generation of *dsrA*$_{Bb}$ mutants

We hypothesized that the complementary sequences of $DsrA_{Bb}$ and the upstream region of *rpoS* mRNA interact and base-pair with each other to regulate RpoS translation. To determine the function of $DsrA_{Bb}$, we deleted 65 nucleotides of the sequence encoding the region of *rpoS* complementarity (Fig. 1A) of *dsrA*$_{Bb}$ on the chromosome (Fig. 6A). The 65 nucleotides were replaced with either a kanamycin resistance (Bono *et al*., 2000) or streptomycin resistance (Frank *et al*., 2003) cassette; note that 27 nucleotides of the hypothetical open reading frame *bb0577* are also deleted (Fig. 6A). The sequence was deleted in low-passage infectious strain B31–A3 (Elias *et al*., 2002) with both markers and in a low-passage infectious strain 297 clone (Hübner *et al*., 2001) with the kanamycin resistance cassette. We confirmed, by PCR, the insertion of the antibiotic resistance cassettes and their orientations in the transformants (Fig. 6B). Northern blot analysis of total RNA, using the same single-stranded probe as described above, demonstrated that none of the multiple $DsrA_{Bb}$ species were present in the *dsrA*$_{Bb}$ mutant strain (Fig. 6C).

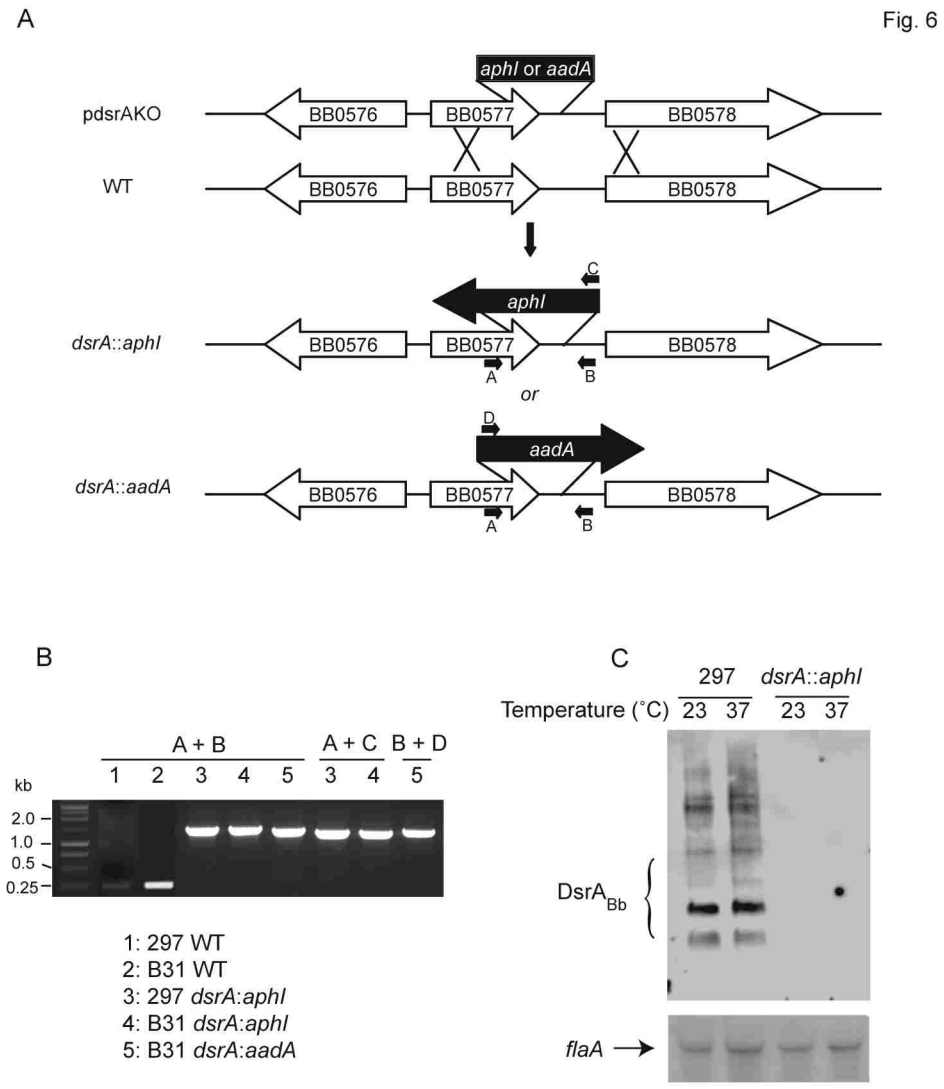

Fig. 6. Mutation of *dsrA*$_{Bb}$. (A) Strategy for deleting the *rpoS* complementary sequence of DsrA$_{Bb}$. Sixty-five nucleotides complementary to the *rpoS* upstream region were replaced with either a kanamycin (*flgB*p-*aphI*) or streptomycin (*flgB*p-*aadA*) resistance cassette (pdsrAKO). Transformation into wild-type (WT) *B. burgdorferi* and homologous recombination generated *dsrA*$_{Bb}$ mutant strains. The X's indicate approximate positions of crossover events. The small arrows denote oligonucleotide primers used for PCR analysis. (B) PCR analysis of wild type (WT) and *dsrA*$_{Bb}$ transformants. The letter combinations designate primer pairs used for the PCR. (C) The *dsrA*$_{Bb}$ mutant strains do not transcribe DsrA$_{Bb}$. Parental 297 and mutant *dsrA*$_{Bb}$ strains were grown at 23°C or temperature shifted to and grown at 37°C. Equal amounts of RNA (15 μg) were separated on a 6% TBE-Urea gel, electroblotted to a nylon membrane, and hybridized with a single-stranded RNA probe for either *dsrA*$_{Bb}$ or *flaA* (top and bottom panels, respectively).

Regulation of RpoS and OspC in the *dsrA*$_{Bb}$ mutant

Temperature, pH, and growth phase are well characterized factors that regulate levels of RpoS and OspC *in vitro*. Therefore, we examined the role of DsrA$_{Bb}$ in the regulation of RpoS and OspC in response to these factors. *B. burgdorferi* low-passage wild-type and *dsrA*$_{Bb}$ mutant strains were temperature shifted to 37°C and grown to low cell density (1-3 × 10^7 cells ml^{-1}). Western blot analyses of whole-cell lysates demonstrate that wild-type B31 and 297 display the characteristic temperature-induced synthesis of RpoS, while the *dsrA*$_{Bb}$ mutants have reduced levels of RpoS (Fig. 7A).

Fig. 7

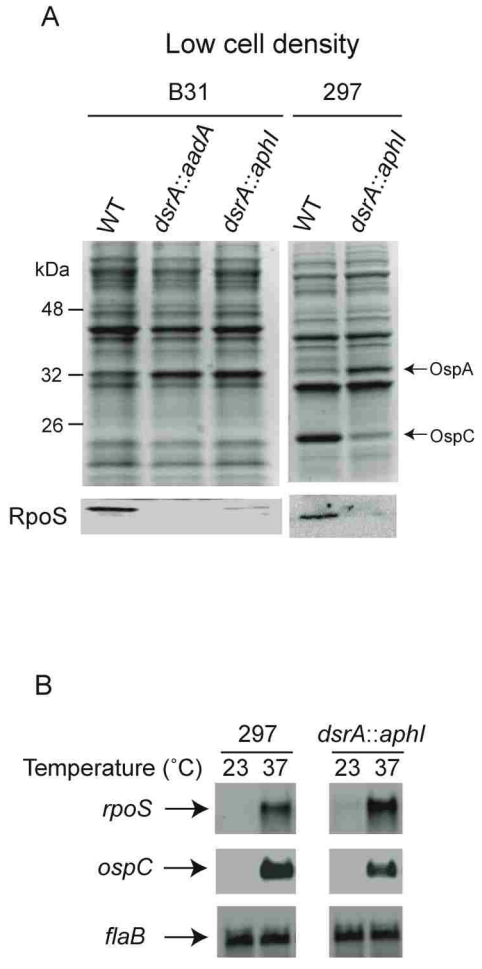

Fig. 7. DsrA$_{Bb}$ regulates RpoS levels posttranscriptionally in response to a temperature shift. Deletion of the DsrA$_{Bb}$ sequence complementary to *rpoS* reduces the levels of RpoS protein after a temperature shift at a low cell density. Immunoblot analyses of whole-cell lysates of B31–A3 wild type, 297 wild type and *dsrA*$_{Bb}$ mutants after a temperature shift to 37°C at low cell density of ~2 × 10^7 cells ml^{-1} (A). The top panel is a Coomassie brilliant blue-stained SDS-PAGE gel and the bottom panel is an immunoblot probed with anti-RpoS antibody. OspC, which is dependent on RpoS, does not stain well in strain B31. Three independent experiments were performed and representative data are shown. Northern blot analyses of total RNA (15 μg) fractionated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized with an *rpoS*, *ospC,* or *flaB* single-stranded RNA probe (B). Two independent experiments were performed and representative data are shown; the signal was quantified on a FujiFilm LAS-3000.

To further understand the mechanism of temperature regulation of RpoS by $DsrA_{Bb}$, we examined the steady-state levels of *rpoS* and *ospC* mRNA in the *dsrA*$_{Bb}$ mutant strain by Northern blot analyses. One class of sRNAs base-pair with target mRNA to affect translation and/or the stability of the mRNA (Majdalani *et al.*, 2005; Repoila *et al.*, 2003). We hypothesized that $DsrA_{Bb}$ acts post-transcriptionally by stimulating translation of *rpoS* mRNA. As shown by Northern blot analysis, the steady-state *rpoS* mRNA level was 1.5-fold higher in the *dsrA*$_{Bb}$ mutant compared to the wild-type strain after a temperature shift to 37°C (Fig. 7B). Therefore, the *dsrA*$_{Bb}$ mutant strain has high levels of *rpoS* transcript (Fig. 7B), but low levels of RpoS protein (Fig. 7A) suggesting that the temperature-induced regulation of RpoS by $DsrA_{Bb}$ is post-transcriptional. In addition, the Northern blot data indicate that $DsrA_{Bb}$ does not stabilize the *rpoS* transcript.

We hypothesized that the reduced level of RpoS protein in the *dsrA*$_{Bb}$ mutant strain (Fig. 7A) should be reflected by a decreased level of *ospC* mRNA in the mutant because *ospC* transcription is dependent on RpoS. The *ospC* steady-state mRNA level was two-fold lower in the *dsrA*$_{Bb}$ mutant strain compared to the wild-type strain (Fig. 7B). These data suggest that the *rpoS* mRNA in the *dsrA*$_{Bb}$ mutant strain is either not translated efficiently or the RpoS protein is degraded at an increased rate.

The predominate species of the *rpoS* transcript at high cell density does not contain the sequence complementary to $DsrA_{Bb}$ (Fig. 1C and D) and, therefore, we hypothesized that $DsrA_{Bb}$ would not regulate RpoS in response to this environmental stimulus. *B. burgdorferi* low-passage wild-type and *dsrA*$_{Bb}$ mutant strains were temperature shifted to 37°C and grown to a high cell density (1×10^8 cells ml^{-1}). As

shown by Western blot analysis, RpoS levels are similar in both the wild-type and mutant strains at a high cell density (Fig. 8A).

Fig. 8

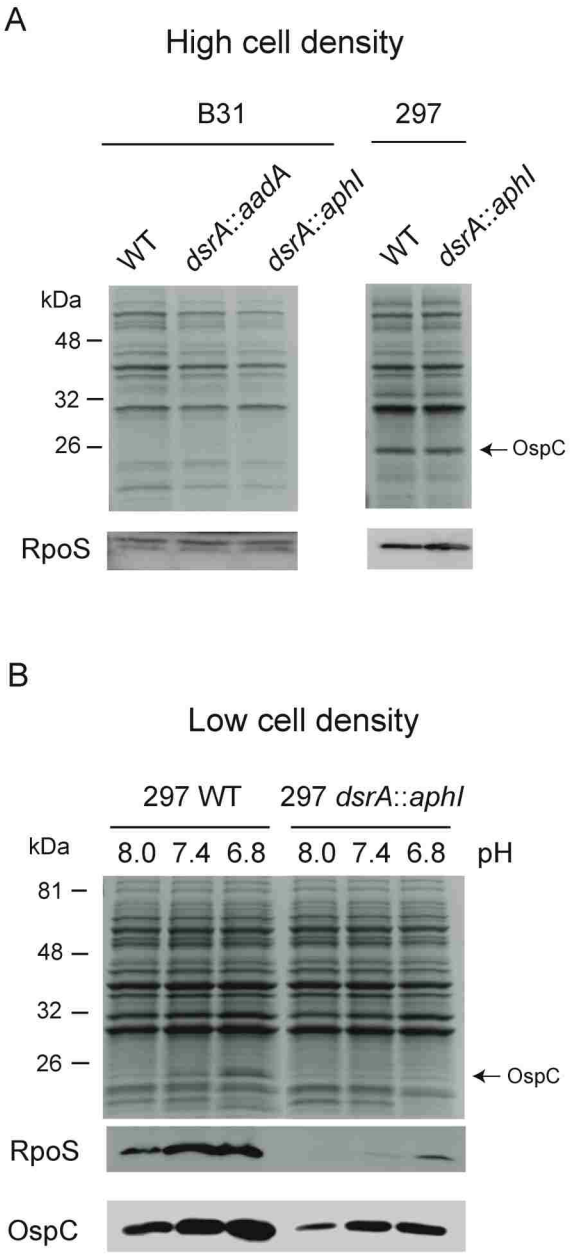

Fig. 8. $DsrA_{Bb}$ does not affect the regulation of RpoS and OspC in response to changes in cell density or pH at 37°C. Immunoblot analyses of whole-cell lysates of B31-A3 wild type, 297 wild type and $dsrA_{Bb}$ mutants temperature shifted to 37°C and grown to a high cell density of ~1×10^8 cells ml^{-1} (A). Immunoblot analysis of wild-type (WT) 297 and the $dsrA_{Bb}$ mutant temperature shifted to 37°C and grown at pH 8.0, 7.4 or 6.8 to a low cell density of ~2×10^7 cells ml^{-1} (B). Whole-cell lysates were fractionated by SDS-PAGE and stained with Coomassie brilliant blue (top panels) or electroblotted to a PVDF membrane and probed with anti-OspC or anti-RpoS antibody (bottom panels). Three independent experiments were performed and representative data are shown.

Furthermore, we observed that the pH-dependent regulation of RpoS and OspC are not affected in the *dsrA*$_{Bb}$ mutant. A decrease in pH, corresponding to the change in pH of the tick midgut after a blood meal, results in an increase in RpoS and OspC (Carroll *et al.*, 1999; Yang *et al.*, 2000). However, regulation of RpoS and OspC by pH was observed in the *dsrA*$_{Bb}$ mutant strain, although the overall level of each remained reduced in the mutant strain (Fig. 8B). Therefore, regulation of RpoS and OspC at a high cell density and changes in pH are not dependent on DsrA$_{Bb}$.

Complementation of *dsrA*$_{Bb}$

To establish that the loss of RpoS and OspC synthesis was the result of the *dsrA*$_{Bb}$ deletion, we *trans*-complemented the mutant with a wild-type copy of *dsrA*$_{Bb}$. However, complementing *dsrA*$_{Bb}$ was complicated because the precise location of the *dsrA*$_{Bb}$ promoter was neither known nor readily identifiable and the *dsrA*$_{Bb}$ gene overlaps in sequence with the upstream hypothetical open reading frame *bb0577* (Fig. 1B). Therefore, either the promoter and transcriptional start site of DsrA$_{Bb}$ are within the *bb0577* gene, somewhat similar to the *gac* promoter within the *gyrA* gene (Knight and Samuels, 1999), or DsrA$_{Bb}$ is the product of RNA processing of a transcript including both *bb0577* and DsrA$_{Bb}$. Five different shuttle vectors derived from pKFSS1 (Frank *et al.*, 2003) were constructed to complement the *dsrA*$_{Bb}$ mutant strains and localize the region containing the *dsrA*$_{Bb}$ promoter (Table 2 and Fig. 9A).

Table 2. Plasmids used for *dsrA*$_{Bb}$ mutant complementation.

Plasmid name	Insert
pKFSS1	None
pKFSC	*dsrA*$_{Bb}$ and part of the BB0577 ORF
pKFMC	*dsrA*$_{Bb}$ and the entire BB0577 ORF
pKFLC	*dsrA*$_{Bb}$, the putative BB0577 promoter, and the entire BB0577 ORF
pKFG	The putative BB0577 promoter and the entire BB0577 ORF
pKFLCS	Identical to pKFLC, but with a stop codon in the BB0577 ORF

The largest species of DsrA$_{Bb}$ is 352 nucleotides and this sequence encompasses the three other species (Fig. 3C), therefore this species was used as the reference for DsrA$_{Bb}$ in the complementation assay. pKFSC carries *dsrA*$_{Bb}$ and the 150 nucleotides upstream (within the *bb0577* ORF); pKFMC carries *dsrA*$_{Bb}$ and the entire *bb0577* ORF; pKFLC carries *dsrA*$_{Bb}$, the *bb0577* ORF, and the *bb0577* putative promoter (82 nucleotides upstream of the start codon); pKFG carries the *BB0577* putative promoter and ORF, and the first 114 nucleotides of *dsrA*$_{Bb}$; and pKFLCS is identical to pKFLC except that it has an engineered early stop codon in the *bb0577* ORF (Fig. 9A). All five plasmids and the parent vector pKFSS1 were transformed into the 297 *dsrA*$_{Bb}$::*aphI* mutant strain. Transformants were screened by PCR and xenodiagnosis (data not shown).

We hypothesized that 1) the promoter of *dsrA*$_{Bb}$ is within the 150 nucleotides upstream of DsrA$_{Bb}^{352}$; 2) the reduced levels of RpoS and OspC in the *dsrA*$_{Bb}$ mutant strain is a consequence of the deletion of DsrA$_{Bb}$, but not due to the partial deletion of BB0577 and 3) the putative promoter of *bb0577* and the BB0577 protein would not be

required for transcription or activity of $DsrA_{Bb}$. Therefore, we expected that the *dsrA*$_{Bb}$ mutant strains carrying all the constructs except pKFG and the control pKFSS1 would express $DsrA_{Bb}$ and complement the mutant phenotype thus restoring the wild-type temperature-induced synthesis of RpoS and OspC. Northern blot analysis demonstrates that $DsrA_{Bb}$ is transcribed in all the constructs except pKFG and the pKFSS1 control (Fig. 9B), suggesting that the *dsrA*$_{Bb}$ promoter is within 150 base pairs of the gene. However, Western blot analyses of whole-cell lysates from these strains demonstrate that RpoS and OspC were induced after a temperature shift only in the strains containing either pKFMC and pKFLC, but not the other constructs or the pKFSS1 control (Fig. 9C). These data suggest that BB0577 is necessary, but not sufficient, for complementation (pKFLCS and pKFG do not synthesize RpoS and OspC). Surprisingly, the strain carrying pKFMC, which lacks the sequence upstream of the *bb0577* ORF and was presumed to not express *bb0577*, did induce RpoS and OspC. Therefore, we postulate that pKFMC expresses *bb0577* either from an intrinsic vector promoter or that the *bb0577* promoter is within the annotated ORF. Northern blot analysis demonstrated that *bb0577* is transcribed in the strain carrying pKFMC (Fig. 9D). Taken together, these data suggest that both $DsrA_{Bb}$ and BB0577 are required for the temperature-induced regulation of RpoS and OspC.

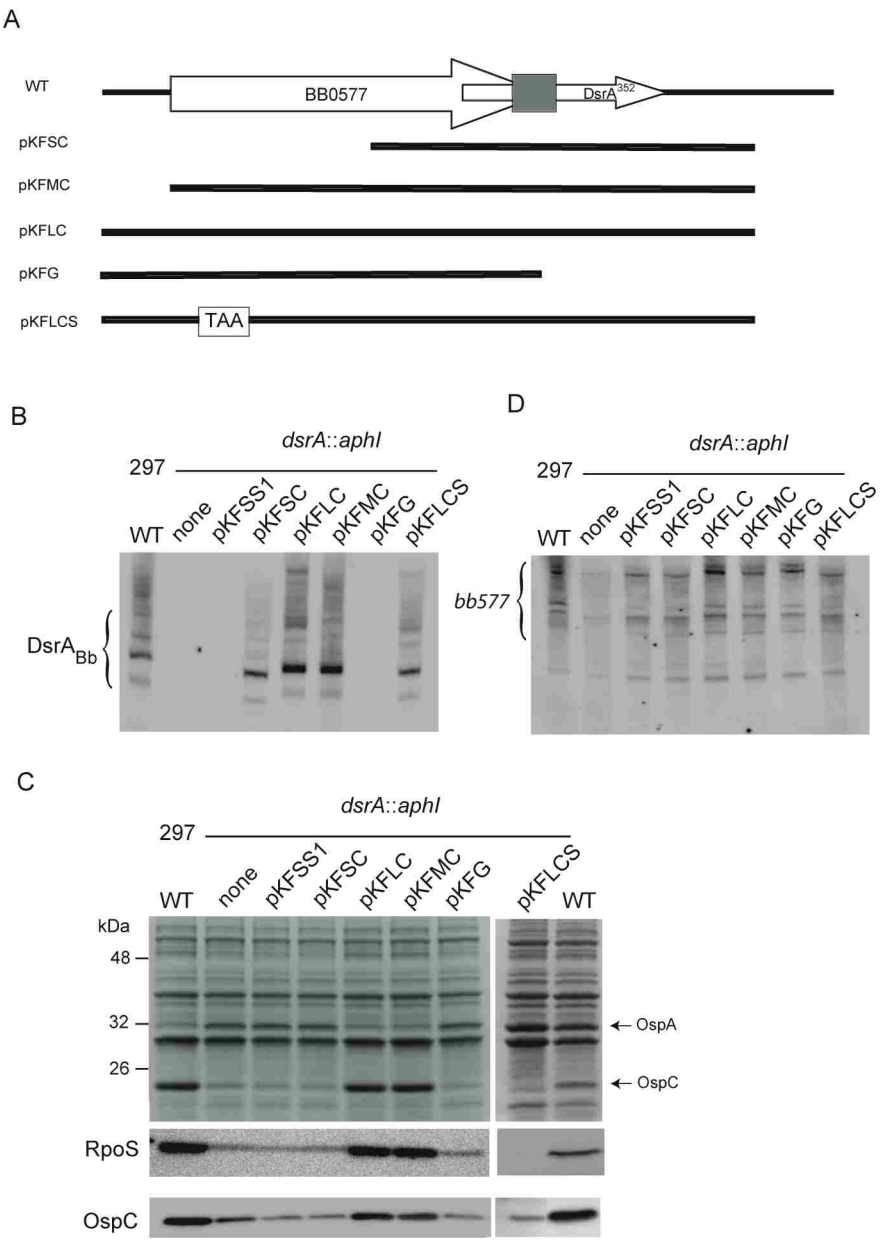

Fig. 9. Complementation of $dsrA_{Bb}$ restores the wild-type phenotype. (A) Schematic of a series of shuttle vector inserts used for $dsrA_{Bb}$ complementation. All inserts were cloned into the shuttle vector pKFSS1. The gray box denotes the 65 nucleotides of the *rpoS* complementary sequence removed in the $dsrA_{Bb}$ mutant strains. (B) $DsrA_{Bb}$ Northern blot of wild-type (WT) 297 and $dsrA_{Bb}$ mutants transformed with the various complementation vectors. Equal amounts of total RNA (15 μg), confirmed by ethidium bromide staining (data not shown), were fractionated on a 6% TBE-Urea gel, electroblotted to a nylon membrane and hybridized with a $dsrA_{Bb}$ single-stranded RNA probe. All cultures were temperature shifted to 37°C and grown to a low cell density of ~2×10^7 cells ml^{-1}. (C) Coomassie brilliant blue-stained SDS-PAGE gel (top panel) and immunoblots (bottom two panels) of whole-cell lysates of wild-type (WT) 297 and $dsrA_{Bb}$ mutants transformed with the various complementation vectors. All cultures were temperature shifted to 37°C and grown to a low cell density of ~2×10^7 cells ml^{-1}. (D) *bb0577* Northern blot of wild-type (WT) 297 and $dsrA_{Bb}$ mutants transformed with the various complementation vectors. Equal amounts of total RNA (15 μg), confirmed by ethidium bromide staining (data not shown), were fractionated on a 6% TBE-Urea gel, electroblotted to a nylon membrane and hybridized with a *bb0577* single-stranded RNA probe. All cultures were temperature shifted to 37°C and grown to a low cell density of ~2×10^7 cells ml^{-1}.

The $dsrA_{Bb}^{-}$ phenotype is suppressed in high-passage strains

Serial passage of the *B. burgdorferi* $dsrA_{Bb}$ mutant strains results in a reversal of the phenotype from the low-passage $dsrA_{Bb}$ mutant strains. *B. burgdorferi* cultures were serially passaged at 23°C and then temperature shifted to 37°C and grown to a low cell density. Western blot analyses demonstrate that both RpoS and OspC syntheses are increased in the mutant compared to the wild type after serial passage, as early as passage six (Fig. 10).

Fig. 10

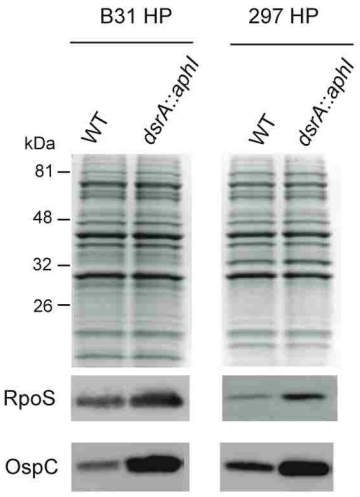

Fig. 10. RpoS and OspC synthesis is increased in the $dsrA_{Bb}$ mutant strains compared to the wild type in high-passage strains. Coomassie brilliant blue-stained SDS-PAGE gel (top panel) and immunoblots (bottom panels) of whole-cell lysates of high-passage (HP) *B. burgdorferi* strains B31–A3 (left panels) and 297 (right panels) wild type (WT) and $dsrA_{Bb}$ mutants. Cultures were temperature shifted to 37°C and grown to a low cell density of ~2×10^7 cells ml^{-1}.

We initially hypothesized that the high-passage $dsrA_{Bb}$ mutant strains gained a suppressor mutation in the upstream region of the *rpoS* mRNA, which would result in a structural change that would allow efficient translation of RpoS without $DsrA_{Bb}$. However, no mutations were found in the upstream region of *rpoS* in ten high-passage colonies. We then hypothesized that the long, presumably $DsrA_{Bb}$-dependent, *rpoS* transcript (-171) was not present in the high-passage $dsrA_{Bb}$ mutant strains, resulting in *rpoS* translation independent of $DsrA_{Bb}$. 5′ RACE identified only the short transcript of *rpoS* (-50) in a high-passage $dsrA_{Bb}$ mutant strain after a temperature shift at low cell density. The

absence of the long *rpoS* transcript (-171) in this *dsrA*$_{Bb}$ mutant strain may be the cause of the reversal in phenotype, but the mechanism for the change in the *rpoS* transcription start site is unknown.

Chapter 5

DsrA and the upstream region of *rpoS* interact *in vitro*

Here in we demonstrate that $DsrA_{Bb}$ and the *rpoS* mRNA upstream region interact *in vitro*. In addition, we present our efforts to identify and characterize the primary sequence and secondary structural elements required for the *in vitro* interaction. Furthermore, we have identified a potential non-canonical Hfq analog in *B. burgdorferi*, which we hypothesize is required for $DsrA_{Bb}$ activity *in vivo*, via the $DsrA_{Bb}$::*rpoS* interaction.

$DsrA_{Bb}$ and *rpoS* interact *in vitro*

We hypothesized that $DsrA_{Bb}$ stimulates RpoS translation via a direct interaction with the upstream region of the *rpoS* mRNA. Our model is that the *rpoS* mRNA is essentially maintained in an inactive form via folding into a structure that sequesters the Shine-Dalgarno and start codon in stem-loops, rendering them inaccessible to the ribosome. We hypothesized that temperature directly affects the structure of $DsrA_{Bb}$: at 23°C $DsrA_{Bb}$ is folded into a structure that is unable to interact with the *rpoS* upstream region, while at 37°C the structure changes so that $DsrA_{Bb}$ and *rpoS* mRNA interact. As a result, the Shine-Dalgarno region and start site of *rpoS* transcript are freed from their secondary structures and are accessible to the ribosome (Fig. 11).

Fig. 11

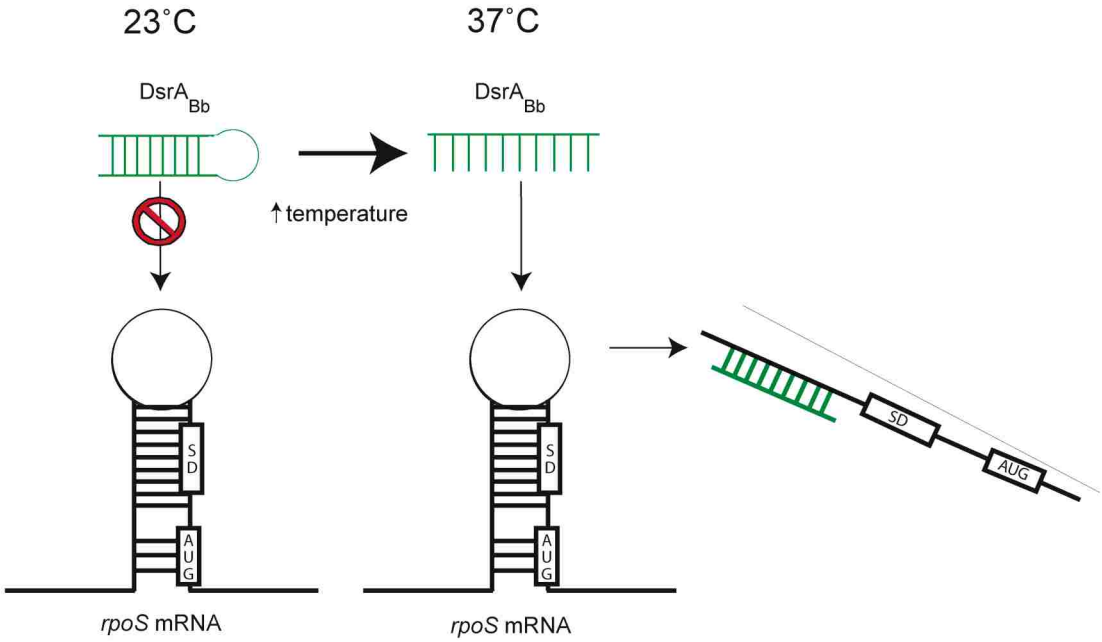

Fig. 11. Model for $DsrA_{Bb}$ thermoregulation of *rpoS* translation. We hypothesize that the *rpoS* mRNA upstream region folds into a stable stem-loop structure that sequesters the Shine-Dalgarno region (SD), rendering it inaccessible to the ribosome. We propose that the secondary structure of $DsrA_{Bb}$ at 23°C does not allow for base-pairing with the *rpoS* transcript, but after a temperature shift, the secondary structure changes to allow for the two RNAs to bind. The binding of $DsrA_{Bb}$ to the *rpoS* upstream region alters the structure of the transcript, the SD becomes available, and translation can be initiated.

Our model of $DsrA_{Bb}$ regulation predicts that the two RNAs specifically interact with each other via complementary sequences (an antisense mechanism) and that the interaction is dependent on temperature. To determine if the RNAs interact in a temperature-dependent manner, we performed electrophoretic mobility shift assays (EMSAs) with *in vitro* transcribed RNAs. All four species of DsrA were generated and assayed for binding to *rpoS* under several different conditions.

We hypothesized that $DsrA_{Bb}$ and *rpoS* RNA, transcribed *in vitro*, would interact at 37°C but not at 23°C. The RNAs were suspended separately in 1X dimer buffer (50

mM Tris-HCl pH 7.5, 300 mM KCl, 5 mM $MgCl_2$), heated to 65°C for 10 min and slow-cooled to 23°C to allow for proper folding of the RNAs. The two RNAs were then mixed together and either incubated at 23°C or shifted to 37°C for 40 min. As a positive control, the two RNAs were mixed together before the initial 65°C and subjected to the same protocol. The reactions were resolved on a 0.8% agarose gel and stained with ethidium bromide. Initially, we chose to assay the interaction under permissive conditions, which included running the gel at 4°C in the presence of $MgCl_2$ and the absence of EDTA. All four species of DsrA (213, 275, 290, and 352 nt.) interact with *rpoS* and appear to do so in a temperature-dependent manner under permissive conditions (Fig. 12).

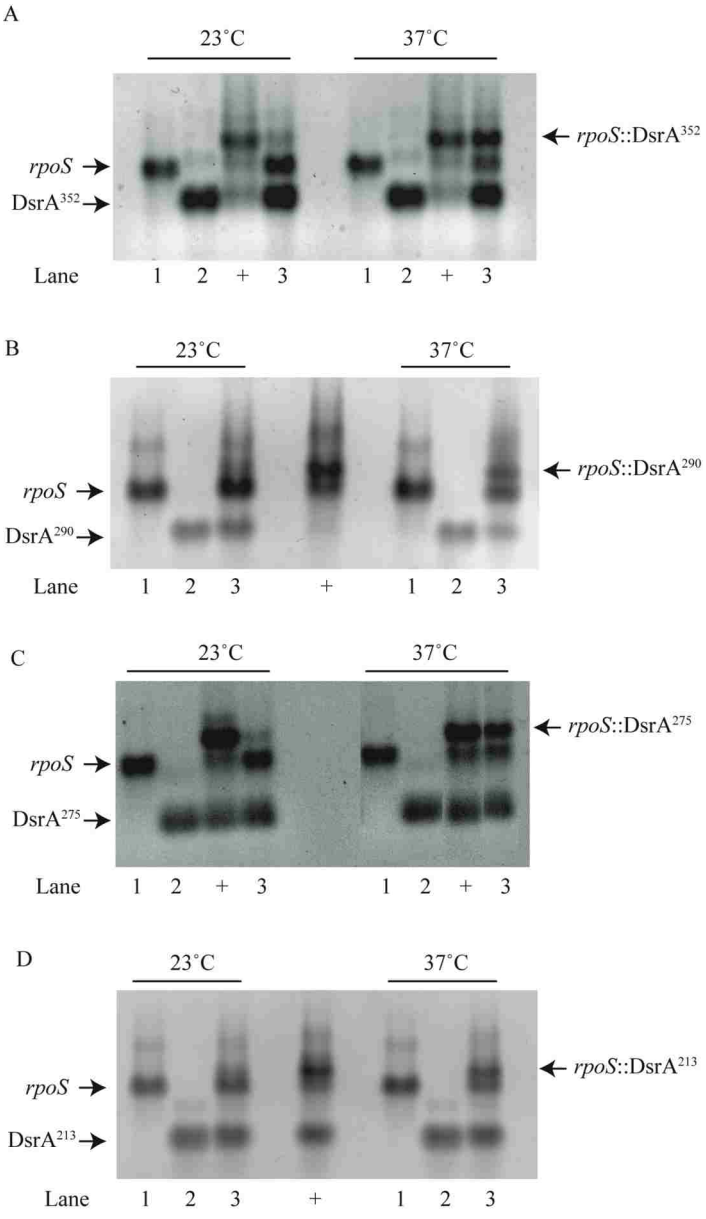

Fig. 12. $DsrA_{Bb}$ binds to the upstream region of the *rpoS* mRNA under permissive conditions. RpoS RNA and the four different species of DsrA (352, 290, 275, and 213 nt. shown in panels A, B, C, and D, respectively) were generated by *in vitro* transcription, incubated separately at 65°C for 10 min and then slow-cooled to 23°C to allow for proper folding. The *rpoS* RNA and DsrA RNA were mixed together at 23°C and incubated at 23°C or 37°C for 40 min (lanes 3). The RNAs were fractionated at 4°C on a 0.8% agarose gel in 0.5X TB with 0.1 mM $MgCl_2$ and stained with ethidium bromide. *rpoS* RNA and DsrA RNA were each incubated separately to visualize any self-dimerization (lanes 1 and 2, respectively). As a positive control, the two RNAs were mixed together before the initial incubation at 65°C (lanes +).

By contrast, under restrictive conditions (the absence of $MgCl_2$ and presence of EDTA in the running buffer) $DsrA^{213}$ does not interact with *rpoS* at 23°C or 37°C, while the other three species still bind to *rpoS* in a temperature dependent manner (Fig. 13). These data suggest that the *rpoS* RNA and $DsrA_{Bb}$ RNA interact extensively at 37°C, but the interaction is minimal at 23°C.

Fig. 13

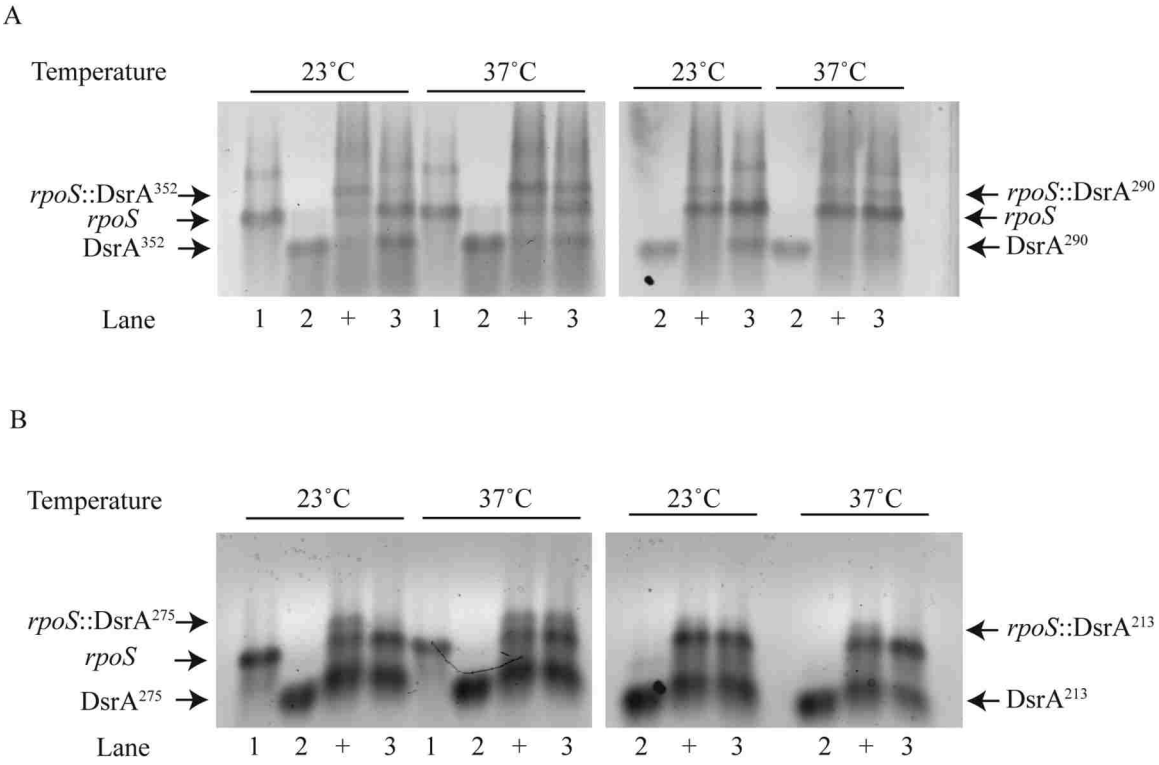

Fig. 13. The three largest species of $DsrA_{Bb}$ bind to the upstream region of the *rpoS* mRNA under restrictive conditions. RpoS RNA and the four different species of DsrA (352, 290, 275, and 213 nt. shown in panels A, B, C, and D, respectively) were generated by *in vitro* transcription, incubated separately at 65°C for 10 min and then slow-cooled to 23°C to allow for proper folding. The *rpoS* RNA and DsrA RNA were mixed together at 23°C and incubated at 23°C or 37°C for 40 min (lanes 3). The RNAs were fractionated at 4°C on a 0.8% agarose gel in 0.5X TBE and stained with ethidium bromide. *rpoS* RNA and DsrA RNA were each incubated separately to visualize any self-dimerization (lanes 1 and 2, respectively). As a positive control, the two RNAs were mixed together before the initial incubation at 65°C (lanes +).

We hypothesized that the structures of $DsrA_{Bb}$ and *rpoS* at 23°C do not allow for the two RNAs to interact; however, the effect of temperature on the interaction may be entirely or partially due to the effect of temperature on the rate of the interaction. We initially hypothesized that at 23°C the two RNAs would not bind regardless of the incubation time. To test this hypothesis we incubated the RNAs together at 23°C for 24 h instead of 40 min. Under permissive conditions $DsrA^{352}$ and *rpoS* interact extensively after 24 h at 23°C (Fig. 14A). A time course assay was then performed to further elucidate the effect of time on the *rpoS*::DsrA interaction at both 23°C and 37°C. The DsrA::*rpoS* interaction is essentially complete by 10 min at 37°C (Fig 14B). However, the *rpoS*::DsrA interaction is still minimal and a large amount of free *rpoS* and $DsrA^{352}$ remain at 23°C after 4 h (Fig 14C).

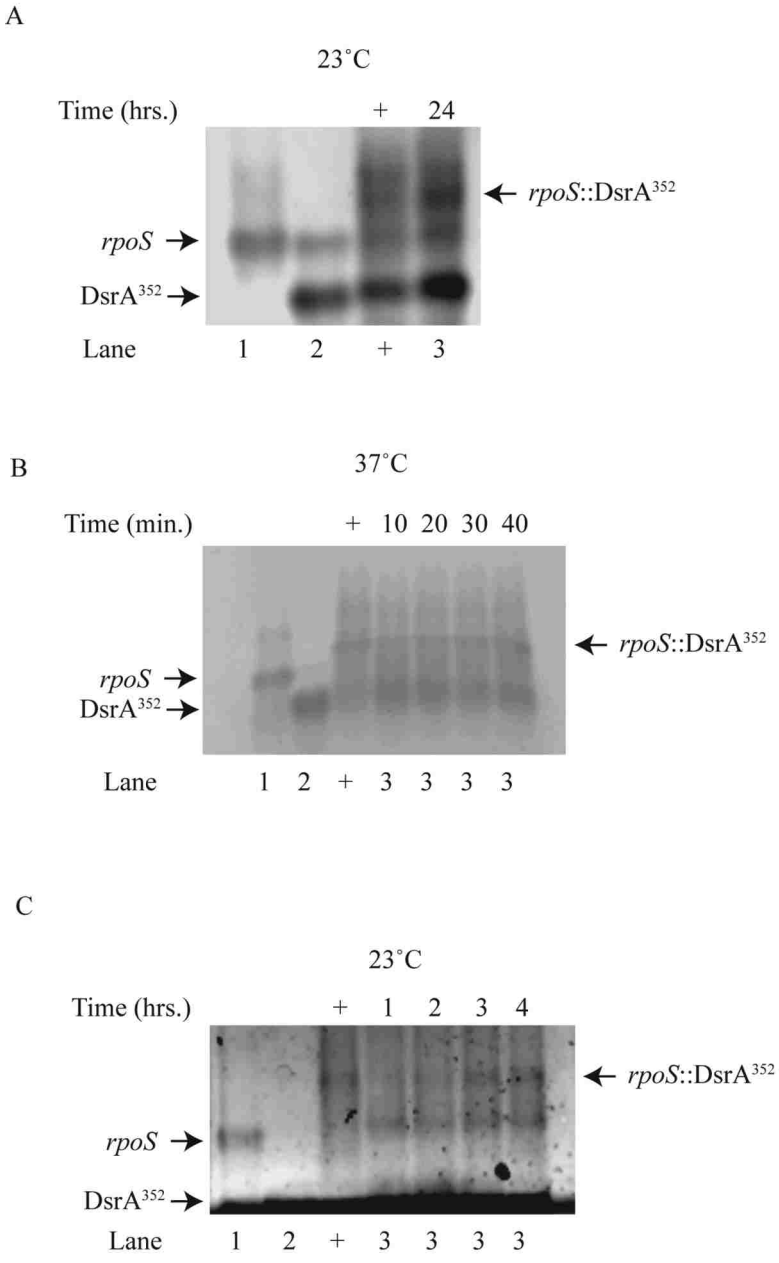

Fig. 14. Kinetics of *rpoS*::DsrA complex formation at 23°C and 37°C. *rpoS* RNA and DsrA^{352} RNA were generated by *in vitro* transcription, incubated separately at 65°C for 10 min and then slow cooled to 23°C to allow for proper folding. The *rpoS* RNA and DsrA RNA were mixed together at 23°C and incubated at 23°C (A and C) or 37°C (B) for the indicated times (lanes 3). The RNAs were fractionated at 4°C on a 0.8% agarose gel in 0.5X TB with 0.1 mM $MgCl_2$ and stained with ethidium bromide. *rpoS* RNA and DsrA RNA were each incubated separately to visualize any self-dimerization (lanes 1 and 2,

We tried several different protocols in an attempt to mitigate the effect of temperature on the rate of the complex formation. First, we slow-cooled the RNAs separately to either 37°C or 23°C to allow for proper folding. The RNAs were then mixed together and incubated at 23°C for 40 min. We hypothesized that the RNAs folded at 37°C would interact more extensively than the RNAs folded at 23°C. The reactions were fractionated under permissive conditions at 4°C. The DsrA::*rpoS* interaction is approximately the same regardless of the temperature of RNA folding (Fig 15A). We hypothesized that the structure of the RNA at 37°C may be stable during the slow cooling and a further decrease in the temperature to 23°C may not change the structure. Consequently, the structure at 23°C after slow cooling may not be representative of the native structure at 23°C. Therefore, in an effort to test this hypothesis, we snap-cooled the RNAs instead of slow cooling. The RNAs were incubated separately at 94°C for 5 min, directly placed on ice for 2 min and then mixed together and allowed to fold at 23°C or 37°C for two h (the amount of time required to slow cool). The RNAs were then mixed together and allowed to bind at 23°C to diminish the effect of temperature on the rate of the reaction. The reactions were performed under permissive conditions and the gel was run at 4°C. Under these conditions the amount of DsrA::*rpoS* complex was similar despite the different folding temperatures (Fig 15B). Taken together our data suggest that the temperature-dependent differences in DsrA::*rpoS* duplex formation *in vitro* may be entirely or partially due to the kinetic properties at the different temperatures.

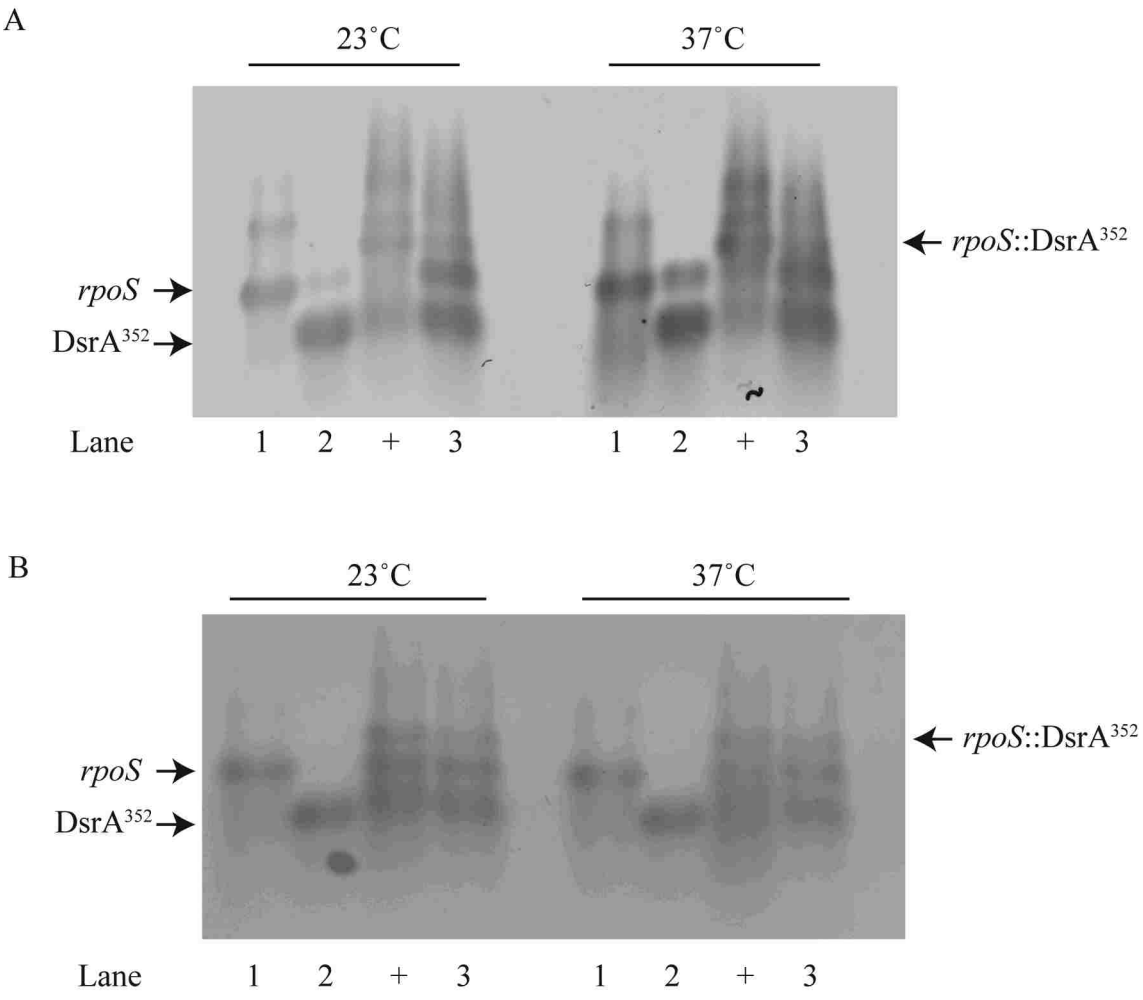

Fig. 15. *rpoS*::DsrA complex formation at 23°C. *rpoS* RNA and DsrA^{352} RNA were generated by *in vitro* transcription, incubated separately at 65°C for 10 min and then slow-cooled to 23°C or 37°C (A) or incubated separately at 95°C for 5 min, snap-cooled on ice for 2 min and then moved to 23°C or 37°C (B) to allow for proper folding. The *rpoS* RNA and DsrA RNA were mixed together and incubated at 23°C for 40 min (lanes 3). The RNAs were fractionated at 4°C on a 0.8% agarose gel in 0.5X TB with 0.1 mM $MgCl_2$ and stained with ethidium bromide. *rpoS* RNA and DsrA RNA were incubated separately to visualize any self-dimerization (lanes 1 and 2, respectively). As a positive control, the two RNAs were mixed together before the initial incubation at 65°C (lanes +).

In vitro characterization of the DsrA::*rpoS* interaction

Herein we endeavored to dissect the primary sequence and secondary structural elements required for the DsrA::*rpoS* interaction. Complimentary RNA/RNA interactions are frequently initiated by two hairpin loops forming "kissing complexes" (Brunel *et al.*, 2002). Also intrinsic to the function of anti-sense sRNAs is their ability to base pair with their target mRNA. MFOLD models of the secondary structures of *rpoS* and DsrA (Figs. 4 and 5) demonstrate that the regions of complementarity form stable stem-loop structures with a single-stranded four base-pair loop. We hypothesized that i) the primary complementary sequence, ii) the single-stranded four base stem loop, and iii) the overall secondary structure would be necessary for the DsrA::*rpoS* interaction. To test these hypotheses and elucidate the elements required for *rpoS*::DsrA complex formation, we chose to mutate various regions of the stem-loop in DsrA, generate the compensatory mutation on *rpoS,* and assay for binding with EMSAs. We constructed three different pairs of mutants based on our hypotheses and used MFOLD to confirm the predicted secondary structures of these mutants (Fig. 16).

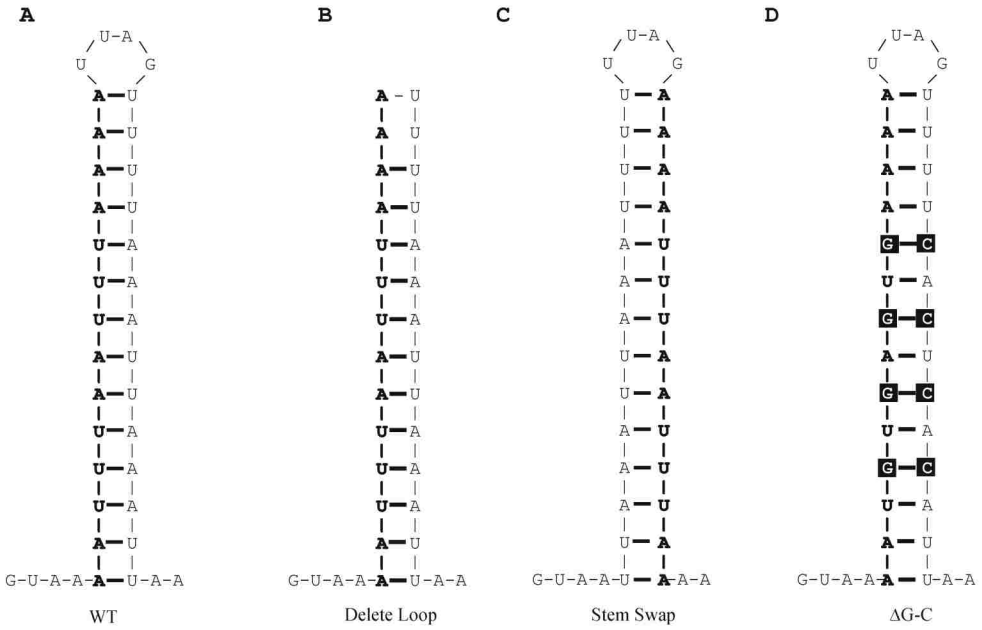

Fig. 16. Predicted secondary structures (MFOLD) of the site-directed mutations of the $DsrA_{Bb}$ stem-loop. (A) Wild-type sequence, (B) loop deletion (dloop), (C) stem swap (St/Sw), and (D) A:U to G:C (dGC) mutagenesis.

First, we deleted the four bases of the hairpin loop to determine if it was required for the initial recognition step between the two RNAs (Fig. 16B). Three different EMSA conditions were used to assay for binding, but the DsrA::*rpoS* complex was visualized regardless of the reaction stringency. Briefly, the samples were heated to 65°C, slow-cooled to 23°C, mixed together and then incubated at 37°C for 40 min. Initially, the reactions were fractionated at 4°C under permissive conditions (TB + $MgCl_2$ in running buffer); under these conditions there was no noticeable effect of the mutant RNAs on the complex formation (Fig 17A). Next, we fractionated the reactions under restrictive conditions (0.5X TBE as running buffer) at 4°C. Again, there was no discernable difference in the complex formation with mutant RNAs (Fig. 17B). Next, we

fractionated the reactions under even more stringent conditions at 23°C with TBE as the running buffer; under these conditions, none of the RNAs, including wild-type RNAs, formed complexes (data not shown). Finally, we fractionated the reactions at 23°C under permissive conditions. Still, the deletion of the stem loop showed no effect on the formation of the DsrA::*rpoS* complex (Fig. 17C).

Fig. 17

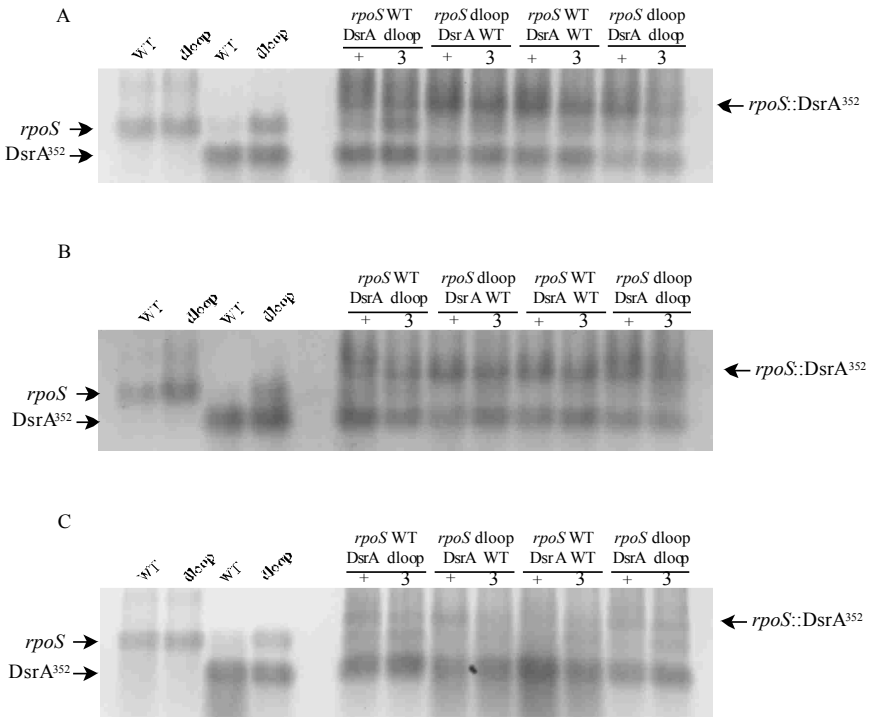

Fig. 17. Deletion of the hairpin loop does not eliminate *rpoS*::DsrA complex formation. Wild-type (WT) and dloop versions of the *rpoS* RNA and DsrA^{352} WT were generated by *in vitro* transcription, incubated separately at 65°C for 10 min and then slow-cooled to 23°C to allow for proper folding. The *rpoS* RNAs and DsrA RNAs were mixed together at 23°C and incubated at 37°C for 40 min (lanes 3). The RNAs were fractionated at 4°C on a 0.8% agarose gel in 0.5X TB with 0.1 mM $MgCl_2$ (A), 0.5X TBE (B) or at 23°C in 0.5X TB with 0.1 mM $MgCl_2$ (C) and stained with ethidium bromide. WT and dloop *rpoS* and DsrA RNAs were each incubated separately to visualize any self-dimerization. As a positive control, the two RNAs were mixed together before the initial incubation at 65°C (lanes +).

To assay the requirement of the complementary base-pairing between *rpoS* and DsrA in complex formation, we swapped the two sides of the stem-loop, thus eliminating the complementary base-pairing to *rpoS*, but maintaining the secondary structure of the stem-loop (Fig. 16C). Notably, only the 31 of 34 nucleotides of complementarity were abolished in this mutation; the less extensive base-pairing of the flanking regions remained intact. The EMSAs were performed using the three conditions as described above for the loop deletion mutant. There was no detectable difference in complex formation between the mutant stem/swap RNAs and wild-type RNAs at any of the tested conditions (Fig 18).

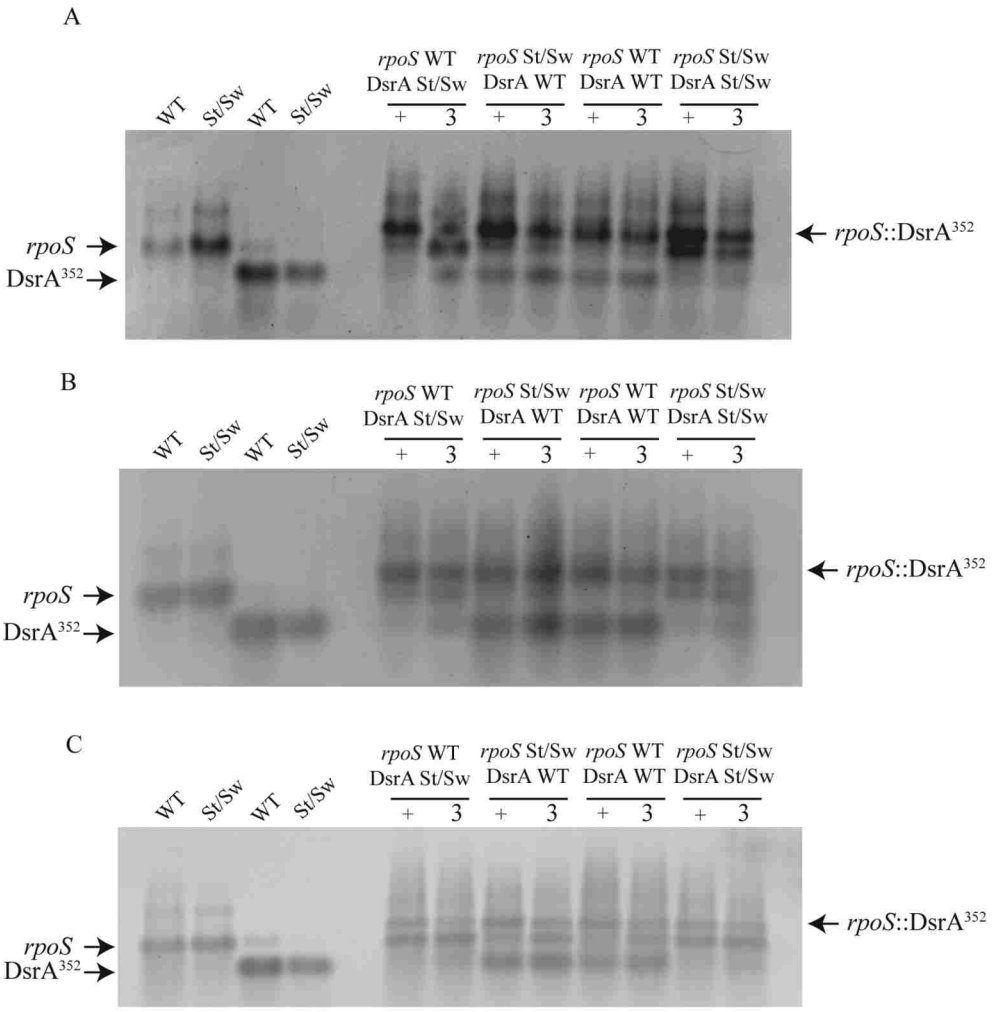

Fig. 18. Swapping the stem loop does not eliminate *rpoS*::DsrA complex formation. Wild-type (WT) and St/Sw versions of the *rpoS* RNA and $DsrA^{352}$ WT were generated by *in vitro* transcription, incubated separately at 65°C for 10 min and then slow-cooled to 23°C to allow for proper folding. The *rpoS* RNAs and DsrA RNAs were mixed together at 23°C and incubated at 37°C for 40 min (lanes 3). The RNAs were fractionated at 4°C on a 0.8% agarose gel in 0.5X TB with 0.1 mM $MgCl_2$ (A), 0.5X TBE (B) or at 23°C in 0.5X TB with 0.1 mM $MgCl_2$ (C) and stained with ethidium bromide. WT and St/Sw *rpoS* and DsrA RNAs were each incubated separately to visualize any self-dimerization. As a positive control, the two RNAs were mixed together before the initial incubation at 65°C (lanes +).

Finally, we hypothesized that the strength of the base-pairing within the stem of the stem-loop contributes to the stability of the structure, which would affect the temperature-dependence of the binding. Therefore, we mutated four A:U base pairs to four G:C base-pairs within the stem (Fig. 16D), which we predicted would strengthen the stability of the stem-loop. We reasoned that this may "recalibrate the DsrA thermometer" specifically we hypothesized that increased stability of the stem-loop would require higher temperatures to melt the stem. The binding reactions were fractionated under permissive and restrictive conditions at 4°C and demonstrated that a DsrA::*rpoS* complex formed regardless of the mutations (Fig. 19). However, the WT::mutant and mutant::mutant complexes fractionated differently than the WT::WT complexes, suggesting different secondary structures of DsrA (dGC), *rpoS* (dGC), and *rpoS*::DsrA heterocomplexes compared to the wild-type RNAs and the wild-type homocomplex.

Taken together, our data suggest that the *rpoS*::DsrA interaction *in vitro* does not depend entirely on any of the primary sequences or secondary structures that we tested under these conditions.

Fig. 19

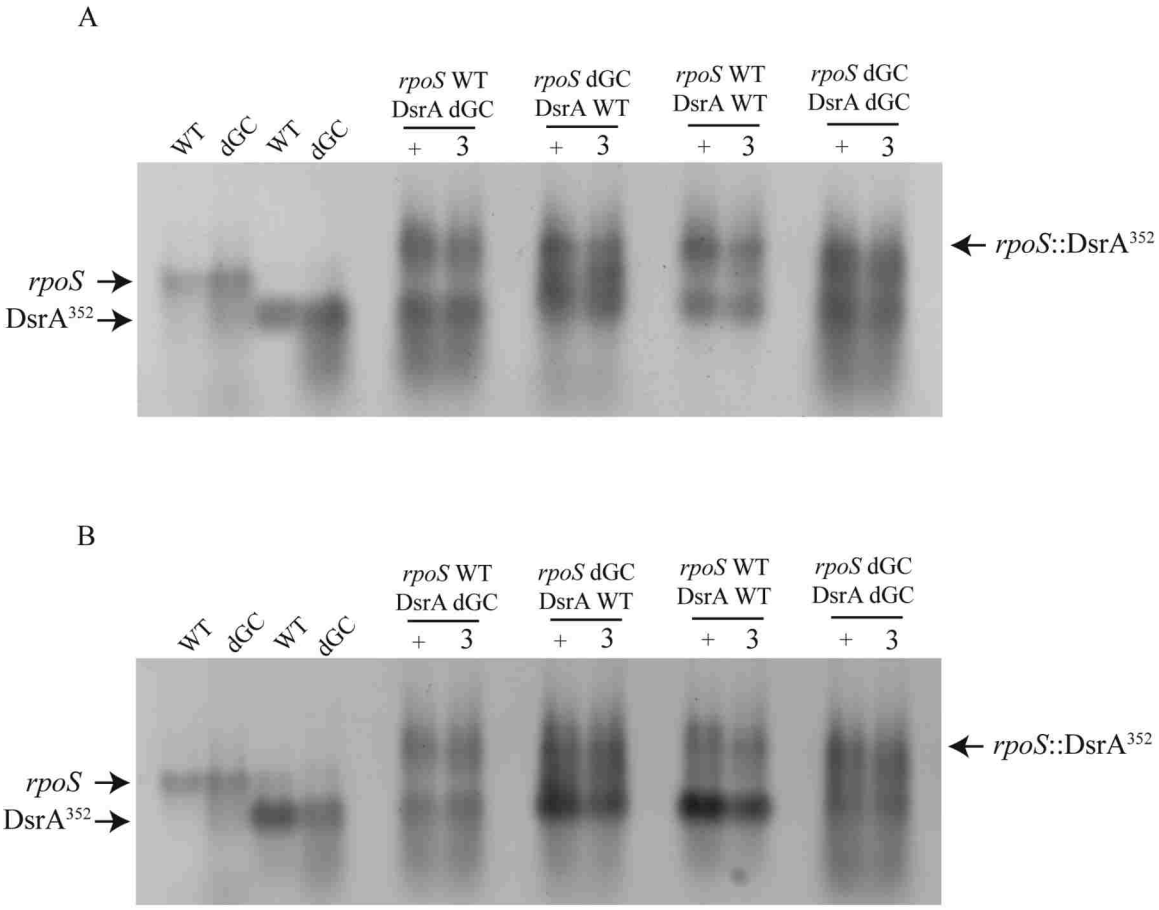

Fig. 19. Stabilizing the stem-loop with G:C base-pairs does not affect *rpoS*::DsrA complex formation. Wild-type (WT) and dGC versions of the *rpoS* RNA and DsrA^{352} WT were generated by *in vitro* transcription, incubated separately at 65°C for 10 min and then slow-cooled to 23°C to allow for proper folding. The *rpoS* RNAs and DsrA RNAs were mixed together at 23°C and incubated at 37°C for 40 min (lanes 3). The RNAs were fractionated at 4°C on a 0.8% agarose gel in 0.5X TB with 0.1 mM $MgCl_2$ (A), or 0.5X TBE (B) and stained with ethidium bromide. WT and dGC *rpoS* and DsrA RNAs were each incubated separately to visualize any self-dimerization. As a positive control, the two RNAs were mixed together before the initial incubation at 65°C (lanes +).

Identification of a non-canonical Hfq anolog in *B. burgdorferi*

We present here preliminary data that suggest the conserved hypothetical protein *bb0268* is a non-canonical Hfq analog in *B. burgdorferi*. Hfq is an RNA chaperone that is required for activity of antisense or complementary *trans* sRNAs *in vivo* (Majdalani *et al.*, 2005). Many bacteria do not have annotated Hfq homologs, including *B. burgdorferi*.

In collaboration with Dr. Andrew Feig, we identified two putative Hfq analog candidates in *B. burgdorferi* with a BLAST search using a *Streptococcus pneumoniae* non-canonical Hfq protein sequence. The *S. pneumoniae* non-canonical Hfq homolog was identified by computational threading to the *E. coli* Hfq structure performed by the Feig laboratory (unpublished data). *B. burgdorferi* conserved hypothetical open reading frame *bb268* showed 31% similarity and 18% identity with the *S. pneumoniae* sequence. In addition, an Sm-1 like motif was identified in the N-terminal region of BB268 and there are sequence similarities with several other RNA binding proteins, including an RNA helicase as identified by a BLAST search using the BB268 sequence. Another conserved hypothetical open reading frame, *bb260,* showed 27% similarity and 14% identity with the *S. pneumoniae* sequence, but was much larger than the *E. coli* Hfq and did not have any readily identifiable Sm motifs.

We hypothesized that BB268 is the Hfq analog and functions as a sRNA chaperone in *B. burgdorferi*. Hfq is required for DsrA to stimulate translation of *rpoS* in response to a decrease in temperature and entrance into stationary phase in *E. coli*. Evidence suggests that Hfq co-localizes *rpoS* and DsrA, likely presenting the two RNAs in favorable positions for complex formation. To determine if BB268 functions as a RNA chaperone, similar to Hfq in *E. coli*, we assayed its ability to *trans*-complement an

hfq null mutant of *E. coli*. The *bb268* and *bb260* open reading frames were inserted into the arabinose-inducible vector pBAD24. The empty pBAD24 vector was used as a negative control and *E. coli hfq* cloned into the pBAD24 vector was used as a positive control. DsrA and Hfq are both needed for growth-phase related regulation of RpoS::LacZ at 30°C in *E. coli*. The efficient translation of an RpoS::LacZ translational fusion, in response to changes in cell density, was measured with a β-galactosidase assay. Therefore, all cultures were grown at 30°C and assayed for LacZ production at various cell densities related to growth phase. BB268 partially restores the efficient translation of RpoS::LacZ at 30°C (Fig. 20), suggesting that it has an RNA chaperone-like activity in *E. coli*. By contrast, BB260 did not complement the *hfq* null mutant in *E. coli*. Five independent experiments with BB268 and three independent experiments with BB260 yielded similar results. The Hfq binding site on small RNAs was identified as single stranded A-U stretch followed or preceded by a stem-loop (Moll *et al.*, 2003; Moller *et al.*, 2002; Zhang *et al.*, 2002). Notably, we identified a putative Hfq binding site on $DsrA_{Bb}$ (Fig. 5).

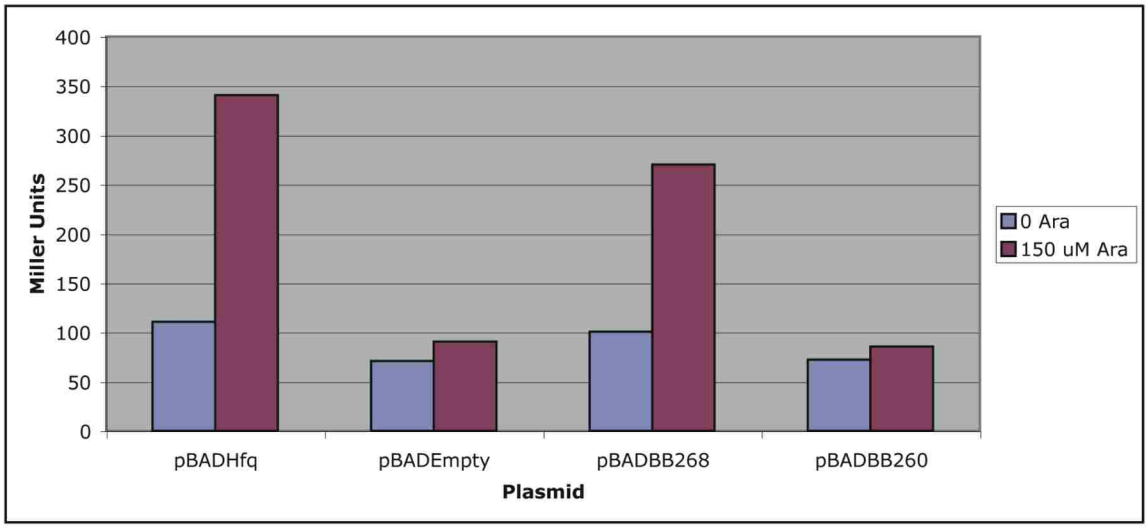

Fig. 20. BB268 partially complements the *hfq-1* null mutant of *E. coli*. An *hfq*-1 mutant strain (a derivative of *E. coli* K-12 strain SG20250) containing an RpoS::LacZ translational fusion carrying the indicated plasmids were assayed for total ß-galactosidase units. pBADHfq expresses recombinant Hfq under control of the *araBAD* promoter. pBAD24 is the empty *araBAD* vector. pBADBB268 and pBADBB260 have the *B. burgdorferi bb268* and *bb260* open reading frames under the control of the *araBAD* promoter. Cultures were grown at 30˚C, and samples were taken at various times during logarithmic growth. Total ß-galactosidase units were determined as described by Miller (1972). Three independent experiments (BB260) and five independent experiments (BB268) were performed and yielded similar results.

Chapter 6

Discussion

Small non-coding RNAs have emerged as major regulatory factors in the expression of many bacterial genes, including virulence factors and stress response modulators (Gottesman, 2004; Hengge-Aronis, 2002; Lease and Belfort, 2000; Majdalani *et al.*, 2005; Narberhaus *et al.*, 2006; Repoila *et al.*, 2003; Storz *et al.*, 2004). We identified and characterized the first small regulatory RNA in *Borrelia burgdorferi*. Our data demonstrate that (i) $DsrA_{Bb}$ and BB0577 play a role in the temperature-induced increase in RpoS levels (during log phase growth), (ii) the regulation occurs at the post-transcriptional level, and (iii) $DsrA_{Bb}$ and the upstream region of *rpoS* directly interact *in vitro*. The analogous sRNA in *E. coli* is DsrA, which regulates RpoS levels in response to temperature. Therefore, we chose to term this sRNA $DsrA_{Bb}$. We hypothesize that $DsrA_{Bb}$ regulates RpoS synthesis by stimulating its translation and that BB0577 regulates the activity of $DsrA_{Bb}$, either directly or indirectly. In addition, we postulate that temperature directly regulates the structure and, as a result, the activity of DsrA. Furthermore, we present preliminary data that suggest BB268 is a non-canonical Hfq-like RNA chaperone in *B. burgdorferi* and we postulate that BB268 is required for $DsrA_{Bb}$ regulation of RpoS. Our model of temperature-dependent $DsrA_{Bb}$ regulation represents a novel mechanism of *trans*-acting sRNAs.

Structure of the *rpoS* transcript

Our data indicate that the *rpoS* transcript exists with two 5′ ends that are differentially transcribed or processed based on the cell density of the culture (Fig. 1C and D). We observe that *B. burgdorferi* grown to either a low or high cell density after a

temperature upshift correlates well to mid-logarithmic or stationary growth phase, respectively (data not shown). One caveat is that we have not extensively buffered pH in these experiments and the pH of culture medium decreases with cell growth, so we can not formally exclude pH effects. However, the importance of growth phase in the enzootic life cycle of *B. burgdorferi* remains unknown. The 5′ end of *rpoS* that we identified at a high cell density (-50) was also recently described by Smith *et al.* (Smith *et al.*, 2007). RpoN is the alternative sigma factor responsible for the transcription of the *rpoS* mRNA with the (-50) 5′ end; a consensus RpoN promoter is directly upstream of this 5′ end (Smith *et al.*, 2007). Hübner *et al.* (2001) demonstrated that RpoN is required for the expression of RpoS and OspC, but these studies were all performed at a high cell density. By contrast, the (-171) 5′ end of *rpoS* does not have a readily identifiable RpoN consensus sequence upstream and we originally hypothesized this mRNA is not dependent on RpoN. The data presented in Fischer *et al.* (2005) are supportive of this hypothesis and demonstrate the disconnect between RpoN and RpoS gene regulation. These authors present a set of genes, determined by microarray analysis, that RpoS regulates independently of RpoN, suggesting an RpoN-independent synthesis of RpoS. However, we performed a preliminary experiment that suggests RpoS protein is not expressed even at low cell densities in an *rpoN*$^-$ strain (data not shown). There is no identifiable RpoD (σ^{70}) or RpoN consensus promoter sequence upstream of the (-171) 5′ end of *rpoS*. The consensus -12/-24 RpoN promoter sequence is 5′-CT**GG**CAC-N_5-TT**GC**A-3′ (with the invariant nucleotides GG/GC in bold). Although the upstream region of the *rpoS* (-171) does not contain a consensus RpoN promoter sequence, we did identify a non-consensus RpoN sequence, which contains the correct binding sequences,

but has an 18 nucleotide spacer instead of the conserved 5 nucleotides. However, the RpoN-dependent promoters studied to date have all demonstrated a strict requirement for the 5 nucleotide spacing in the -12/-24 RpoN consensus sequence (Buck *et al.*, 2000; Cannon *et al.*, 1995; Merrick, 1993). Evidence suggests that RpoN contacts the -12/-24 sequences on the same face of the DNA (Cannon *et al.*, 1995). Therefore, we hypothesize that the (-171) *rpoS* mRNA is either transcribed from an RpoN-independent promoter (at levels difficult to detect *in vitro*) or that the non-consensus RpoN start site is functional in *B. burgdorferi* and RpoN is required for *rpoS* transcription from both start sites. Although extremely intriguing, and possibly paradigm shattering, the understanding of the differential regulation of the two *rpoS* mRNA species is paramount in elucidating the role of RpoS in the enzootic life cycle of *B. burgdorferi*.

We now propose a working model of *B. burgdorferi rpoS* regulation *in vivo*. We propose that a low level of translationally inactive RpoN-independent (-171) *rpoS* transcript exists in *B. burgdorferi* carried by an unfed tick. As the tick begins to take a blood meal, $DsrA_{Bb}$-dependent translation of this *rpoS* transcript rapidly occurs resulting in the timely up-regulation of OspC and down-regulation of OspA. Our lab and others have demonstrated the requirement of OspC to be present for the transmission of *B. burgdorferi* from the tick vector to the mammalian host (Gilbert *et al.*, 2007; Tilly *et al.*, 2006; Tilly *et al.*, 2007; Xu *et al.*, 2007). In addition, several groups have demonstrated the strict requirement for OspC to be down-regulated after *B. burgdorferi* enters the mammalian host (Tilly *et al.*, 2007; Xu *et al.*, 2007); however, other proteins, such as DbpA (decorin-binding protein), likely need to continue to be synthesized. Therefore, we suggest that once in the mammal, factors other than RpoS (possibly DNA supercoiling

and/or a *trans*-acting repressor) down-regulate OspC and the RpoN-dependent *rpoS* transcript is efficiently transcribed and translated independently of $DsrA_{Bb}$. To test this model, we will determine if the (-171) *rpoS* transcript is RpoN-independent. Northern and Western blotting for RpoS will be performed on an *rpoN*⁻ strain of *B. burgdorferi* at both high and low cell densities. We expect that the *rpoN*⁻ strain will produce low levels of *rpoS* (-171) transcript and RpoS protein at a low cell density.

$DsrA_{Bb}$ structure and function

$DsrA_{Bb}$ has the potential to base-pair with 31 of 34 nucleotides or 51 of 68 nucleotides of *rpoS* mRNA (Fig. 1A), which is a more extensive binding than most of the characterized sRNAs in bacteria; in *E. coli* DsrA and RprA base-pair with 26 of 38 and 19 of 30 nucleotides of the *rpoS* mRNA, respectively (Lease *et al.*, 1998; Majdalani *et al.*, 1998; Majdalani *et al.*, 2002).

$DsrA_{Bb}$ exists as four species, determined by the 5′ RACE and RPA assays, as well as visualized by Northern blot analyses (Figs. 2, 3, and 6C). Originally, we thought that some of the larger bands seen in the $DsrA_{Bb}$ Northern blots may have been due to non-specific binding of the RNA probe. However, all of the signals were absent in the $dsrA_{Bb}^{-}$ strain of *B. burgdorferi* (Fig. 6C and Fig. 9B), suggesting that all of the bands correspond to RNA that contains the *rpoS* mRNA complementary sequence. We also postulated that several of the bands larger than 352 nucleotides on the $DsrA_{Bb}$ Northern blots were *bb0577* transcripts containing a large part of $DsrA_{Bb}$, which hybridizes with the probe; however, Northern blot analysis of *bb0577* (Fig. 2B) indicates that this is not the case: the *bb0577* bands are not the same size as the $dsrA_{Bb}$ bands. Therefore, $DsrA_{Bb}$ may exist as more than four species. Three different sizes of DsrA are present in *E. coli*,

but the full-length RNA (85 nucleotides) is thought to be the only RNA species that regulates *rpoS* translation. There is evidence that suggests the two smaller sizes of DsrA in *E. coli* are products of a processing event (Repoila and Gottesman, 2001).

As discussed above, our data indicate that the *rpoS* transcript has two 5′ ends that are differentially transcribed or processed based on the cell density of the culture (Fig. 1C). Notably, the $DsrA_{Bb}$ complementary binding sequence is only contained in the longer *rpoS* 5′ end (-171), which is predominately found at low cell density. Therefore, we hypothesized that $DsrA_{Bb}$ would regulate RpoS levels at low cell density, but not at high cell density. Our data support this hypothesis and indicate that $DsrA_{Bb}$ regulates RpoS levels via an interaction between the complementary sequences (Fig. 7). In addition, the pH regulation of RpoS and OspC was not affected by the loss of $DsrA_{Bb}$ (Fig. 8B). Taken together, these data suggest that $DsrA_{Bb}$ is specifically responsible for stimulating RpoS synthesis after a temperature upshift at mid-logarithmic growth phase, which is likely physiologically relevant during tick to mammal transmission (when the blood meal provides plentiful nutrients). However, we do not exclude the possibility that $DsrA_{Bb}$ has other functions that have yet to be discovered.

In *E. coli,* temperature affects the rate of transcription initiation of the *dsrA* gene and the stability of DsrA RNA (Repoila and Gottesman, 2001). By contrast, $DsrA_{Bb}$ levels remain relatively constant in *B. burgdorferi* despite changes in temperature and cell density (Fig. 2A). Therefore, we hypothesize that temperature either directly affects activity of $DsrA_{Bb}$ at the structural level or indirectly by a *trans*-acting factor. Although we could not identify an *in vitro* condition that increased $DsrA_{Bb}$ levels, mammalian host factors could potentially induce $DsrA_{Bb}$ or tick factors could repress it. $DsrA_{Bb}$, like

DsrA in *E. coli*, may be multifunctional and could be either regulating a different target or responding to a different environmental signal at stationary phase (high cell density).

We predict that the *rpoS* mRNA upstream region folds into a stable stem-loop structure, modeled by MFOLD (Zuker, 2003) (Fig. 4), which sequesters the Shine-Dalgarno region rendering it inaccessible to the ribosome. We propose that the secondary structure of $DsrA_{Bb}$ at 23°C does not allow for base-pairing with the *rpoS* transcript, but the secondary structure changes after a temperature shift and favors hybridization of the two RNAs. Alternatively, temperature could facilitate the interaction between $DsrA_{Bb}$ and the *rpoS* mRNA. Regardless, the binding of $DsrA_{Bb}$ to the *rpoS* mRNA upstream untranslated region would alter the structure of the transcript, the Shine-Dalgarno region would become available, and translation would initiate (Fig. 11).

Increased RpoS and OspC levels are seen in high-passage B31 and 297 $dsrA_{Bb}^{-}$ strains compared to high-passage wild-type strains (Fig. 10). We initially hypothesized that the high-passage $dsrA_{Bb}$ mutant strains had a suppressor mutation in the upstream region of the *rpoS* mRNA. We postulated that these mutations would cause a structural change in the *rpoS* upstream region that would allow the ribosome binding site to be accessible without $DsrA_{Bb}$. However, no mutations were found in the upstream region of *rpoS* in the revertants. Another possibility is that an alternate small RNA exists in *B. burgdorferi* and may be stimulating *rpoS* translation when $DsrA_{Bb}$ is absent, similar to RprA in *E. coli* (Majdalani *et al.*, 2001); however, we do not have evidence for or against this hypothesis. Finally, we hypothesized that the long *rpoS* transcript (-171) was absent in the revertant strain, rendering *rpoS* translation independent of $DsrA_{Bb}$. 5′ RACE analysis revealed that, indeed, the short 5′ end of *rpoS* (-50) was the only 5′ end identified

in the high-passage revertant strain. However, *B. burgdorferi* high-passage strains are often avirulent and lack essential plasmids (Labandeira-Rey and Skare, 2001; Purser and Norris, 2000; Schwan *et al.*, 1988). Therefore, the high-passage phenotype is not likely physiologically relevant in the enzootic life cycle of *B. burgdorferi*.

The sRNA *rrbA* (*rpoS* regulator *Borrelia* A) was recently identified (Östberg *et al.*, 2004) and may be the same RNA or related to the RNA we identified and characterized. The major differences between RrbA and $DsrA_{Bb}$ are the size(s) and number of RNA species, as well as the regulation of the RNA by cell density. RrbA was reported to be soley in the intergenic region between *bb0577* and *bb0578*, while we show $DsrA_{Bb}$ extensively overlaps with the *bb0577* ORF. Suprisingly, the sequence of complementarity between *rpoS* and RrbA was not stated or described, rendering a critical comparison to $DsrA_{Bb}$ impossible. In addition, RrbA was reported as being a single band of approximately 170 bases that was more highly expressed at exponential phase than at stationary phase. In contrast, we report here that $DsrA_{Bb}$ exists as four species, ranging in size from 213 to 352 nucleotides, and does not respond to cell density changes associated with growth phase. The sizes of the four $DsrA_{Bb}$ species were determined by 5′ RACE and RPA and correlate well with sizes obtained by Northern analyses. The size disparities between RrbA and $DsrA_{Bb}$ may be partially due to the type of gel used to fractionate the RNA. Initially we used a 1% agarose-formaldehyde gel, as did Östberg *et al.* (2004), to perform the $DsrA_{Bb}$ Northern blot. However, the data we obtained from these Northern blots were not useful in determining size or number of bands due to broad smeared signals, although we still resolved three bands (data not shown). In order to resolve the bands and obtain an accurate size we used a 6% polyacrylamide TBE-Urea gel (Figs. 2

and 6C). Furthermore, if $DsrA_{Bb}$ and RrbA are the same sRNA, reconciling the disparity regarding the levels of sRNA at stationary growth phase is difficult. Likely, $DsrA_{Bb}$ and RrbA are the same sRNA, but Östberg *et al.* (2004) made several mistakes in characterizing it. However, due to the major differences reported between $DsrA_{Bb}$ and RrbA, we have maintained the $DsrA_{Bb}$ nomenclature.

We first presented the identification and preliminary characterization of $DsrA_{Bb}$, at the Gordon Research Conference on Biology of Spirochetes in January 2004. Coincidently, the paper that identified RrbA was submitted on June 24, 2004. The authors used several different approaches, including direct homology and comparative genomic searches to identify sRNAs in *B. burgdorferi*. These searches only identified three sRNAs and the authors reasoned that the low level of homology found between *E. coli* and *B. burgdorferi* was the reason so few sRNAs were identified using these comparative methods. Therefore, they employed the same technique to find RrbA that we used to identify $DsrA_{Bb}$. They searched for sequences that demonstrated extensive complementarity to the upstream region of likely mRNA targets. One of their main criteria for searching for sRNAs was that the sequence must be in an intergenic region and not within 30 nucleotides of the 5′ or 3′ end of adjacent genes. Based on these criteria, RrbA should not have been considered a good candidate for an sRNA. In addition, the authors state that they searched for other sRNAs by using the same technique as described for *rpoS* for other mRNA targets. Notably, RrbA was the only sRNA identified using this technique and *rpoS* was the only target mRNA. Interestingly, Östberg *et al*. (2004) also suggest that the lack of an annotated Hfq and RNase E in *B. burgdorferi* is likely the reason for the deficiency of sRNAs. Our data are in strong

disagreement given that we have identified an Hfq-like protein in *B. burgdorferi* (discussed below).

BB0577 in the DsrA-dependent temperature-induced regulation of RpoS

The results reported here suggest that BB0577 plays a role in the regulation of RpoS with $DsrA_{Bb}$. The $DsrA_{Bb}$ mutant phenotype is only restored to a wild-type phenotype when both the full length *bb0577* transcript and $DsrA_{Bb}$ are present (Fig. 9). Notably, the sizes of all $DsrA_{Bb}$ species appear smaller in the strains that presumably do not have functional BB0577 protein (Fig. 9C, pKFSC and pKFLCS lanes). A BLAST search revealed that BB0577 has significant homology and 26% identity to an adenine methyl-transferase from *Campylobacter upsaliensis*. Therefore, we hypothesize that BB0577 methylates $DsrA_{Bb}$, which may aid in the correct folding of $DsrA_{Bb}$ or protect it from degradation, increasing its stability and half-life. $DsrA_{Bb}$ methylation may also be required for its interaction with *rpoS* mRNA or the RNA chaperone BB268.

We also hypothesize that the *rpoS* transcript may be methylated by BB0577. The *bb0577* mRNA contains a modest portion of the *rpoS* complementary sequence, which we postulate may direct the *bb0577* mRNA to the *rpoS* transcript. The subsequent translation of *bb0577* would place the protein in close association with *rpoS* mRNA. The methylation of the *rpoS* transcript could regulate the mRNA in the same manner as described above for $DsrA_{Bb}$. RNA-directed DNA methylation has been documented in plant species (Wassenegger, 2000), but there is no precedence for RNA-directed RNA methylation or RNA methylation of sRNAs. RNA methylation is well documented in rRNA, tRNA and capped mRNAs in certain viruses and eukaryotes, but there is no documentation of mRNA methylation in bacteria. Experimental confirmation of these

hypotheses would have broad implications in the area of RNA biochemistry and represent a novel mechanism of RNA regulation. We plan to vigorously pursue these hypotheses and elucidate the role of BB0577 in the $DsrA_{Bb}$-dependent regulation of RpoS. We propose to utilize biochemical and molecular genetic techniques to determine the function of BB0577. We will take a multifaceted approach to test for the methylation of $DsrA_{Bb}$ and the putative methyl-transferase activity of BB0577. First, we expect that BB0577 would use S-adenosylmethionine (AdoMet) as the methyl donor and propose to assay if BB0577 binds AdoMet *in vitro*. There are many experiments that would follow if BB0577 binds AdoMet. In addition to these experiments, we will endeavor to assay if $DsrA_{Bb}$ is methylated. As a preliminary experiment, we will fractionate *in vitro* transcribed $DsrA_{Bb}$ and total RNA isolated from *B. burgdorferi* on a TBE-Urea gel and probe for $DsrA_{Bb}$ with a single-stranded RNA probe. If BB0577 methylates $DsrA_{Bb}$ *in vivo*, and this is the reason for the smaller sizes of $DsrA_{Bb}$ seen in the *bb0577*$^-$ strains in the complementation assay (Fig. 9C), we expect to see a size difference between the *in vitro* transcribed $DsrA_{Bb}$ and $DsrA_{Bb}$ isolated from *B. burgdorferi*.

The results reported here support a model of translational regulation of RpoS by a small RNA in *B. burgdorferi* (Fig. 11). Not surprisingly, the regulation of RpoS is proving to be complex in *B. burgdorferi* and occurs at multiple levels, including transcription, translation initiation, and likely protein stability (Fig. 21).

Fig. 21

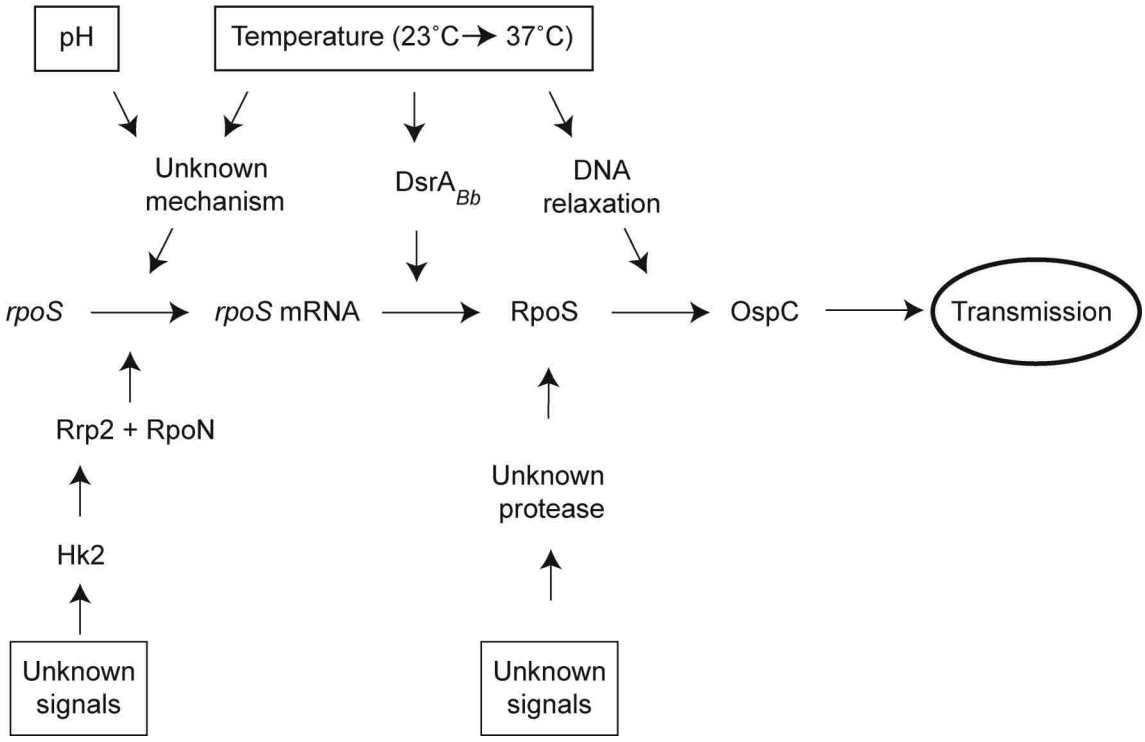

Fig. 21. Multiple levels of RpoS regulation are affected by a variety of different signals and influence OspC synthesis, which is required for the tick to mammal transmission. The Rrp2/RpoN signaling pathway (Yang *et al*., 2003) as well as temperature and pH (Caimano *et al*., 2004) regulate *rpoS* transcription. A temperature shift relaxes DNA supercoiling, which promotes RpoS-mediated transcription of *ospC* (Alverson *et al*., 2003; Yang *et al*., 2005). The results reported here support a model in which $DsrA_{Bb}$ regulates the efficiency of *rpoS* translation in response to temperature. In addition, we propose that RpoS degradation may be regulated (E. Todd, D. Cragun, M. Gilbert and D. Samuels, unpublished data). High cell density *in vitro* increases the levels of RpoS, but the mechanism and level of regulation, as well as the physiological relevance in the enzootic life cycle, remain unknown.

RpoS regulates several differentially expressed proteins, including OspC, DbpA and OspA; therefore, RpoS must be able to integrate a variety of different signals and respond accordingly. Moreover, we hypothesize that RpoS is a key, but not the only, regulator of virulence gene expression during the enzootic life cycle. For example, RpoS is necessary for the transcription of *ospC*, but the level of negative supercoiling affects the activity of

the *ospC* promoter (Alverson *et al.*, 2003; Yang *et al.*, 2005). Caimano *et al*. (2004) report that most RpoS regulation occurs at the level of transcription and that *rpoS* transcript levels were increased by a temperature shift and further induced by a decrease in pH. Our results are in agreement regarding the increase in transcript levels in response to a temperature shift. However, our data demonstrate that the level of *rpoS* transcript is not the sole, nor even the major, factor in determining RpoS protein levels after a temperature shift since $DsrA_{Bb}$ is necessary for RpoS synthesis at a low cell density. *rpoS* transcript levels were higher in the $dsrA_{Bb}^{-}$ strain compared to wild type after a temperature upshift, but the levels of RpoS protein and *ospC* transcript were lower. At a low cell density, likely physiologically relevant in transmission, the predominate form of temperature-mediated induction of RpoS protein is at the post-transcriptional level by $DsrA_{Bb}$. Therefore, we propose that $DsrA_{Bb}$ is a molecular thermometer (Narberhaus *et al.*, 2006; Repoila and Gottesman, 2003) for RpoS induction.

An Hfq analog in *B. burgdorferi*

We provisionally identified BB268 as an Hfq analog and present data that demonstrate BB268 has RNA-chaperone activity in *E. coli*. Hfq is required for DsrA activity in *E. coli* and is necessary for virulence in several bacteria (Christiansen *et al.*, 2004; Ding *et al.*, 2004; Sharma and Payne, 2006; Sittka *et al.*, 2007; Sledjeski *et al.*, 2001; Sonnleitner *et al.*, 2003). By contrast, Bohn *et al*. (2007) report that Hfq in is not necessary for virulence in *Staphylococcus aureus* and they hypothesize that another protein must act as an RNA-chaperone for sRNAs *in vivo*. We identified a potential Hfq-binding site on the predicted secondary structure of $DsrA_{Bb}$ (Fig. 5) and hypothesize that BB268 is necessary for $DsrA_{Bb}$ function. Furthermore, we hypothesize that $DsrA_{Bb}$ and

BB268 are required for *B. burgdorferi* transmission. We have deleted the *bb268* open reading frame, replacing it with a spectinomycin resistance cassette (Frank *et al.*, 2003). The mutant does not synthesize OspC in response to an increase in temperature at low-cell density (data not shown). Although preliminary, the data are consistent with the hypothesis that BB268 is required for $DsrA_{Bb}$ function. Hfq mutants are difficult to complement due to the plieotropic effects of the mutation. The *B. burgdorferi bb268* mutant strain has a slow growth phenotype and increased cell length (data not shown) similar to the phenotype of the *E. coli* Hfq mutant (Muffler *et al.*, 1997b; Tsui *et al.*, 1994). Initially we attempted to complement the *bb268*$^-$ strain in *trans* on a shuttle vector under its own putative promoter. We included the 137 nucleotides upstream of the *bb268* open reading frame, presumably including the promoter. The *bb268* promoter has not been mapped and only four nucleotides separate the upstream open reading frame from the start codon of the *bb268* open reading frame. The *bb268* mutant strain electrocompetent cells arced at the voltage usually used to electroporate *B. burgdorferi*. Therefore, we lowered the voltage and used low levels of DNA during the electroporation. Surprisingly, positive transformants were isolated, but did not complement the phenotype (data not shown), suggesting that the promoter was not within the 137 nucleotides upstream of the open reading frame. Therefore, we hypothesize that the *bb268* gene is in an operon with the upstream flagellar genes and is not being transcribed from the insert in the shuttle vector. Consequently, we are striving to complement the *bb268*$^-$ strain by employing a multifaceted approach. First, we are fusing the *bb268* gene to the inducible *flac* promoter, constructed by our lab (Gilbert *et al.*, 2007), and cloning it into a shuttle vector (pBSV2-*lacI*) that carries *lacI* fused to a

constitutive promoter. This construct will conditionally complement the *bb268*$^{-}$ strain in *trans*. In addition, we are generating a construct that will insert the *flac* promoter directly into the chromosome upstream of *bb268* in a strain of *B. burgdorferi* that produces LacI constitutively (Gilbert *et al.*, 2007). This strain of *B. burgdorferi* would be a conditional *bb268* mutant that circumvents the complementation process. In an effort to further demonstrate the Hfq-like RNA chaperone activity of BB268, we are replacing the *bb268* open reading frame with the *E. coli hfq* gene on the chromosome.

The identification of an Hfq-like RNA chaperone in *B. burgdorferi* suggests that other sRNAs may exist in *B. burgdorferi* and could play central roles in regulation. Hfq has been used to identify numerous sRNAs that were not previously found using the conventional computer programs in other organisms (Zhang *et al.*, 2003). The authors used Hfq to co-immunoprecipitate sRNAs followed by microarray analysis to identify the sRNAs and we could use this approach to identify additional sRNAs in *B. burgdorferi*.

The DsrA$_{Bb}$::*rpoS* interaction *in vitro*

Naturally occurring antisense or complementary RNAs are recognized as an efficient and specific method of regulating gene expression at the post-transcriptional level. Antisense RNAs affect gene regulation by base-pairing with a complementary RNA target. There are two different classes of sRNAs distinguished by the level of complementarity to its target RNA in bacteria (Eguchi *et al.*, 1991; Wagner and Simons, 1994). The first class consists of antisense RNAs from plasmids, phages and transposons, which are fully complementary to their targets because they are transcribed from the same DNA sequence, but in the opposite direction. The second class are sRNAs are only partially complementary to their targets and are transcribed from a chromosomal location distinct

from the target RNA. We hypothesize that $DsrA_{Bb}$ acts as an antisense RNA regulating the efficiency of *rpoS* translation.

Our model of *rpoS* regulation by $DsrA_{Bb}$ dictates a direct interaction between the two complementary regions of the RNAs in a temperature-dependent manner (Fig. 11). Our data clearly demonstrate that $DsrA_{Bb}$ and the upstream region of *rpoS* interact extensively in a temperature-dependent manner *in vitro* (Figs. 12, 13 and 14). However, the effect of temperature on the interaction may be due to simple kinetic constraints at the two temperatures (Figs. 14 and 15). Detailed kinetic measurements on several completely complementary sRNA/RNA interactions *in vivo* and *in vitro* indicate the interaction between RNA molecules is not simply limited by diffusion and that the highly efficient interactions are dependent on the specific structural motifs of the RNAs (Brunel *et al.*, 2002; Eguchi *et al.*, 1991; Wagner and Simons, 1994). In addition, the hybridization process between the two structured RNAs (unzipping and zipping) is not the major factor responsible for the rate of the reaction. Although we have not yet performed careful kinetic experiments at 23°C and 37°C, our data indicate that the rate of the $DsrA_{Bb}$::*rpoS* interaction *in vitro* is considerably slower at 23°C compared to 37°C (Fig. 14). Whether the decreased rate of reaction is purely due to the decreased diffusion rate or if the complex formation is slowed due to the structures of the two RNAs impeding the interaction at 23°C remains unknown. The CopA/CopT (antisense/target RNA) interaction in *E. coli* is one of the most studied regulatory antisense RNA systems. Nordgren *et al*. (Nordgren *et al.*, 2001) used surface plasmon resonance to study the kinetics of the CopA/CopT interaction in real-time. Their data indicate that the association rate constants are not significantly dependent on temperature, but the

dissociation rate constants are dependent on temperature. Using plasmon surface resonance as a means of detecting RNA/RNA interactions at different temperatures may be a viable way for us to assay the temperature-dependent nature of $DsrA_{Bb}$:: *rpoS* interaction. The effect of temperature on the diffusion rate would be mitigated and the kinetics of the interaction could be carefully measured at the same time, which is proving to be necessary to fully dissect the $DsrA_{Bb}$::*rpoS* interaction (as described below).

Antisense RNAs interact with their complementary target RNAs as folded structures. The structures as well as the primary sequence of the RNAs dictate the efficiency and specificity of the interactions. The site of the initial recognition of the target RNA is almost exclusively within loops at the top of stable stems (Brunel *et al*., 2002; Eguchi *et al*., 1991; Wagner and Simons, 1994). We constructed three site-directed mutants of $DsrA_{Bb}$ and the complementary mutants in *rpoS* (Fig. 16) in an effort to identify the primary sequence and secondary structural elements required for the $DsrA_{Bb}$::*rpoS* interaction *in vitro*. We hypothesized that alterations to the intermolecular complementary sequences and the stem-loops would diminish or eliminate complex formation.

Our data indicate that none of the mutations we produced affected the $DsrA_{Bb}$::*rpoS* interaction. Although initially disconcerting, there are several different explanations. First, the loop and stem we mutated in $DsrA_{Bb}$ may simply not be important in the $DsrA_{Bb}$::*rpoS* interaction and complex formation. An unstructured end-segment of one RNA binds to a loop of the other in several systems, (Franch *et al*., 1999; Kittle *et al*., 1989). Another possibility is that the mutations do not abolish the interaction, but instead decrease the rate of the reaction, as seen in some other RNA/RNA

interactions. In these cases, the decreased rates of reaction demonstrated *in vitro* correlate well to a loss of function *in vivo* (Eguchi *et al*., 1991; Wagner and Simons, 1994). The WT $DsrA_{Bb}$:: WT *rpoS* interaction appears to be near completion by 10 min at 37°C (Fig. 14). Unfortunately, we allowed the interactions to proceed for 40 min when studying the effect of the site-directed mutations. Quite possibly, the mutations do have an effect on the rate of the complex formation. Therefore, we are planning on performing the experiments necessary to determine if the rate of the complex formation is decreased with the mutant RNAs. In addition, we are constructing the site-directed mutations in the complementing shuttle vector pKFLC (Fig. 9) to determine if the mutations affect *rpoS* regulation *in vivo*.

Another possibility is that the *in vitro* interaction we observe does not mimic the interaction that occurs naturally within the cell. The putative Hfq analog BB268 may be required for the proper folding or presentation of the $DsrA_{Bb}$ and *rpoS* RNAs. In addition, BB0577 may be methylating $DsrA_{Bb}$ and/or *rpoS in vivo*, which could have significant effects on the RNA structures or interactions.

Concluding Remarks

The recent renaissance of RNA biology is in large part due to the identification and characterization of riboswitches, riboregulators, microRNAs and other small RNA regulators. In contrast to the conventional role of RNA as a passive messenger between the genome and the proteome, the modern RNA has multiple dynamic functions with critical implications in gene regulation. The intrinsic characteristics of RNA chemistry allow for the variety of sRNA functions. First, the structure of RNA is dynamic and RNA can fold into a variety of structures that can bind proteins and other RNAs. Second,

the mechanism of simple base-pairing allows for specific interactions with many different targets. Moreover, RNA structure, unlike protein structure, is labile and can quickly refold or change its structure. Therefore, sRNAs often regulate genes that are required for survival and need to be expressed quickly in response to an environmental signal.

We have characterized the first sRNA in *B. burgdorferi* that potentially transduces an important environmental signal (temperature) and regulates key virulence proteins involved in the enzootic life cycle. In addition, we identified a putative analog of Hfq in *B. burgdorferi* and propose a novel model of temperature regulation via an sRNA.

References

Achsel, T., Brahms, H., Kastner, B., Bachi, A., Wilm, M., and Luhrmann, R. (1999) A doughnut-shaped heteromer of human Sm-like proteins binds to the 3'-end of U6 snRNA, thereby facilitating U4/U6 duplex formation in vitro. *The EMBO journal* **18**: 5789-5802.

Achsel, T., Stark, H., and Luhrmann, R. (2001) The Sm domain is an ancient RNA-binding motif with oligo(U) specificity. *Proc Natl Acad Sci U S A* **98**: 3685-3689.

Akins, D.R., Bourell, K.W., Caimano, M.J., Norgard, M.V., and Radolf, J.D. (1998) A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. *J. Clin. Invest.* **101**: 2240-2250.

Alverson, J., and Samuels, D.S. (2002) *groEL* expression in *gyrB* mutants of *Borrelia burgdorferi*. *J. Bacteriol.* **184**: 6069-6072.

Alverson, J., Bundle, S.F., Sohaskey, C.D., Lybecker, M.C., and Samuels, D.S. (2003) Transcriptional regulation of the *ospAB* and *ospC* promoters from *Borrelia burgdorferi*. *Mol. Microbiol.* **48**: 1665-1677.

Arnqvist, A., Olsén, A., and Normark, S. (1994) σ^S-dependent growth-phase induction of the *csgBA* promoter in *Escherichia coli* can be achieved in vivo by σ^{70} in the absence of the nucleoid-associated protein H-NS. *Mol. Microbiol.* **13**: 1021-1032.

Austin, S., Kundrot, C., and Dixon, R. (1991) Influence of a mutation in the putative nucleotide binding site of the nitrogen regulatory protein NTRC on its positive control function. *Nucleic Acids Res* **19**: 2281-2287.

Baranton, G., and Old, I.G. (1995) The Spirochaetes: a different way of life. *Bull. Inst. Pasteur* **93**: 63-95.

Barbour, A.G., and Garon, C.F. (1987) Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. *Science* **237**: 409-411.

Baril, C., Richaud, C., Baranton, G., and Saint Girons, I. (1989) Linear chromosome of *Borrelia burgdorferi*. *Res. Microbiol.* **140**: 507-516.

Benach, J.L., Bosler, E.M., Hanrahan, J.P., Coleman, J.L., Bast, T.F., Habicht, G.S., Cameron, D.J., Ziegler, J.L., Barbour, A.G., Burgdorfer, W., Edelman, R., and Kaslow, R.A. (1983) Spirochetes isolated from the blood of two patients with Lyme disease. *N. Engl. J. Med.* **308**: 740-742.

Berger, D.K., Narberhaus, F., and Kustu, S. (1994) The isolated catalytic domain of NIFA, a bacterial enhancer-binding protein, activates transcription in vitro: activation is inhibited by NIFL. *Proc Natl Acad Sci U S A* **91**: 103-107.

Boeck, R., Lapeyre, B., Brown, C.E., and Sachs, A.B. (1998) Capped mRNA degradation intermediates accumulate in the yeast spb8-2 mutant. *Mol Cell Biol* **18**: 5062-5072.

Bohn, C., Rigoulay, C., and Bouloc, P. (2007) No detectable effect of RNA-binding protein Hfq absence in *Staphylococcus aureus*. *BMC microbiology* **7**: 10.

Bolotin, A., Mauger, S., Malarme, K., Ehrlich, S.D., and Sorokin, A. (1999) Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. *Antonie van Leeuwenhoek* **76**: 27-76.

Bono, J.L., Elias, A.F., Kupko III, J.J., Stevenson, B., Tilly, K., and Rosa, P. (2000) Efficient targeted mutagenesis in *Borrelia burgdorferi*. *J. Bacteriol.* **182**: 2445-2452.

Bouveret, E., Rigaut, G., Shevchenko, A., Wilm, M., and Seraphin, B. (2000) A Sm-like protein complex that participates in mRNA degradation. *The EMBO journal* **19**: 1661-1671.

Brescia, C.C., Mikulecky, P.J., Feig, A.L., and Sledjeski, D.D. (2003) Identification of the Hfq-binding site on DsrA RNA: Hfq binds without altering DsrA secondary structure. *RNA* **9**: 33-43.

Brown, L., and Elliott, T. (1997) Mutations that increase expression of the *rpoS* gene and decrease its dependence on *hfq* function in *Salmonella typhimurium*. *J. Bacteriol.* **179**: 656-662.

Brunel, C., Marquet, R., Romby, P., and Ehresmann, C. (2002) RNA loop-loop interactions as dynamic functional motifs. *Biochimie* **84**: 925-944.

Buck, M., Gallegos, M.T., Studholme, D.J., Guo, Y., and Gralla, J.D. (2000) The bacterial enhancer-dependent σ^{54} (σ^{N}) transcription factor. *J Bacteriol* **182**: 4129-4136.

Burgdorfer, W., Barbour, A.G., Hayes, S.F., Benach, J.L., Grunwaldt, E., and Davis, J.P. (1982) Lyme disease--a tick-borne spirochetosis? *Science* **216**: 1317-1319.

Burge, C., Tuschi, T., and Sharp, P. (1999) Splicing of precursors to mRNAs by the spliceosomes. *The RNA World* **2nd edn**

Caimano, M.J., Eggers, C.H., Hazlett, K.R., and Radolf, J.D. (2004) RpoS is not central to the general stress response in *Borrelia burgdorferi* but does control expression of one or more essential virulence determinants. *Infect. Immun.* **72**: 6433-6445.

Caimano, M.J., Eggers, C.H., Gonzalez, C.A., and Radolf, J.D. (2005) Alternate sigma factor RpoS is required for the in vivo-specific repression of *Borrelia burgdorferi* plasmid lp54-borne *ospA* and *lp6.6* genes. *J. Bacteriol.* **187**: 7845-7852.

Caimano, M.J., Eggers, C.H., Morton, E.A., Gilbert, M.A., Schwartz, I., and Radolf, J.D. (2007) Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle. *Mol. Microbiol.* **Submitted**.

Cannon, W., and Buck, M. (1992) Central domain of the positive control protein NifA and its role in transcriptional activation. *J Mol Biol* **225**: 271-286.

Cannon, W., Austin, S., Moore, M., and Buck, M. (1995) Identification of close contacts between the σ^{N} (σ^{54}) protein and promoter DNA in closed promoter complexes. *Nucleic Acids Res* **23**: 351-356.

Carroll, J.A., Garon, C.F., and Schwan, T.G. (1999) Effects of environmental pH on membrane proteins in *Borrelia burgdorferi*. *Infect. Immun.* **67**: 3181-3187.

Casjens, S., and Huang, W.M. (1993) Linear chromosomal physical and genetic map of *Borrelia burgdorferi*, the Lyme disease agent. *Mol. Microbiol.* **8**: 967-980.

Casjens, S., Murphy, M., DeLange, M., Sampson, L., van Vugt, R., and Huang, W.M. (1997) Telomeres of the linear chromosomes of Lyme disease spirochaetes: nucleotide sequence and possible exchange with linear plasmid telomeres. *Mol. Microbiol.* **26**: 581-596.

Casjens, S., Palmer, N., van Vugt, R., Huang, W.M., Stevenson, B., Rosa, P., Lathigra, R., Sutton, G., Peterson, J., Dodson, R.J., Haft, D., Hickey, E., Gwinn, M., White, O., and Fraser, C.M. (2000) A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. *Mol. Microbiol.* **35**: 490-516.

Christiansen, J.K., Larsen, M.H., Ingmer, H., Søgaard-Andersen, L., and Kallipolitis, B.H. (2004) The RNA-binding protein Hfq of *Listeria monocytogenes*: role in stress tolerance and virulence. *J. Bacteriol.* **186**: 3355-3362.

Cooper, M., Johnston, L.H., and Beggs, J.D. (1995) Identification and characterization of Uss1p (Sdb23p): a novel U6 snRNA-associated protein with significant similarity to core proteins of small nuclear ribonucleoproteins. *The EMBO journal* **14**: 2066-2075.

Davidson, B.E., MacDougall, J., and Saint Girons, I. (1992) Physical map of the linear chromosome of the bacterium *Borrelia burgdorferi* 212, a causative agent of Lyme disease, and localization of rRNA genes. *J. Bacteriol.* **174**: 3766-3774.

de Haseth, P.L., and Uhlenbeck, O.C. (1980) Interaction of *Escherichia coli* host factor protein with oligoriboadenylates. *Biochemistry* **19**: 6138-6146.

de Silva, A.M., Telford, S.R., 3rd, Brunet, L.R., Barthold, S.W., and Fikrig, E. (1996) *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. *J. Exp. Med.* **183**: 271-275.

Ding, Y., Davis, B.M., and Waldor, M.K. (2004) Hfq is essential for *Vibrio cholerae* virulence and downregulates σ^E expression. *Mol. Microbiol.* **53**: 345-354.

Eggers, C.H., Caimano, M.J., and Radolf, J.D. (2004) Analysis of promoter elements involved in the transcriptional initiation of RpoS-dependent *Borrelia burgdorferi* genes. *J. Bacteriol.* **186**: 7390-7402.

Eggers, C.H., Caimano, M.J., and Radolf, J.D. (2006) Sigma factor selectivity in *Borrelia burgdorferi*: RpoS recognition of the *ospE*/*ospF*/*elp* promoters is dependent on the sequence of the –10 region. *Mol. Microbiol.* **59**: 1859-1875.

Eguchi, Y., Itoh, T., and Tomizawa, J. (1991) Antisense RNA. *Annu Rev Biochem* **60**: 631-652.

Elias, A.F., Bono, J.L., Carroll, J.A., Stewart, P., Tilly, K., and Rosa, P. (2000) Altered stationary-phase response in a *Borrelia burgdorferi rpoS* mutant. *J. Bacteriol.* **182**: 2909-2918.

Elias, A.F., Stewart, P.E., Grimm, D., Caimano, M.J., Eggers, C.H., Tilly, K., Bono, J.L., Akins, D.R., Radolf, J.D., Schwan, T.G., and Rosa, P. (2002) Clonal polymorphism of *Borrelia burgdorferi* strain B31 MI: implications for mutagenesis in an infectious strain background. *Infect. Immun.* **70**: 2139-2150.

Ferdows, M.S., and Barbour, A.G. (1989) Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent. *Proc. Natl. Acad. Sci. U. S. A.* **86**: 5969-5973.

Fikrig, E., and Narasimhan, S. (2006) *Borrelia burgdorferi*–traveling incognito? *Microbes and Infection* **8**: 1390-1399.

Fingerle, V., Laux, H., Munderloh, U.G., Schulte-Spechtel, U., and Wilske, B. (2000) Differential expression of outer surface proteins A and C by individual *Borrelia burgdorferi* in different genospecies. *Med. Microbiol. Immunol.* **189**: 59-66.

Fisher, M.A., Grimm, D., Henion, A.K., Elias, A.F., Stewart, P.E., Rosa, P.A., and Gherardini, F.C. (2005) *Borrelia burgdorferi* σ^{54} is required for mammalian infection and vector transmission but not for tick colonization. *Proc. Natl. Acad. Sci. U. S. A.* **102**: 5162-5167.

Franch, T., Petersen, M., Wagner, E.G., Jacobsen, J.P., and Gerdes, K. (1999) Antisense RNA regulation in prokaryotes: rapid RNA/RNA interaction facilitated by a general U-turn loop structure. *J Mol Biol* **294**: 1115-1125.

Frank, K.L., Bundle, S.F., Kresge, M.E., Eggers, C.H., and Samuels, D.S. (2003) *aadA* confers streptomycin resistance in *Borrelia burgdorferi*. *J Bacteriol* **185**: 6723-6727.

Franze de Fernandez, M.T., Eoyang, L., and August, J.T. (1968) Factor fraction required for the synthesis of bacteriophage Qbeta-RNA. *Nature* **219**: 588-590.

Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., White, O., Ketchum, K.A., Dodson, R., Hickey, E.K., Gwinn, M., Dougherty, B., Tomb, J.-F., Fleischmann, R.D., Richardson, D., Peterson, J., Kerlavage, A.R., Quakenbush, J., Salzberg, S., Hanson, M., van Vugt, R., Palmer, N., Adams, M.K., Gocayne, J., Weidman, J., Utterback, T., Watthey, L., McDonald, L., Artiach, P., Bowman, C., Garland, S., Fujii, C., Cotton, M.D., Horst, K., Roberts, K., Hatch, B., Smith, H.O., and Venter, J.C. (1997) Genomic sequence of a Lyme disease spirochete, *Borrelia burgdorferi*. *Nature* **390**: 580-586.

Geissmann, T.A., and Touati, D. (2004) Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *The EMBO journal* **23**: 396-405.

Gilbert, M.A., Morton, E.A., Bundle, S.F., and Samuels, D.S. (2007) Artificial regulation of *ospC* expression in *Borrelia burgdorferi*. *Mol. Microbiol.* **63**: 1259-1273.

Gottesman, S. (2004) The small RNA regulators of *Escherichia coli*: roles and mechanisms. *Annu. Rev. Microbiol.* **58**: 303-328.

Grimm, D., Tilly, K., Byram, R., Stewart, P.E., Krum, J.G., Bueschel, D.M., Schwan, T.G., Policastro, P.F., Elias, A.F., and Rosa, P.A. (2004) Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. *Proc Natl Acad Sci U S A* **101**: 3142-3147.

Guiney, D.G. (1997) Regulation of bacterial virulence gene expression by the host environment. *J. Clin. Invest.* **99**: 565-569.

Guo, B.P., Norris, S.J., Rosenberg, L.C., and Höök, M. (1995) Adherence of *Borrelia burgdorferi* to the proteoglycan decorin. *Infect. Immun.* **63**: 3467-3472.

Hajnsdorf, E., and Regnier, P. (2000) Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I. *Proc Natl Acad Sci U S A* **97**: 1501-1505.

Hefty, P.S., Jolliff, S.E., Caimano, M.J., Wikel, S.K., Radolf, J.D., and Akins, D.R. (2001) Regulation of OspE-related, OspF-related, and Elp lipoproteins of *Borrelia burgdorferi* strain 297 by mammalian host-specific signals. *Infect. Immun.* **69**: 3618-3627.

Hengge-Aronis, R. (2002) Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* **66**: 373-395.

Hermann, H., Fabrizio, P., Raker, V.A., Foulaki, K., Hornig, H., Brahms, H., and Luhrmann, R. (1995) snRNP Sm proteins share two evolutionarily conserved sequence motifs which are involved in Sm protein-protein interactions. *The EMBO journal* **14**: 2076-2088.

Hinnebusch, J., Bergström, S., and Barbour, A.G. (1990) Cloning and sequence analysis of linear plasmid telomeres of the bacterium *Borrelia burgdorferi*. *Mol. Microbiol.* **4**: 811-820.

Hinnebusch, J., and Barbour, A.G. (1991) Linear plasmids of *Borrelia burgdorferi* have a telomeric structure and sequence similar to those of a eukaryotic virus. *J. Bacteriol.* **173**: 7233-7239.

Hopper, S., and Bock, A. (1995) Effector-mediated stimulation of ATPase activity by the σ^{54}-dependent transcriptional activator FHLA from *Escherichia coli*. *J Bacteriol* **177**: 2798-2803.

Hübner, A., Yang, X., Nolen, D.M., Popova, T.G., Cabello, F.C., and Norgard, M.V. (2001) Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. *Proc. Natl. Acad. Sci. U. S. A.* **98**: 12724-12729.

Kambach, C., Walke, S., Young, R., Avis, J.M., de la Fortelle, E., Raker, V.A., Luhrmann, R., Li, J., and Nagai, K. (1999) Crystal structures of two Sm protein complexes and their implications for the assembly of the spliceosomal snRNPs. *Cell* **96**: 375-387.

Kazmierczak, M.J., Wiedmann, M., and Boor, K.J. (2005) Alternative sigma factors and their roles in bacterial virulence. *Microbiol. Mol. Biol. Rev.* **69**: 527-543.

Kittle, J.D., Simons, R.W., Lee, J., and Kleckner, N. (1989) Insertion sequence IS10 anti-sense pairing initiates by an interaction between the 5' end of the target RNA and a loop in the anti-sense RNA. *J Mol Biol* **210**: 561-572.

Knight, S.W., and Samuels, D.S. (1999) Natural synthesis of a DNA-binding protein from the C-terminal domain of DNA gyrase A in *Borrelia burgdorferi*. *EMBO J.* **18**: 4875-4881.

Kramer, A. (1996) The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annu Rev Biochem* **65**: 367-409.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F., Danchin, A., and et al. (1997) The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390**: 249-256.

Labandeira-Rey, M., and Skare, J.T. (2001) Decreased infectivity in *Borrelia burgdorferi* strain B31 is associated with loss of linear plasmid 25 or 28-1. *Infect. Immun.* **69**: 446-455.

Lane, R.S., Piesman, J., and Burgdorfer, W. (1991) Lyme borreliosis: relation of its causative agent to its vectors and hosts in North America and Europe. *Annual Review of Entomology* **36**: 587-609.

Lease, R.A., Cusick, M.E., and Belfort, M. (1998) Riboregulation in *Escherichia coli*: DsrA RNA acts by RNA:RNA interactions at multiple loci. *Proc. Natl. Acad. Sci. U. S. A.* **95**: 12456-12461.

Lease, R.A., and Belfort, M. (2000) Riboregulation by DsrA RNA: *trans*-actions for global economy. *Mol. Microbiol.* **38**: 667-672.

Lease, R.A., and Woodson, S.A. (2004) Cycling of the Sm-like protein Hfq on the DsrA small regulatory RNA. *J Mol Biol* **344**: 1211-1223.

Lee, J.H., Scholl, D., Nixon, B.T., and Hoover, T.R. (1994) Constitutive ATP hydrolysis and transcription activation by a stable, truncated form of *Rhizobium meliloti* DCTD, a σ^{54}-dependent transcriptional activator. *The Journal of biological chemistry* **269**: 20401-20409.

Leuba-Garcia, S., Martinez, R., and Gern, L. (1998) Expression of outer surface proteins A and C of *Borrelia afzelii* in *Ixodes ricinus* ticks and in the skin of mice. *Zentralbl. Bakteriol.* **287**: 475-484.

Lin, B., Short, S.A., Eskildsen, M., Klempner, M.S., and Hu, L.T. (2001) Functional testing of putative oligopeptide permease (Opp) proteins of *Borrelia burgdorferi*: a complementation model in opp(-) *Escherichia coli*. *Biochim. Biophys. Acta* **1499**: 222-231.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and Gottesman, S. (1998) DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc. Natl. Acad. Sci. U. S. A.* **95**: 12462-12467.

Majdalani, N., Chen, S., Murrow, J., St. John, K., and Gottesman, S. (2001) Regulation of RpoS by a novel small RNA: the characterization of RprA. *Mol. Microbiol.* **39**: 1382-1394.

Majdalani, N., Hernandez, D., and Gottesman, S. (2002) Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Mol. Microbiol.* **46**: 813-826.

Majdalani, N., Vanderpool, C.K., and Gottesman, S. (2005) Bacterial small RNA regulators. *CRC Crit. Rev. Biochem. Mol. Biol.* **40**: 93-113.

Marchler-Bauer, A., Anderson, J.B., DeWeese-Scott, C., Fedorova, N.D., Geer, L.Y., He, S., Hurwitz, D.I., Jackson, J.D., Jacobs, A.R., Lanczycki, C.J., Liebert, C.A., Liu, C., Madej, T., Marchler, G.H., Mazumder, R., Nikolskaya, A.N., Panchenko, A.R., Rao, B.S., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Vasudevan, S., Wang, Y., Yamashita, R.A., Yin, J.J., and Bryant, S.H. (2003) CDD: a curated Entrez database of conserved domain alignments. *Nucleic Acids Res* **31**: 383-387.

Massé, E., Majdalani, N., and Gottesman, S. (2003) Regulatory roles for small RNAs in bacteria. *Curr. Opin. Microbiol.* **6**: 120-124.

Medrano, M., Ding, Y., Wang, X.-G., Lu, P., Coburn, J., and Hu, L.T. (2007) Regulators of expression of the oligopeptide permease A proteins of *Borrelia burgdorferi*. *J. Bacteriol.* **189**: 2653-2659.

Mekalanos, J.J. (1992) Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* **174**: 1-7.

Merrick, M.J. (1993) In a class of its own--the RNA polymerase sigma factor σ^{54} (σ^{N}). *Mol Microbiol* **10**: 903-909.

Mikulecky, P.J., Kaw, M.K., Brescia, C.C., Takach, J.C., Sledjeski, D.D., and Feig, A.L. (2004) *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs. *Nature Structural & Molecular Biology* **11**: 1206-1214.

Moll, I., Leitsch, D., Steinhauser, T., and Blasi, U. (2003) RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep* **4**: 284-289.

Moller, T., Franch, T., Hojrup, P., Keene, D.R., Bachinger, H.P., Brennan, R.G., and Valentin-Hansen, P. (2002) Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol Cell* **9**: 23-30.

Montgomery, R.R., Malawista, S.E., Feen, K.J., and Bockenstedt, L.K. (1996) Direct demonstration of antigenic substitution of *Borrelia burgdorferi* ex vivo: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. *J. Exp. Med.* **183**: 261-269.

Muffler, A., Barth, M., Marschall, C., and Hengge-Aronis, R. (1997a) Heat shock regulation of turnover: a role for DnaK and relationship between stress responses mediated by σ^S and σ^{32} in *Escherichia coli*. *J Bacteriol* **179**: 445-452.

Muffler, A., Traulsen, D.D., Fischer, D., Lange, R., and Hengge-Aronis, R. (1997b) The RNA-binding protein HF-I plays a global regulatory role which is largely, but not exclusively, due to its role in expression of the σ^S subunit of RNA polymerase in *Escherichia coli*. *J Bacteriol* **179**: 297-300.

Narberhaus, F., Waldminghaus, T., and Chowdhury, S. (2006) RNA thermometers. *FEMS Microbiology Reviews* **30**: 3-16.

Nordgren, S., Slagter-Jager, J.G., and Wagner, G.H. (2001) Real time kinetic studies of the interaction between folded antisense and target RNAs using surface plasmon resonance. *J Mol Biol* **310**: 1125-1134.

Okamoto, K., and Freundlich, M. (1986) Mechanism for the autogenous control of the *crp* operon: transcriptional inhibition by a divergent RNA transcript. *Proc Natl Acad Sci U S A* **83**: 5000-5004.

Orloski, K.A., Hayes, E.B., Campbell, G.L., and Dennis, D.T. (2000) Surveillance for Lyme disease--United States, 1992-1998. *MMWR CDC Surveill Summ* **49**: 1-11.

Östberg, Y., Bunikis, I., Bergström, S., and Johansson, J. (2004) The etiological agent of Lyme disease, *Borrelia burgdorferi*, appears to contain only a few small RNA molecules. *J. Bacteriol.* **186**: 8472-8477.

Padula, S.J., Sampieri, A., Dias, F., Szczepanski, A., and Ryan, R.W. (1993) Molecular characterization and expression of p23 (OspC) from a North American strain of *Borrelia burgdorferi*. *Infect. Immun.* **61**: 5097-5105.

Paget, M.S., and Helmann, J.D. (2003) The σ^{70} family of sigma factors. *Genome biology* **4**: 203.

Pal, U., Li, X., Wang, T., Montgomery, R.R., Ramamoorthi, N., de Silva, A.M., Bao, F., Yang, X., Pypaert, M., Pradhan, D., Kantor, F.S., Telford, S., Anderson, J.F., and Fikrig, E. (2004a) TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. *Cell* **119**: 457-468.

Pal, U., Yang, X., Chen, M., Bockenstedt, L.K., Anderson, J.F., Flavell, R.A., Norgard, M.V., and Fikrig, E. (2004b) OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. *J Clin Invest* **113**: 220-230.

Perez-Martin, J., and de Lorenzo, V. (1996) In vitro activities of an N-terminal truncated form of XylR, a σ^{54}-dependent transcriptional activator of *Pseudomonas putida*. *J Mol Biol* **258**: 575-587.

Pillai, R.S., Grimmler, M., Meister, G., Will, C.L., Luhrmann, R., Fischer, U., and Schumperli, D. (2003) Unique Sm core structure of U7 snRNPs: assembly by a specialized SMN complex and the role of a new component, Lsm11, in histone RNA processing. *Genes Dev* **17**: 2321-2333.

Purser, J.E., and Norris, S.J. (2000) Correlation between plasmid content and infectivity in *Borrelia burgdorferi*. *Proc. Natl. Acad. Sci. U. S. A.* **97**: 13865-13870.

Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X.F., Fish, D., Anguita, J., Norgard, M.V., Kantor, F.S., Anderson, J.F., Koski, R.A., and Fikrig, E. (2005) The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature* **436**: 573-577.

Ramamoorthy, R., and Scholl-Meeker, D. (2001) *Borrelia burgdorferi* proteins whose expression is similarly affected by culture temperature and pH. *Infect. Immun.* **69**: 2739-2742.

Rathinavelu, S., and de Silva, A.M. (2001) Purification and characterization of *Borrelia burgdorferi* from feeding nymphal ticks (*Ixodes scapularis*). *Infect. Immun.* **69**: 3536-3541.

Repoila, F., and Gottesman, S. (2001) Signal transduction cascade for regulation of RpoS: temperature regulation of DsrA. *J. Bacteriol.* **183**: 4012-4023.

Repoila, F., and Gottesman, S. (2003) Temperature sensing by the *dsrA* promoter. *J. Bacteriol.* **185**: 6609-6614.

Repoila, F., Majdalani, N., and Gottesman, S. (2003) Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli*: the RpoS paradigm. *Mol. Microbiol.* **48**: 855-861.

Romeo, T. (1998) Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol Microbiol* **29**: 1321-1330.

Samuels, D.S., Marconi, R.T., Huang, W.M., and Garon, C.F. (1994) *gyrB* mutations in coumermycin A_1-resistant *Borrelia burgdorferi*. *J. Bacteriol.* **176**: 3072-3075.

Samuels, D.S. (1995) Electrotransformation of the spirochete *Borrelia burgdorferi*. In *Electroporation Protocols for Microorganisms*. Vol. 47. Nickoloff, J.A. (ed). Totowa, New Jersey: Humana Press, pp. 253-259.

Schwan, T.G., Burgdorfer, W., and Garon, C.F. (1988) Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. *Infect. Immun.* **56**: 1831-1836.

Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., and Rosa, P.A. (1995) Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc. Natl. Acad. Sci. U. S. A.* **92**: 2909-2913.

Schwan, T.G., and Piesman, J. (2000) Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. *J. Clin. Microbiol.* **38**: 382-388.

Senear, A.W., and Steitz, J.A. (1976) Site-specific interaction of Qbeta host factor and ribosomal protein S1 with Qbeta and R17 bacteriophage RNAs. *The Journal of biological chemistry* **251**: 1902-1912.

Seraphin, B. (1995) Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. *The EMBO journal* **14**: 2089-2098.

Sharma, A.K., and Payne, S.M. (2006) Induction of expression of *hfq* by DksA is essential for *Shigella flexneri* virulence. *Mol. Microbiol.* **62**: 469-479.

Singh, S.K., and Girschick, H.J. (2004) Molecular survival strategies of the Lyme disease spirochete *Borrelia burgdorferi*. *The Lancet Infectious Diseases* **4**: 575-583.

Sittka, A., Pfeiffer, V., Tedin, K., and Vogel, J. (2007) The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. *Mol. Microbiol.* **63**: 193-217.

Sledjeski, D., and Gottesman, S. (1995) A small RNA acts as an antisilencer of the H-NS-silenced *rcsA* gene of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **92**: 2003-2007.

Sledjeski, D.D., Gupta, A., and Gottesman, S. (1996) The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in *Escherichia coli*. *EMBO J.* **15**: 3993-4000.

Sledjeski, D.D., Whitman, C., and Zhang, A. (2001) Hfq is necessary for regulation by the untranslated RNA DsrA. *J. Bacteriol.* **183**: 1997-2005.

Smith, A.H., Blevins, J.S., Bachlani, G.N., Yang, X.F., and Norgard, M.V. (2007) Evidence that RpoS (σ^S) in *Borrelia burgdorferi* is controlled directly by RpoN (σ^{54}/σ^N). *J. Bacteriol.* **189**: 2139–2144.

Sonnleitner, E., Hagens, S., Rosenau, F., Wilhelm, S., Habel, A., Jager, K.E., and Blasi, U. (2003) Reduced virulence of a *hfq* mutant of *Pseudomonas aeruginosa* O1. *Microb Pathog* **35**: 217-228.

Spielman, A. (1994) The emergence of Lyme disease and human babesiosis in a changing environment. *Ann N Y Acad Sci* **740**: 146-156.

Steere, A.C., Grodzicki, R.L., Kornblatt, A.N., Craft, J.E., Barbour, A.G., Burgdorfer, W., Schmid, G.P., Johnson, E., and Malawista, S.E. (1983) The spirochetal etiology of Lyme disease. *N. Engl. J. Med.* **308**: 733-740.

Steere, A.C., Coburn, J., and Glickstein, L. (2004) The emergence of Lyme disease. *J. Clin. Invest.* **113**: 1093-1101.

Steere, A.C. (2006) Lyme borreliosis in 2005, 30 years after initial observations in Lyme Connecticut. *Wiener Klinische Wochenschrift* **118**: 625-633.

Stevenson, B., Schwan, T.G., and Rosa, P.A. (1995) Temperature-related differential expression of antigens in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infect. Immun.* **63**: 4535-4539.

Storz, G., Opdyke, J.A., and Zhang, A. (2004) Controlling mRNA stability and translation with small, noncoding RNAs. *Curr. Opin. Microbiol.* **7**: 140-144.

Sun, X., Zhulin, I., and Wartell, R.M. (2002) Predicted structure and phyletic distribution of the RNA-binding protein Hfq. *Nucleic Acids Res* **30**: 3662-3671.

Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A.K., Melefors, O., Georgellis, D., Babitzke, P., and Romeo, T. (2002) Regulatory circuitry of the CsrA/CsrB and BarA/UvrY systems of *Escherichia coli*. *J Bacteriol* **184**: 5130-5140.

Tharun, S., He, W., Mayes, A.E., Lennertz, P., Beggs, J.D., and Parker, R. (2000) Yeast Sm-like proteins function in mRNA decapping and decay. *Nature* **404**: 515-518.

Tilly, K., Krum, J.G., Bestor, A., Jewett, M.W., Grimm, D., Bueschel, D., Byram, R., Dorward, D., Vanraden, M.J., Stewart, P., and Rosa, P. (2006) *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection. *Infect. Immun.* **74**: 3554-3564.

Tilly, K., Bestor, A., Jewett, M.W., and Rosa, P. (2007) Rapid clearance of Lyme disease spirochetes lacking OspC from skin. *Infect. Immun.* **75**: 1517-1519.

Tomizawa, J., Itoh, T., Selzer, G., and Som, T. (1981) Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc Natl Acad Sci U S A* **78**: 1421-1425.

Toro, I., Thore, S., Mayer, C., Basquin, J., Seraphin, B., and Suck, D. (2001) RNA binding in an Sm core domain: X-ray structure and functional analysis of an archaeal Sm protein complex. *The EMBO journal* **20**: 2293-2303.

Toro, I., Basquin, J., Teo-Dreher, H., and Suck, D. (2002) Archaeal Sm proteins form heptameric and hexameric complexes: crystal structures of the Sm1 and Sm2 proteins from the hyperthermophile *Archaeoglobus fulgidus*. *J Mol Biol* **320**: 129-142.

Tsui, H.C., Leung, H.C., and Winkler, M.E. (1994) Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12. *Mol Microbiol* **13**: 35-49.

Vytvytska, O., Moll, I., Kaberdin, V.R., von Gabain, A., and Blasi, U. (2000) Hfq (HF1) stimulates *ompA* mRNA decay by interfering with ribosome binding. *Genes Dev* **14**: 1109-1118.

Wagner, E.G., von Heijne, J., and Nordstrom, K. (1987) Control of replication of plasmid R1: translation of the 7k reading frame in the *RepA* mRNA leader region counteracts the interaction between CopA RNA and CopT RNA. *The EMBO journal* **6**: 515-522.

Wagner, E.G., and Simons, R.W. (1994) Antisense RNA control in bacteria, phages, and plasmids. *Annu Rev Microbiol* **48**: 713-742.

Wassarman, K.M., and Storz, G. (2000) 6S RNA regulates *E. coli* RNA polymerase activity. *Cell* **101**: 613-623.

Wassenegger, M. (2000) RNA-directed DNA methylation. *Plant Mol Biol* **43**: 203-220.

Will, C.L., and Luhrmann, R. (1997) Protein functions in pre-mRNA splicing. *Curr Opin Cell Biol* **9**: 320-328.

Xu, Q., McShan, K., and Liang, F.T. (2007) Identification of an *ospC* operator critical for immune evasion of *Borrelia burgdorferi*. *Mol Microbiol* **64**: 220-231.

Yang, X., Goldberg, M.S., Popova, T.G., Schoeler, G.B., Wikel, S.K., Hagman, K.E., and Norgard, M.V. (2000) Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. *Mol. Microbiol.* **37**: 1470-1479.

Yang, X.F., Alani, S.M., and Norgard, M.V. (2003) The response regulator Rrp2 is essential for the expression of major membrane lipoproteins in *Borrelia burgdorferi*. *Proc. Natl. Acad. Sci. U. S. A.* **100**: 11001-11006.

Yang, X.F., Pal, U., Alani, S.M., Fikrig, E., and Norgard, M.V. (2004) Essential role for OspA/B in the life cycle of the Lyme disease spirochete. *J. Exp. Med.* **199**: 641-648.

Yang, X.F., Lybecker, M.C., Pal, U., Alani, S.M., Blevins, J., Revel, A.T., Samuels, D.S., and Norgard, M.V. (2005) Analysis of the *ospC* regulatory element controlled by the RpoN-RpoS regulatory pathway in *Borrelia burgdorferi*. *J. Bacteriol.* **187**: 4822-4829.

Zhang, A., Altuvia, S., Tiwari, A., Argaman, L., Hengge-Aronis, R., and Storz, G. (1998) The OxyS regulatory RNA represses *rpoS* translation and binds the Hfq (HF-I) protein. *The EMBO journal* **17**: 6061-6068.

Zhang, A., Wassarman, K.M., Ortega, J., Steven, A.C., and Storz, G. (2002) The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell* **9**: 11-22.

Zhang, A., Wassarman, K.M., Rosenow, C., Tjaden, B.C., Storz, G., and Gottesman, S. (2003) Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol* **50**: 1111-1124.

Zhang, X., and Bremer, H. (1995) Control of the *Escherichia coli rrnB* P1 promoter strength by ppGpp. *The Journal of biological chemistry* **270**: 11181-11189.

Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. **31**: 1-10.