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Translational efficiency of *rpoS* mRNA from *Borrelia burgdorferi*: Effects of the length and sequence of the mRNA leader region

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ABSTRACT

Regulation of the enzootic cycle in *Borrelia burgdorferi* requires a shift to the RNA polymerase alternative sigma factor, RpoS. We used *in vitro* and *in vivo* assays to assess the relative importance of the putative Shine–Dalgarno sequence and its sequestration for the translational efficiency of *rpoS*. We created mutant leader regions in which we either removed the Shine–Dalgarno sequence, disrupted the secondary structure or both. Binding assays and toeprint assays demonstrated that both the presence and the availability of the Shine–Dalgarno sequence are important to the efficiency and specificity of ribosome binding. Adding a $DsrA_{Bb}$ mimic in the form of a single-stranded DNA oligonucleotide increased the level and specificity of binding ribosomes to the transcript with an extended leader, presumably by making the Shine–Dalgarno sequence available for binding. In *in vivo* assays we confirmed that the Shine–Dalgarno sequence must be both present and un-sequestered in order for translation to proceed efficiently. The longer transcript was significantly better translated in *B. burgdorferi* at 37 °C than at 26 °C, lending support to the hypothesis that $DsrA_{Bb}$ acts as a temperature-dependent stimulator of translation. These studies demonstrate that translational regulation of gene expression in *B. burgdorferi* may be an important mechanism for responding to environmental signals important in the enzootic cycle.

1. Introduction

The spirochete, *Borrelia burgdorferi*, is the causative agent of Lyme disease [1–4]. In nature, the spirochete undergoes significant shifts in gene expression patterns as it cycles between an *Ixodes* tick vector and a vertebrate host [5–8]. Temperature [9,10], pH [4,11–13] and mammalian host factors [14,15] play a role in the shifts in gene expression patterns (reviewed by Samuels [7]).

The alternative sigma factor rpoS is induced during tick feeding and is responsible for global changes in gene expression associated with transmission of the spirochete to a vertebrate host [7,8,16,17]. Two *rpoS* transcripts have been identified in *B. burgdorferi* [18]. At high cell density ($\sim 1 \times 10^8$ cells/ml) a transcript, RS, with a 5′ end located 50 nucleotides upstream of the start codon is found [18]. At lower cell density ($1–3 \times 10^7$ cells/ml), a longer transcript, RL, with a 5′ end 171 nucleotides upstream of the start codon, is found [18].

The two transcripts are predicted to have different translational efficiencies (Model presented in Fig. 1) In *Escherichia coli*, the interaction of the 3′ end of the 16S rRNA in the 30S ribosomal subunit with the Shine–Dalgarno sequence (SD) [19] of the mRNA may be rate determining in translational initiation [20]. A strong inverse correlation between translational efficiency and the thermodynamic stability of secondary structure that sequesters the SD sequence has been observed [20–25]. In the model presented by Lybecker and Samuels, the RS transcript is predicted to have an unstructured leader sequence allowing the SD sequence to interact with the 3′ end of the 16S rRNA in the 30S ribosomal subunit for efficient translational initiation (Fig. 1A) [18]. The RL transcript is predicted to have extensive secondary structure that involves the base-pairing of the ribosome binding site to an upstream region in the transcript's 5′ untranslated region (UTR) [18] to form a stable helix that inhibits translational initiation (Fig. 1B).

The RL transcript contains a region that is complementary to a small non-coding RNA, $DsrA_{Bb}$ [18]. $DsrA_{Bb}$, in the presence of a *B. burgdorferi*-specific Hfq_{Bb} chaperone [26], up-regulates *rpoS* expression post-transcriptionally with temperature upshift by an as yet unknown mechanism [7,18,26]. Base-pairing of $DsrA_{Bb}$ to the 5′ UTR of the mRNA is predicted to change the conformation of the mRNA and increase translational efficiency (Fig. 1C) [18]. In *B. burgdorferi*, two alternative pairings of the 3′ end of the 16S rRNA to the leader region of the *rpoS* mRNA are possible (Fig. 2A). Although both are further from the start codon than what is typically observed (<13 nucleotides) [27] for a SD sequence, these pairings share 5 nucleotides in common which will be considered the putative SD sequence for the purpose of these studies.

Abbreviations: $DsrA_{BbDNA}$, $DsrA_{Bb}$ DNA oligonucleotide; $30S_{Ec}$, *E. coli* 30S ribosomal subunits; SD, Shine–Dalgarno; UTR, untranslated region.

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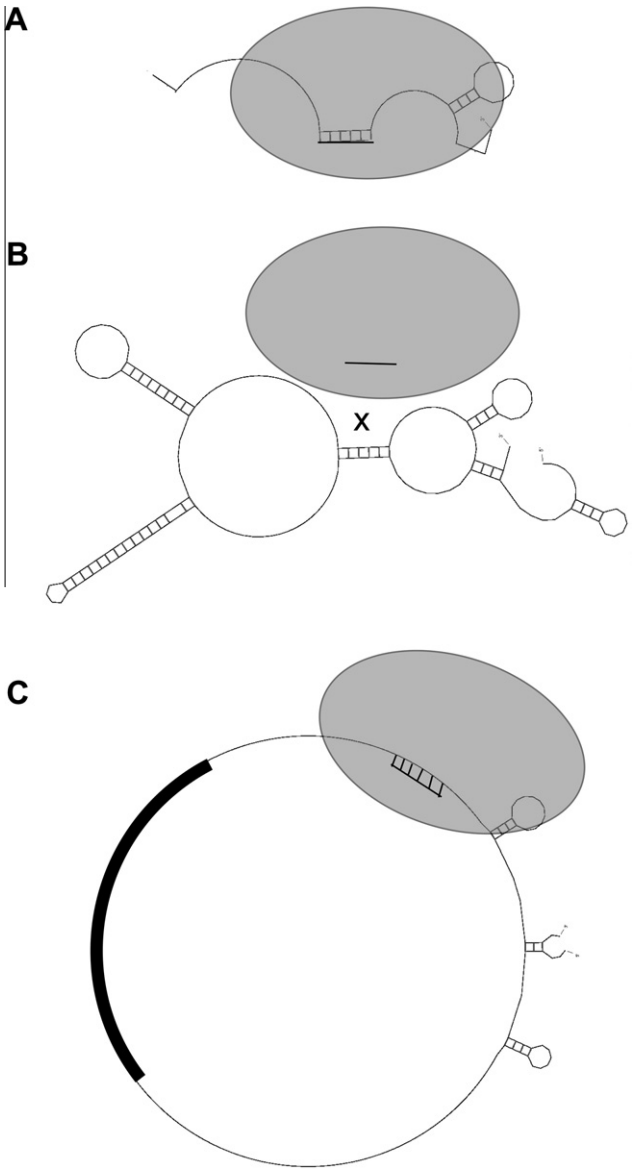

Fig. 1. Model: Translational Regulation of the *Borrelia burgdorferi rpoS* mRNA transcript. In this model, the 30S ribosomal subunit is represented by a grey oval. The 3′ end of the 16S rRNA is represented as a line within the "ribosome". Trace drawings [32], of the *rpoS* mRNA leader region are represented as a line drawing. (A) The −50 RS mRNA, expressed at high cell density, lacks an extended upstream sequence and is predicted to have an accessible Shine–Dalgarno sequence that pairs to the 3′ end of the 16S rRNA. (B) The −171 RL mRNA, expressed at low cell density, is predicted to have an extended structure that sequesters the Shine–Dalgarno sequence in an intramolecular base-pairing interaction, preventing the intermolecular interaction with the 16S rRNA. (C) At low cell density and elevated temperature, the −171 RL mRNA transcript is predicted to interact with the non-coding RNA $DsrA_{Bb}$ (represented by a dark bar along the mRNA). This interaction is expected to increase the extent of the interaction with the 30S ribosomal subunit, and therefore, the translational efficiency of the mRNA.

The role of the length, sequence and structure of the *B. burgdorferi rpoS* 5′ UTR on translational efficiency is the subject of these studies. We have examined the translational efficiency of *E. coli* 30S ribosomal subunits ($30S_{Ec}$) with $rpoS_{Bb}$ variants (Fig. 2B) using toe-printing assays and compared the levels of luciferase expression controlled by $rpoS_{Bb}$ leader variants in *E. coli* and in *B. burgdorferi*. Our results indicate that the presence of the putative SD sequence is essential for strong ribosome affinity and that sequestration of the sequence reduces translational efficiency. Our results support the models of Lybecker and Samuels [7,18,26] that the RL transcript is poorly translated and that translational efficiency is modulated by a conformational change in the mRNA facilitated by base-pairing a nucleic acid to the 5′ UTR of *rpoS* mRNA.

2. Materials and methods

Bacterial culture conditions and construction of variants used in these studies are described in the Supplementary Materials and Methods. All reagents were Molecular Biology grade or better.

2.1. rpoS and variant mRNA transcripts

RNA transcripts were prepared using T7 RNA polymerase (Promega, Madison, WI) from PCR fragments and purified as described previously [25]. 1 μM RNA was denatured in standard renaturation buffer (30 mM Tris–Cl (pH 7.3) and 100 mM potassium acetate) at 65 °C for 5 min and slow cooled to temperatures below 37 °C. In assays involving primer extension, the appropriate 5′ γ-^{32}P labeled primer was added to the RNA before denaturation. In assays involving the $DsrA_{Bb}$-like DNA oligonucleotide, the $DsrA_{Bb}$-like DNA oligonucleotide was added to the RNA to a final concentration of 1 μM prior to denaturation.

2.2. 30S subunits

E. coli 30S subunits were purified as previously described [25]. $30S_{Ec}$ were renatured in binding buffer (20 mM Tris–Cl pH 7.5 at 4 °C, 100 mM NH_4Cl, 10.5 mM magnesium acetate, 0.5 mM EDTA, 3 mM 2-mercaptoethanol) at 37 °C for 20 min before use.

2.3. Toeprint assays

In toeprint assays [28], varying concentrations of $30S_{Ec}$ (from 100–1000 nM), 1 μM $tRNA_f^{Met}$ and renatured mRNA transcripts (typically 2 pmol) were incubated together in toeprint buffer (100 mM potassium acetate, 30 mM Tris–Cl, 1 mM Spermidine, 10 mM $MgCl_2$, 1 mM DTT) for 15 min at 37 °C. 0.5–1 units of AMV Reverse Transcriptase (Promege, Madison, WI) were added and reactions were incubated another 20 min. Sequencing reactions consisted of ~2 pmol of renatured mRNA transcript annealed to the appropriate radioactive primer, 1 unit of AMV Reverse Transcriptase, ddNTP mix (48 μM each NTP, 4.8 μM each ddNTP) (USB/Affymetrix, Santa Clara, CA), 50 mM Tris–HCl, pH 8.3, 10 mM $MgCl_2$, 0.5 mM spermidine, and 10 mM DTT). Reactions were incubated for 20 min at 42 °C. All reactions were quenched on ice. All reactions were placed in loading buffer (95% Formamide, 18 mM EDTA, 0.025% SDS, and Bromophenol Blue), heated to 90 °C for 3 min and run on 10% (0.5× TBE) denaturing acrylamide gels. Gels were dried and exposed overnight to phosphorimager plates. The plates were scanned with a Fuji FLA-5000 imaging system.

2.4. Luciferase assay

Luciferase assays were performed in a Glomax Multi Jr. luminometer using a Luciferase Assay System kit (Promega, Madison, WI). Transformed DH5α *E. coli* were grown to an OD590 of 0.4 to 0.6 at 37 °C with shaking. *B. burgdorferi* were grown to a density of 7.5×10^6 to 8.5×10^7 cells/ml at 35 °C or 26 °C and 5% CO_2. Cells were harvested by centrifugation in aliquots of 4×10^7 cells, and treated according to the manufacturer's protocol. Luciferase activity was measured in relative luminescence units (RLUs) for 10 s after a 2 s delay.

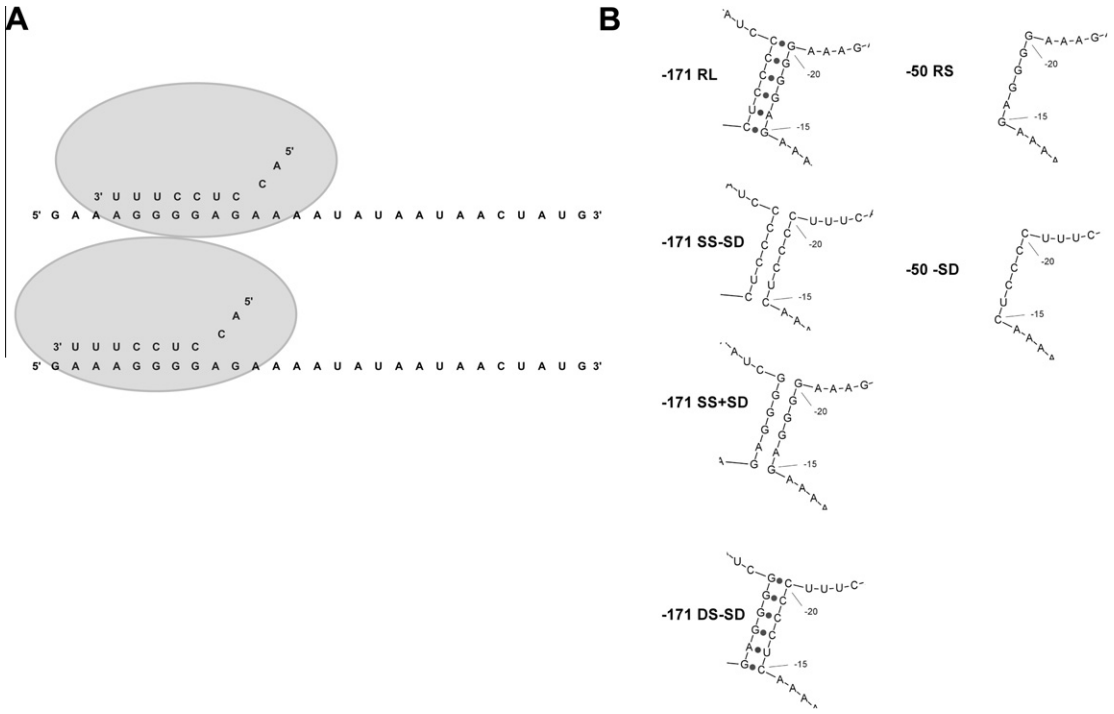

Fig. 2. Predicted Shine–Dalgarno Interactions in *rpoS* mRNA Sequence Variants. (A) The 5′ leader region of *rpoS* mRNA [18] has two potential Shine–Dalgarno (SD) sequences that would allow pairing to the 3′ end of the 16S rRNA in the 30S ribosomal subunit (grey oval) of *B. burgdorferi*. (B) The putative SD sequence is predicted to be base-paired to an upstream sequence in the long *rpoS* transcript RL. Shown is the sequence and potential base-pairing of this helix for RL and for a series of sequence variants that alter the sequence of the putative SD region and/or the trans-acting upstream sequence which sequesters it. Sequences predicted to lack secondary structure upstream of the initiation codon are RS, −50 SS−SD, −171 S−SD and −171 SS+SD. RL and −171 DS−SD are predicted to have a paired helix upstream of the initiation codon. Constructs RS, RL, and −171 SS+SD are expected to have functional SD sequences.

3. Results and discussion

Induction of rpoS expression is required for transmission of *B. burgdorferi* from a tick to a vertebrate host [7,8,15,17]. It has been proposed that the folded RL transcript, found at low cell density and temperature mimicking the midgut of an unfed tick host, has poor ribosome binding site accessibility and that interactions with $DsrA_{Bb}$ increase its translational efficiency [18]. Additionally, the RS transcript, which does not form the same inhibitory structure and is found at higher cell density and temperature, should have a relatively high translational efficiency [18] (Fig. 1).

To examine the effects of the putative SD sequence and the mRNA secondary structure on translational efficiency, a series of sequence variants were constructed. The sequence variants were designed to replace the SD sequence with its complement (designated by -SD) or to replace the 5′ UTR sequence that pairs to the putative SD sequence with its complement. The double variant (restoring the base-pairing) was also generated (Fig. 2B). The variants were named by the position of their 5′ end (−171 or −50), the expectation that the sequence would be single stranded (SS) or double stranded (DS) and the presence (+SD) or absence of the SD sequence (−SD).

To determine that ribosome binding is occurring at the initiation codon and to determine the level of translational efficiency *in vitro*, we performed toeprint assays. The relative abundance of the signal from the toeprint fragment to the signal from the full length fragment is a measure of the translational efficiency in single time point kinetic assays. We observed that primer extension of RS or RL in the absence of either $tRNA_f^{met}$ or $30S_{Ec}$ resulted in a full length primer extension product (Fig. 3). The pattern and intensity of toeprint signals depends upon the secondary structure of the mRNA. For the single stranded constructs containing a SD sequence, RS and −171 SS+SD, intense toeprint signals are observed at the expected position, +16 (numbered with +1 corresponding to the translation start site AUG) (e.g. Figs. 3 and S3). The RL transcript results in two low intensity toeprint signals at residues +16 and +31 relative to the initiation codon, corresponding to occupancy of the downstream AUG alternative ribosome binding site (Figs. 3 and S3). Variants missing the SD sequence (−171 SS−SD, −50 SS−SD, −171 DS−SD) all showed toeprint signals at +16 corresponding to occupancy of the initiation codon of the *rpoS* gene, although at reduced intensity, and at position +31 (Fig. 3). These data suggest that under our conditions, the SD sequence is not essential, but is helpful for formation of a stable *in vitro* translation initiation complex.

The toeprint signals at positions +16 and +31 require an AUG codon 16 or 15 nucleotides respectively upstream (Fig. S3). Other toeprint signals, further downstream of additional AUG sequences in the coding region of the *rpoS* gene were also observed (data not shown). Although alternative ribosome binding sites complicate quantitative analysis of translational efficiency, the intensity of the toeprint signal arising from occupancy at the *rpoS* initiation codon is always higher for constructs with limited secondary structure and functional SD sequences (RS and SS+SD) at all concentrations of $30S_{Ec}$ tested. Our results indicate that sequestering the SD sequence reduces translational efficiency. They also support a hypothesis that the ribosome occupies a stand-by site on the mRNA of a highly structured message to facilitate rapid occupancy of the appropriate ribosome binding site on the time scale associated with the kinetics of secondary structure shifts in the mRNA [29].

A DNA oligonucleotide that mimicked the action of the *E. coli* DsrA increased ribosome accessibility on *E. coli rpoS* leader sequences [25]. We observe a similar effect here. Annealing the

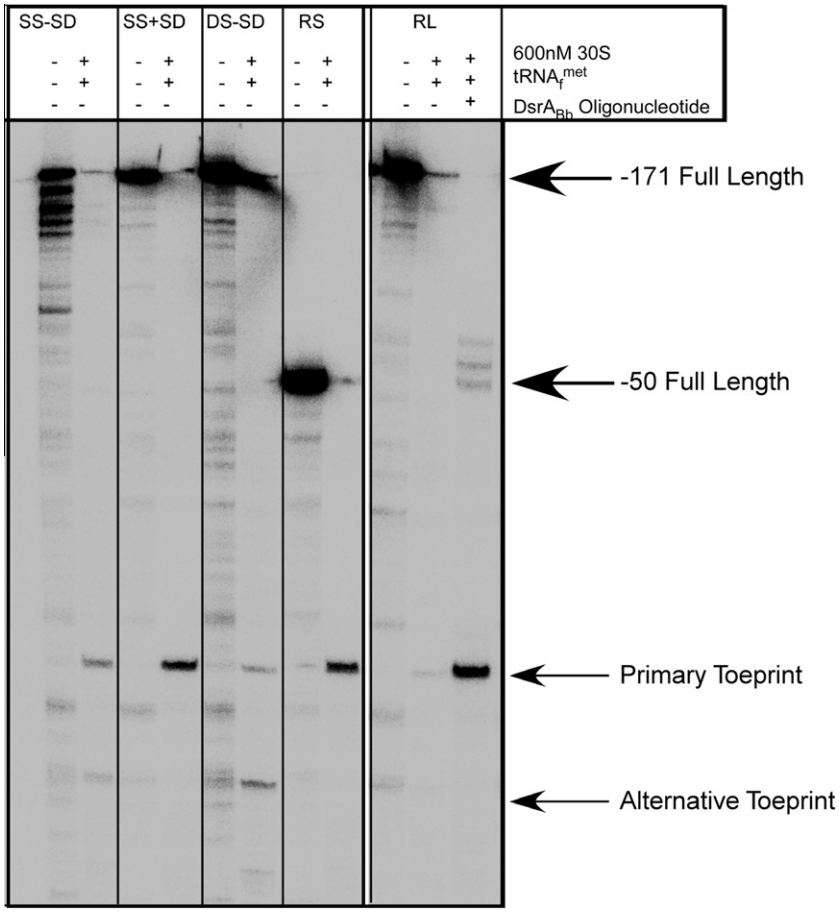

Fig. 3. Interactions of *E. coli* 30S subunits with Sequence and Structure Variants of *rpoS* mRNA. Sequence variants altering the secondary structure and/or eliminating the presence of the Shine–Dalgarno sequence influence the intensities of the primary and alternative toeprint signals. Annealing the DsrA$_{Bb}$-like DNA oligonucleotide increases the signal of the primary toeprint. The location of the full length transcripts for the −171 constructs (RL, FC and SC) and the −50 construct (RS) are indicated as well as the location of the primary toeprint located at +16 and a secondary, alternative toeprint located at position +31.

DsrA$_{Bb}$-like DNA oligonucleotide(DsrA$_{BbDNA}$) to RL before primer extension results in inhibition of transcription of the full length product and in formation of truncated primer extension products corresponding to the annealing site of the rpoS: DsrA$_{BbDNA}$duplex (Figs. 3 and S3). This result suggests that the DNA:RNA hybrid is resistant to dissociation by AMV Reverse Transcriptase. In the presence of 30S$_{Ec}$ and initiator tRNA$_f^{met}$, toeprint signals appear downstream of the initiation codon. The intensity of the toeprint signal arising from occupancy of the initiation codon is increased and the signal arising from occupancy of the downstream site (the alternative toeprint signal) is reduced in the presence of DsrA$_{BbDNA}$ (Figs. 3 and S3), suggesting that melting out mRNA secondary structure by interacting with the DsrA$_{BbDNA}$ increases 30S$_{Ec}$ binding to the initiation codon and reduces the binding at the alternative ribosome binding site. These data strongly suggest that the ribosome accessibility for RS and the RL:DsrA$_{BbDNA}$ duplex are higher than for RL. Experiments performed with 30S$_{Ec}$ indicate a larger fraction of the mRNA participates in ribosome binding for the RS fragment and for the RL fragment annealed to DsrA$_{BbDNA}$ than to RL (Fig. S1 and Table SR1). Affinities are similar to those previously reported [30]. Structure mapping data further supports the hypothesis that DsrA$_{BbDNA}$ alters the mRNA secondary structure to increase the single-stranded nature of the SD sequence (Fig. S2).

In order to assess the importance of sequences and the potential for forming secondary structures that sequester the SD sequence *in vivo*, translational fusions were constructed using the leader sequences of the *rpoS* variants described above. The *rpoS* leader regions were subcloned into the NdeI site of pJSB541 downstream of a *flaB* promoter and upstream of a luciferase gene codon optimized for expression in *B. burgdorferi*[31] as described in Supplementary Materials and Methods. The levels of luciferase expression were measured for the RL, RS, −171 SS+SD, −171 SS−SD, −171 DS−SD and −50 SS−SD translational fusion constructs in both *E. coli* DH5α and in *B. burgdorferi* B31 cfarm (Fig. 4). As expected, the SD sequence and the predicted secondary structure of the mRNA played a significant role in determining the amount of luciferase expressed. In *E. coli* and *B. burgdorferi*, high levels (nearly identical) of luciferase were made from constructs containing SD sequences and that were predicted to be single stranded (RS and −171 SS+SD). In *E. coli*, very low levels of luciferase were expressed from constructs lacking a SD sequence (−171 DS−SD, −171 SS−SD, −50 SS−SD) or predicted to have significant secondary structure (RL, −171 DS−SD). Results in *B. burgdorferi* were similar to the results in *E. coli* with the exception of expression from the construct containing the RL sequence. In *B. burgdorferi*, but not *E. coli*, RL levels were significantly higher than constructs lacking a SD sequence. *B. burgdorferi* contains the trans-acting regulators DsrA$_{Bb}$ and Hfq$_{Bb}$ [7,18,26] necessary for melting out the secondary structure which sequesters the SD sequence in this construct. This effect is specific to the RL construct and not observed in constructs lacking a SD sequence or significant secondary structure (RS, −171 DS−SD, −50 SS−SD, −171 SS−SD, −171 SS+SD).

Lybecker and Samuels have postulated that the effects of DsrA$_{Bb}$ on *rpoS* expression are temperature dependent [18]. They proposed

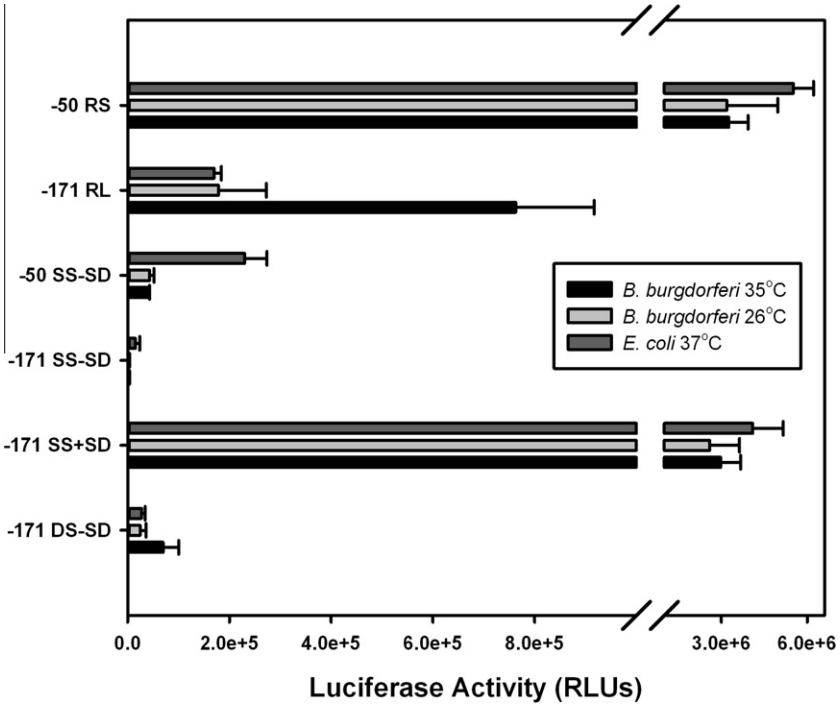

Fig. 4. *In vivo* expression data for translational fusions controlling luciferase expression. Translational efficiency of *rpoS* leader region variants as measured by luciferase activity is shown for *B. burgdorferi* grown at 35 °C ($n = 4$) and 26 °C (−50RS: $n = 5$, −171RL: $n = 4$, others: $n = 3$) and for *E. coli* at 37 °C (−171RL: $n = 3$, others: $n = 4$). Luciferase activity was measured in relative luminescence units (RLUs). Constructs in which the Shine–Dalgarno sequence was expected to be available for ribosome binding (−50 RS, −171 SS+SD) showed high levels of expression. In constructs in which the Shine–Dalgarno sequence was either missing (−50 SS−SD, −171 SS−SD, −171 DS−SD) or expected to be sequestered (−171 RL), expression levels were greatly reduced. The exception to this trend is seen in the −171 RL construct in *B. burgdorferi* grown at 35 °C, where expression levels are somewhat enhanced relative to the same construct in *E. coli* and in *B. burgdorferi* at 26 °C.

that at elevated temperatures (35 °C) $DsrA_{Bb}$ has a positive effect on expression that is not present at lower temperatures. Within the uncertainty of our experiments, only the constructs which are predicted to be double stranded (RL and −171 DS−SD) show a difference (>4-fold and >2-fold respectively) in the expression levels at 35 °C and 26 °C (Fig. 4) indicating that the temperature-dependent *rpoS* induction requires the presence of an extended 5′ leader region containing the binding site for $DsrA_{Bb}$ and involves base pairing interactions that sequester the region upstream of the *rpoS* start codon.

Samuels and coworkers have reported that in an RpoN mutant (where RS is not transcribed) rpoS levels are low suggesting poor translational efficiency of RL, even in the presence of $DsrA_{Bb}$ and Hfq_{Bb}[7]. Our *in vitro* results indicate that RL hybridized to a $DsrA_{Bb}$-like oligonucleotide has a high translational efficiency with $30S_{Ec}$: the toeprint signal arising from occupancy of the initiation codon of RL hybridized to $DsrA_{BbDNA}$ is nearly the same as that observed in the RS construct. Although our *in vitro* experiments use $30S_{Ec}$, the 3′ ends of the 16S rRNA in *E. coli* (5′…tggatcacctcctta 3′ NCBI Reference Sequence: NC_000913.2) and in *B. burgdorferi* (5′…tggatcacctccttt 3′ NCBI Reference Sequence: NC_001318.1) are identical in all but the terminal nucleotide. Thus we expect that the first steps in translational initiation, 30S:mRNA interactions and binding of the initiator tRNA [20], are likely to be similar in *E. coli* and *B. burgdorferi*. It is more likely that our high levels of translational efficiency observed *in vitro* result from the use of $DsrA_{BbDNA}$. $DsrA_{BbDNA}$ does have a larger effect on translational efficiency than a $DsrA_{Bb}$ transcript (data not shown), suggesting that the hybridization *in vitro* with this synthetic oligonucleotide is more efficient than the natural regulator. *In vivo*, expression of RL translational fusions is lower than RS-fusions, but still moderately high. Our translational fusions may be slightly more efficient than the natural transcript because they are truncated at the initiation codon and may lack sequences that are essential for translational regulation *in vivo* or that influence rates of mRNA degradation or processing. Regardless, the data presented indicate that the secondary structure of RL sequesters a sequence that is important for translational efficiency, and that altering the structure of the RNA through annealing a nucleic acid to the upstream region of the mRNA where $DsrA_{Bb}$ binds increases translational efficiency.

Very few mechanisms for transcriptional regulation in *B. burgdorferi* have been identified despite extensive efforts [7]. Our studies demonstrate that translational regulation of gene expression in *B. burgdorferi* may be an important mechanism for responding to environmental signals important in the enzootic cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.063.

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