A Dual Luciferase Reporter System for *B. burgdorferi* Measures Transcriptional Activity during Tick-Pathogen Interactions

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Knowledge of the transcriptional responses of vector-borne pathogens at the vector-pathogen interface is critical for understanding disease transmission. *Borrelia (Borreliella) burgdorferi*, the causative agent of Lyme disease in the United States, is transmitted by the bite of infected *Ixodes* sp. ticks. It is known that *B. burgdorferi* has altered patterns of gene expression during tick acquisition, persistence and transmission. Recently, we and others have discovered *in vitro* expression of RNAs found internal, overlapping, and antisense to annotated open reading frames in the *B. burgdorferi* genome. However, there is a lack of molecular genetic tools for *B. burgdorferi* for quantitative, strand-specific, comparative analysis of these transcripts in distinct environments such as the arthropod vector. To address this need, we have developed a dual luciferase reporter system to quantify *B. burgdorferi* promoter activities in a strand-specific manner. We demonstrate that constitutive expression of a *B. burgdorferi* codon-optimized *Renilla reniformis* luciferase gene (rluc$_{Bb}$) allows normalization of the activity of a promoter of interest when fused to the *B. burgdorferi* codon-optimized *Photinus pyralis* luciferase gene (fluc$_{Bb}$) on the same plasmid. Using the well characterized, differentially regulated, promoters for flagellin (*flaBp*), outer surface protein A (*ospAp*) and outer surface protein C (*ospCp*), we document the efficacy of the dual luciferase system for quantitation of promoter activities during *in vitro* growth and in infected ticks. Cumulatively, the dual luciferase method outlined herein is the first dual reporter system for *B. burgdorferi*, providing a novel and highly versatile approach for strand-specific molecular genetic analyses.

**Keywords:** *Borrelia (Borreliella) burgdorferi*, Lyme disease, tick-pathogen interactions, bioluminescence reporter, *Photinus reniformis* luciferase, *Photinus pyralis* luciferase

**INTRODUCTION**

Vector-borne illnesses account for 17% of worldwide infectious diseases, amounting to over one billion cases yearly (World Health Organization, 2016). Ticks are notorious for delivering a diversity of infectious agents to their hosts during the blood meal. Of these pathogens the *Borrelia burgdorferi* sensu lato complex or *Borreliella* genus (Adeolu and Gupta, 2014), the spirochete group...
that causes Lyme disease, contributes the highest incidence of arthropod-transmitted bacterial infection worldwide (Schotthoefer and Frost, 2015). Particular to the United States, Borrelia (Borrelia) burgdorferi interaction with and colonization of Ixodes species is highly specific (de Silva et al., 2009), with no other natural arthropod vector identified to date.

Newly hatched larval ticks are not colonized with B. burgdorferi, as there is currently no evidence to support transovarial transmission of the pathogen (Rollend et al., 2013). Rather, larvae can become infected by feeding on one of the numerous small vertebrates that serve as reservoirs for B. burgdorferi in nature, such as the white-footed mouse Peromyscus leucopus. Larval ticks then undergo an approximate month-long morphogenesis process and molt into nymphs. All the while, B. burgdorferi reside in the tick midgut. Like the larvae, the infected nymphs take a single blood meal from a vertebrate followed by morphogenesis to adults. During nymph feeding, B. burgdorferi migrate from the midgut to the tick salivary glands and are transmitted to the vertebrate host, maintaining the spirochete in its enzootic cycle (Radolf et al., 2012). Therefore, it has been proposed that B. burgdorferi undergoes three major tick-related events that require complex genetic regulation: acquisition, persistence, and transmission (Iyer et al., 2015; Caimano et al., 2016).

Survival of B. burgdorferi in the tick requires that the spirochete overcome a number of environmental stress conditions, such as starvation and assault from tick immune factors (Radolf et al., 2012; Caimano et al., 2016). Recently, open reading frame-based microarray analysis has provided insight into the gene expression changes that occur in the B. burgdorferi transcriptome in fed larvae, fed nymphs, and under mammalian host-like conditions in dialysis membrane chambers (Iyer et al., 2015). The unusual structure of B. burgdorferi’s genome and its scarcity of characterized transcription factors, further contribute to interest in understanding the mechanisms of stress adaptation and gene regulation that the spirochete employs during its interaction with the tick vector. The B. burgdorferi segmented genome, in characterized type strain B31, is composed of an approximate 900 kb linear chromosome and 21 plasmids of size ranges 5–56 kb that include many annotated open reading frames (ORFs) of unknown function (Fraser et al., 1997; Casiens et al., 2012). A recent global examination and 5′ end mapping of the B. burgdorferi transcriptome by our laboratory has revealed that the spirochete is rich with “overlapping transcripts” where 63% of total RNA species are transcribed internal and 13% antisense to annotated open reading frames (Adams et al., 2017). Other recent RNA-seq based applications have also described the presence of these transcripts in B. burgdorferi (Arnold et al., 2016; Popitsch et al., 2017). These findings are supported by similar analyses in other bacteria, which have revealed complex transcriptomes that include a variety of antisense, intragenic, intergenic, and orphan transcripts, which in some cases represent the majority of transcript types as opposed to mRNAs for annotated open reading frames (Sharma et al., 2010; Kroger et al., 2012; Thomason et al., 2015). These discoveries drive the need for the development of new molecular genetic tools for investigating the expression patterns and functional roles of novel RNA transcripts in a strand-specific manner.

For over three decades, researchers have been isolating, expressing, and adapting bioluminescence genes for biomedical applications (de Wet et al., 1987; Lorenz et al., 1991). These techniques are based on the enzymatic (i.e., luciferase) oxidation of a substrate (i.e., luciferin) to generate light. Transcriptional reporters using bioluminescence read-outs have proven to be robust and sensitive molecular tools for investigating transcript expression (Andreu et al., 2011). Infectious disease-based research has resulted in the development of multiple luciferase systems for a variety of pathogens, and demonstrated that relative luciferase units of constitutively expressed bioluminescence reporters correlate to bacterial numbers (Andreu et al., 2011). Advanced and high-throughput adaptations for transcriptional reporters utilize multiple luciferase enzymes with unique substrates, which are compatible within the same experimental setup. In this manner, one luciferase serves as an experimental readout of promoter activity and the other as the normalization control for cell number (McNabb et al., 2005; Wright et al., 2005). A previously engineered B. burgdorferi codon-optimized Photinus pyralis (firefly) luciferase gene (Blevins et al., 2007), when fused to a constitutive promoter, has been successful for in vivo live imaging of B. burgdorferi dissemination during mouse infection (Hyde et al., 2011; Wager et al., 2015). Furthermore, this luciferase reporter has been used to characterize the promoters for a variety of annotated ORFs and novel RNAs during in vitro cultivation, in vivo mouse infection, and in infected mouse tissues ex vivo (Skare et al., 2016; Adams et al., 2017). However, this reporter plasmid is limited in that it does not contain a constitutive control reporter to allow normalization and quantitation of the data. In order to expand the utility of this approach, we engineered a dual luciferase plasmid that carries both a constitutively expressed B. burgdorferi codon-optimized Renilla reniformis (sea pansy) luciferase gene and the B. burgdorferi codon-optimized Photinus pyralis (firefly) luciferase gene driven by a promoter of interest. Luciferin, the substrate of Photinus pyralis luciferase, emits yellow-green photons (550–570 nm) of light (Marques and Esteves da Silva, 2009), whereas coelenterazine, the substrate of Renilla reniformis luciferase, produces light in the blue spectrum (470 nm) (Woo et al., 2008). Functioning on the premise that each luciferase enzyme requires unique substrates for bioluminescence readout, this approach provides a method for quantitative measurement of strand-specific transcription, in an environment of interest. It has been previously demonstrated that coelenterazine-based luciferase reporters are ineffective for in vivo live imaging detection of bacterial pathogens during murine infection (Andreu et al., 2010), despite successful in vivo applications for mammalian tumor systems (Bhaumik and Gambhir, 2002). Herein, our studies demonstrate the efficacy of the B. burgdorferi dual luciferase system for genetic studies during in vitro cultivation of spirochetes and analysis of transcriptional activity that occurs at the tick-pathogen interface, which is critical for understanding the interactions of B. burgdorferi with the tick vector for the development of novel therapeutic strategies for Lyme disease.
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

*B. burgdorferi* clones used in this study were derived from strain B31. For genetic manipulations infectious low-passage clone A3-68Δbbb02 was utilized, which lacks cp9, lp56, and gene bbe02 on lp25 (Rego et al., 2011), and herein referred to as wild type. Spirochetes were cultivated in liquid Barbour-Stoenner-Kelly (BSK) II medium supplemented with gelatin and 6% rabbit serum (Barbour, 1984) and grown at 35°C with 2.5% CO₂. Luciferase plasmids were engineered in DH5α. Transformants were selected by resuspending in PBS to a density of 2 × 10⁸ spirochetes/ml and grown in BSKII medium and pelleted at 3,210 × g for 10 min. Cells were washed with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and resuspended in 300 µl of PBS. Eighty microliters of each sample was used to measure the optical density at 600 nm (OD₆₀₀) using a BioTek Synergy 4. This resulted in an average OD₆₀₀ value of 0.25 for logarithmic phase spirochetes and 0.36 for stationary phase spirochetes. One hundred microliters of each sample was loaded into a black, solid bottom 96-well plate (Corning) and combined with 700 µM D-luciferin (Regis) in PBS or 3.5 mM water soluble native coelenterazine (NanoLight Technology) in PBS. For samples containing coelenterazine, one well was left empty, in all directions around each sample, to decrease signal overlap between samples. For determining *B burgdorferi* Photinus luciferase (FlucBb) and Renilla luciferase (RlucBb) sensitivity, spirochetes containing pCFA801 were grown to logarithmic phase in 15 ml of BSKII medium, cell density determined using an EnVision 2104 Multilabel Reader (PerkinElmer), following measuring photon emission in each well for 1 s, 10 times using the average RLUs for PBS alone plus 3 or 10 standard deviations, or Rluc units/10⁶ cells/ml. Samples were serial diluted 10-fold and 100 µl of each dilution was loaded into a black, solid bottom 96-well plate (Corning) and combined with 700 µM D-luciferin or 3.5 mM coelenterazine. The relative luciferase units (RLUs) for FlucBb and RlucBb were determined by measuring photon emission in each well for 1 s, 10 times using the EnVision 2104 Multilabel Reader (PerkinElmer), following the addition of luciferin or coelenterazine substrate, respectively. Background relative FlucBb or RlucBb units, the average RLUs of the PBS control for either substrate, was subtracted from all experimental measurements, as appropriate. Background-subtracted relative FlucBb units were then normalized to the OD₆₀₀ value or 10⁸ background-subtracted relative RlucBb units of the same sample, when applicable (e.g., 4 × 10⁴ FlucBb units/0.1 OD₆₀₀ value = 4 × 10⁵ relative FlucBb units/OD₆₀₀; 4 × 10⁴ FlucBb units/0.06 10⁸ relative RlucBb units = 6.4 × 10⁵ relative FlucBb units/10⁸ RlucBb units). The limit of detection (LoD) and quantification (LoQ) for FlucBb and RlucBb were established as the average RLUs for PBS alone plus 3 or 10 standard deviations, respectively. All experiments were conducted in biological triplicate.

Construction of the Dual Luciferase Plasmids

The *Renilla reniformis* luciferase gene (Lorenz et al., 1991) was codon-optimized for *B. burgdorferi* (rlucBb) with the OptimumGene algorithm, synthesized, and cloned into the *E. coli* vector pUC18 (Genscript) (Genebank accession number MF043582). All primer sequences are listed in Table 1. The rlucBb gene was PCR amplified from pUC18-rlucBb plasmid DNA using Phusion High-fidelity DNA polymerase (NEB) and primer pair 1732 and 1733. This also resulted in the addition of 27 bp of DNA from the 3′ of the flaB promoter to the 5′ of rlucBb. Concurrently, a DNA fragment containing the flaB promoter sequence with a 24 bp overhang from the 5′ of the rlucBb gene was Phusion-PCR amplified using B31 A3 genomic DNA and primer pair 1730 and 1731. The PCR fragments were ligated together by combining Gibson Assembly® Master Mix (NEB) and 0.16 pmol of each PCR fragment and incubating the reaction at 50°C for 1 h. One microliter of assembled product (flaBp-rlucBb) was Phusion-PCR amplified using primers 1730 and 1733 and the DNA fragment gel extracted using the QIAquick Gel Extraction kit (Qiagen), and ligated to the Zero Blunt PCR cloning cassette using T4 DNA ligase (NEB), generating plasmids pCFA701, pCFA801, pCFA802, and pCFA803. All plasmid constructs were verified by PCR, restriction digest, and Sanger sequencing.

### TABLE 1 | Oligonucleotide primers used in this study.

| Number | Name | Sequence (5′-3′) |
|--------|------|----------------|
| 1730   | flaBp 5′ | TGTCTGTCCCTCCCTTGTG |
| 1731   | flaBp 5′, 24 bp overlay rlucBb 5′ | AGGATCATAAATTCCTTGGCTCATGATTGAAACATATATATTCCCTCA |
| 1732   | rlucBb 5′, 27 bp overlay flaBp 3′ | TGCAAGAATTATATGATTACATCAGCAAGTAAGTTTATCCATC |
| 1733   | rlucBb 3′ | TATATTTCTCTTCCAAATCTG |
| 1850   | rlucBb 3′ KpnI | CTAAAGGATACCTTTGATTTTCCATATCCTGTC |
| 1910   | flaBp 5′ BamHI | GTGCGCGATCTGGCTTCGGCGTCCTTGTGGC |
Ethics Statement
The University of Central Florida is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and were reviewed and approved by the University of Central Florida Institutional Animal Care and Use Committee.

*B. burgdorferi* Infection of Ticks
One week prior to inoculation, and throughout the duration of the study, mice were treated with 5 mg/ml streptomycin and 1 mg/ml Equal® sweetener in their water to maintain selection for the luciferase plasmid in the *B. burgdorferi* clones. Using *B. burgdorferi* carrying pJSB175, pCFA701, pCFA801, pCFA802, or pCFA803, groups of two 6–8 week old female C3H/HeN mice (Envigo) per clone were inoculated with 1 × 10^5 spirochetes per mouse 80% intraperitoneally and 20% subcutaneously. The inoculum doses were verified by colony forming unit (CFU) counts in solid BSKII medium. All inoculum were PCR verified to contain the endogenous *B. burgdorferi* plasmids of the parent clone as previously described (Elias et al., 2002; Jewett et al., 2007). Three weeks post inoculation mouse infection was confirmed by positive seroreactivity against *B. burgdorferi* protein lysate as previously described (Schwan et al., 1989; Jewett et al., 2007). Groups of approximately 200 naïve *Ixodes scapularis* larvae each (Centers for Disease Control, BEI resources) were fed to repletion on the *B. burgdorferi* infected mice (Jewett et al., 2009). Mice were further confirmed for infection by reisolation of spirochetes from bladder and joint tissues, as described (Showman et al., 2016). Larvae were analyzed for infection (Grimm et al., 2005; Jewett et al., 2009). Briefly, ticks were individually surface sterilized by sequential washes in 100 µl of 3% H2O2, 70% ethanol, and sterile H3O. Subsets of larvae were analyzed for infection by reisolation of spirochetes in BSKII medium containing RPA cocktail (60 µM rifampicin, 110 µM phosphomycin, and 2.7 µM amphotericin B), immediately post feeding to repletion. Approximately 2 weeks following feeding, additional subsets of larvae were crushed and plated in solid BSKII containing RPA cocktail and 50 µg/ml streptomycin to determine CFU counts/larva. The remaining larvae were maintained and allowed to molt into nymphs. Two groups of 10–18 infected nymphs per *B. burgdorferi* clone were fed to repletion on naïve 6–8 week old female C3H/HeN mice (Envigo). These mice were treated with 5 mg/ml streptomycin and 1 mg/ml Equal® sweetener in their water 1 week prior to the feeding, to help sustain the luciferase plasmids in *B. burgdorferi* within the feeding nymphs. Throughout the duration of the study, ticks were stored in glass desiccation jars containing saturated potassium sulfate for to maintain appropriate humidity.

**In vivo** Tick Dual Luciferase Assay
Approximately 2 weeks post feeding to repletion triplicate groups of 24 fed larvae or 8 fed nymphs per *B. burgdorferi* clone were crushed with a sterile pestle in 250 µl of PBS to generate tick extracts. For tick extracts, which were also plated for CFU counts, the ticks were first surface sterilized as described above, with a final wash in sterile PBS instead of H2O. Tick debris was allowed to settle and 100 µl of sample was removed and incubated with 700 µM D-luciferin (Regis) in PBS or 3.5 mM water soluble native coelenterazine (NanoLight Technology) in PBS. RLUs were measured as described for *in vitro* grown spirochetes. The limit of quantification (LoQ) for Fluc<sub>Bb</sub> was established as the average relative Fluc<sub>Bb</sub> units for PBS alone plus 10 standard deviations. The LoQ for Rluc<sub>Bb</sub> was established as the average relative Rluc<sub>Bb</sub> units for infected tick extracts with spirochetes containing pJSB175, which lacks the rlu<sub>Bb</sub> gene, plus 10 standard deviations. Samples with relative Fluc<sub>Bb</sub> units below the LoQ were given a value of zero; whereas, samples with relative Rluc<sub>Bb</sub> units below the LoQ were removed from the analysis. Relative Fluc<sub>Bb</sub> units were normalized to 10^8 relative Rluc<sub>Bb</sub> units for each sample. One microliter of each fed nymph extract was also plated for CFUs in solid BSKII containing RPA cocktail and 50 µg/ml streptomycin.

**Statistical Analysis**
GraphPad Prism version 7.02 was used for all statistical analyses. One-way ANOVA was used for analysis of all luciferase assays. For statistical comparison of the relative Fluc<sub>Bb</sub> units normalized to OD<sub>600</sub> or 10<sup>8</sup> relative Fluc<sub>Bb</sub> units, which had an extremely wide distribution (∼10<sup>−1</sup>–10<sup>7</sup>), all values were first square root transformed prior to statistical analysis. Following ANOVA, all samples were compared to the *B. burgdorferi* clones carrying the promoterless *fluc<sub>Bb</sub>* control plasmid pJSB161 or pCFA701 using Dunnett’s multiple comparison test. To compare two groups (i.e., the same clone in logarithmic versus stationary phase) following ANOVA, Bonferroni’s multiple comparison test was applied to determine significance. For association analysis, Pearson correlation coefficient (r) was determined. p ≤ 0.05 was considered statistically significant for all statistical tests.

**RESULTS**
**Generation of the *B. burgdorferi* Dual Luciferase Plasmid**
The *B. burgdorferi* shuttle vector pJSB161 (Blevins et al., 2007) contains a promoterless *B. burgdorferi* codon-optimized *Photinus pyralis* luciferase gene (*fluc<sub>Bb</sub>* with a BlgII restriction site upstream of a ribosome binding site (RBS) for *fluc<sub>Bb</sub>* (Figure 1A). This reporter plasmid allows a cloned promoter of interest to be analyzed for activity in a strand-specific manner via a bioluminescence detection method (Blevins et al., 2007; Skare et al., 2016; Adams et al., 2017). However, this approach does not allow for quantitative comparative analysis of promoter activity in different environments or between multiple promoters in the same environment due to the lack of an endogenous means to control for spirochete number across samples and conditions. Therefore to improve upon this technique for quantiative applications, we engineered a dual luciferase reporter system to constitutively express *Renilla reniformis* luciferase (*rluc*) (Lorenz et al., 1991), while maintaining *fluc<sub>Bb</sub>* for quantifying the activity of a promoter of interest. Codon usage in *B. burgdorferi* is biased (Fraser et al., 1997; Nakamura et al., 2000), as the A/T nucleotide frequency is at 71.8% across the genome (Fraser et al., 1991).
Codon optimization has been shown to improve production and activity of non-\(B.\) burgdorferi proteins expressed in \(B.\) burgdorferi (Blevins et al., 2007; Hayes et al., 2010). Therefore to prevent rare codons interfering with the Renilla luciferase reporter, the OptimumGene\textsuperscript{TM} algorithm (GenScript) was used to refine the codon adaption index (CAI) of \(rluc\) (Lorenz et al., 1991) for \(B.\) burgdorferi from 0.64 to 0.85 (where a CAI value of 1.0 indicates the highest proportion of the most abundant codons) and synthesized (GenScript). This codon-optimized \(rluc\) gene (\(rluc_{BB}\)) (Genebank accession number MF043582) was cloned into \(pJSB161\) (Blevins et al., 2007), for use in the dual luciferase reporter system under control of the constitutive promoter \(flaBp\) and corresponding ribosome binding site, generating \(pCFA701\) (Figure 1B).

\(B.\) burgdorferi survival in the tick vector is essential for maintenance of the pathogen in its enzootic cycle. The spirochete is known to change its transcriptional profile at different stages of tick colonization including: acquisition, persistence during the molt, and transmission to the mammalian host (Iyer et al., 2015; Caimano et al., 2016). Because of our interest in applying the dual luciferase reporter system to quantitative analysis of \(B.\) burgdorferi promoter activities in the tick, we selected three well characterized promoters with distinct patterns of expression in the tick environment for proof of principle analysis. The flagellar protein promoter, \(flaBp\), is constitutively active (Ge et al., 1997). The promoter for outer surface protein A (\(ospA\)) is active during \textit{in vitro} culture and in the tick during acquisition and persistence. In contrast, the promoter for outer surface protein C (\(ospC\)) is active in the feeding tick during transmission and the mammalian host during the early stages of infection (Schwan et al., 1995; Schwan and Piesman, 2000; Schwan, 2003; Srivastava and de Silva, 2008). The \(flaBp-rluc_{BB}\) cassette was cloned into three previously constructed plasmids, each containing one of these promoters driving the expression of \(fluc_{BB}\) (Blevins et al., 2007; Adams et al., 2017), generating plasmids \(pCFA801\), \(pCFA802\), and \(pCFA803\), respectively (Figure 1B). Spirochetes carrying \(pCFA701\), \(pCFA801\), \(pCFA802\), or \(pCFA803\) had no observed \textit{in vitro} growth defect in BSKII medium compared to the wild type \(B.\) burgdorferi carrying \(flaBp-fluc_{BB}\) alone (\(pJSB175\)) (Blevins et al., 2007, Figure 1C).

**RLuc\textsubscript{BB} Selectivity and Limit of Quantification**

\(Photinus pyralis\) luciferase (Fluc) and \(Renilla reniformis\) luciferase (Rluc) are compatible for a dual reporter due to the specificity of each enzyme for distinct substrates (Bhaumik and Gambhir, 2002; McNabb et al., 2005). Therefore, we verified the selectivity of the Fluc\textsubscript{BB} and Rluc\textsubscript{BB} enzymes to recognize luciferin and coelenterazine, respectively. Based on our previous work using the \(flaBp-fluc_{BB}\) reporter (Adams et al., 2017), we...
performed these analyses with approximately $3 \times 10^6$ spirochetes harvested during log phase growth. As expected, the negative control, spirochetes not expressing $\text{fluc}_{Bb}$ or $\text{rluc}_{Bb}$ ($+\text{pJSB161}$), demonstrated no significant relative luciferase units for either substrate compared to wild type. Spirochetes expressing $\text{fluc}_{Bb}$ alone ($+\text{pJSB175}$) demonstrated robust activity when incubated with luciferin, but no significant activity above the background of $\text{B. burgdorferi}$ carrying pJSB161 when exposed to coelenterazine (Figure 2A). Conversely, spirochetes expressing $\text{rluc}_{Bb}$ alone ($+\text{pCFA701}$) demonstrated strong activity when incubated with coelenterazine, but no significant activity above the negative control background when exposed to luciferin (Figure 2A). Spirochetes which express both $\text{fluc}_{Bb}$ and $\text{rluc}_{Bb}$ ($+\text{pCFA801}$) demonstrated significant relative luciferase units compared to spirochetes containing pJSB161 for both luciferin and coelenterazine. The background relative luciferase units for wild type and negative control spirochetes exposed to coelenterazine were found to be approximately 10-fold higher than those of the same spirochetes incubated with luciferin. Collectively, these data validated the ability of the codon-optimized Fluc$_{Bb}$ enzyme to effectively oxidize coelenterazine and confirmed the specificity of the Fluc$_{Bb}$ and Rluc$_{Bb}$ enzymes for their respective substrates.

The utility of the dual luciferase reporter system not only depends on the substrate specificity of Fluc$_{Bb}$ and Rluc$_{Bb}$, but also the sensitivity of detecting and quantifying spirochetes expressing $\text{rluc}_{Bb}$. The limit of detection (LoD) and limit of quantification (LoQ) were established as the number of spirochetes required to achieve relative Rluc$_{Bb}$ units greater than that of phosphate-buffered saline (PBS) alone plus three standard deviations and 10 standard deviations, respectively. Analysis of triplicate samples of 10-fold serially diluted spirochetes, $2 \times 10^8$ to $2 \times 10^0$, harvested during log phase growth and incubated with coelenterazine, demonstrated $2 \times 10^3$ spirochetes to be the lowest detectable number of flaBp-fluc$_{Bb}$ expressing spirochetes in the assay (Figure 2B). However, the LoQ fell between $2 \times 10^3$ and $2 \times 10^4$ spirochetes. Saturation of the bioluminescence signal was never reached under the conditions examined, with a linear increase in relative Rluc$_{Bb}$ units from $2 \times 10^3$ to $2 \times 10^9$ spirochetes ($y = 0.0404x; R^2 = 0.9997$). Extrapolating from this linear equation, the LoQ was calculated to be $4.8 \times 10^3$ spirochetes. These data indicate that a minimum of $\sim 1 \times 10^4$ flaBp-rluc$_{Bb}$ expressing spirochetes are needed to achieve quantifiable relative Rluc$_{Bb}$ units in the assay. Similar to what has been reported previously (Hyde et al., 2011), $2 \times 10^3$ spirochetes was also found to be the lowest detectable number of flaBp-fluc$_{Bb}$ expressing spirochetes (data not shown).

**The flaBp-rluc$_{Bb}$ Reporter is a Robust Constitutive Control for Measuring B. burgdorferi Promoter Activities during In vitro Growth**

Previously, we reported quantification of in vitro active $B. burgdorferi$ promoters by normalizing relative luciferase units (RLUs) from fluc$_{Bb}$ expressing cells to the optical density of the bacterial sample measured at 600 nm (OD$_{600}$) (Adams et al., 2017). In this manner, the OD$_{600}$ measurement reflects the number of spirochetes in the sample allowing normalization of RLUs across samples and assay conditions. To establish the flaBp-rluc$_{Bb}$ reporter as an effective alternative for OD$_{600}$ readings in

![Figure 2](image-url)

**Figure 2** Selectivity and sensitivity of the dual luciferase assay in B. burgdorferi. (A) B. burgdorferi clones were grown to mid-logarithmic phase, and the in vitro luciferase assay performed with 700 µM D-luciferin or 3.5 mM coelenterazine. Relative luciferase units were normalized to optical density at 600 nm (OD$_{600}$) and presented as the mean relative luciferase units/OD$_{600}$ ± standard deviation for biological triplicate samples. The data were square root transformed and analyzed with a one-way ANOVA followed by Dunnnett’s multiple comparison test compared to B. burgdorferi containing the promoterless fluc$_{Bb}$ (+pJSB161) for each substrate. Unless indicated, means were not significantly different from the control. Significant differences are indicated with asterisks (*p < 0.0001). (B) Mid-logarithmic phase grown B. burgdorferi expressing both flaBp-fluc$_{Bb}$ and flaBp-rluc$_{Bb}$ (+pCFA801) were serially diluted from $2 \times 10^6$ to $2 \times 10^0$ spirochetes, and incubated with 3.5 mM coelenterazine. The limit of detection (LoD) was established as the mean relative luciferase units for PBS alone plus 3 standard deviations (gray dotted line). The limit of quantitation (LoQ) was established as the mean relative luciferase units for PBS alone plus 10 standard deviations (red dotted line). Data are presented as the mean relative luciferase units ± standard deviation for biological triplicate samples.
our assay, first, relative $\text{RLuc}_{BB}$ units were measured for all $\text{RLuc}_{BB}$-expressing $B.\ burgdorferi$ clones and normalized to the number of spirochetes in the assay by OD$_{600}$ (Figure 3A). All clones demonstrated consistent, robust relative $\text{RLuc}_{BB}$ units, ranging from $5 \times 10^7$ to $1.68 \times 10^8$. There was no significant difference among clones except for $B.\ burgdorferi$ carrying pCFA802, which demonstrated higher relative $\text{RLuc}_{BB}$ units/OD$_{600}$ compared to all other clones as well as a difference between logarithmic and stationary phase growth. The same $\text{RLuc}_{BB}$-expressing clones, were also incubated with luciferin and relative $\text{Fluc}_{BB}$ units were determined by normalizing to OD$_{600}$ (Figure 3B). All $\text{Fluc}_{BB}$ promoter fusions displayed the expected relative $\text{Fluc}_{BB}$ units/OD$_{600}$, given the known expression patterns of their corresponding mRNA during logarithmic and stationary phase growth (Arnold et al., 2016). Both the $\text{flaB} (+\text{pCFA801})$ and $\text{ospA} (+\text{pCFA802})$ promoters demonstrated significant activity above the promoterless $\text{fluc}_{BB}$ control ($+\text{pCFA701}$) for both logarithmic and stationary phase growth. The activity of the $\text{ospC}$ promoter ($+\text{pCFA803}$) during logarithmic phase growth was no different than the promoterless $\text{fluc}_{BB}$ control ($+\text{pCFA701}$). Whereas, the $\text{ospC}$ promoter activity underwent significant induction from logarithmic to stationary phase growth (Figure 3B). Normalization of the relative $\text{Fluc}_{BB}$ units to $10^8$ relative $\text{RLuc}_{BB}$ units for each clone demonstrated no difference in the trend of the data resulting from this method of analysis compared to the data resulting from $\text{Fluc}_{BB}$ units normalized to OD$_{600}$ (Figure 3B). Together these findings establish $\text{flaB}-\text{rLuc}_{BB}$, as an effective constitutive control reporter, whose quantitation is reflective of spirochete number and is a robust means to normalize data obtained from $\text{fluc}_{BB}$ promoter fusions using the dual luciferase reporter system.

**The Dual Luciferase Reporter System Quantifies Promoter Activities during Tick-Spirochete Interactions**

Having established the dual luciferase reporter system for use with *in vitro* grown spirochetes, we examined the efficacy of the reporter system for measuring *B. burgdorferi* promoter activities in the tick vector. Naïve *Ixodes scapularis* larval ticks were infected with *B. burgdorferi* carrying the dual luciferase reporter plasmids or $\text{flaBp-fluc}_{BB}$, lacking $\text{rLuc}_{BB}$ (+pJSB175) by feeding on groups of mice infected with the reporter clones via needle inoculation. Immediately following feeding to repletion, the percent of infected larvae per experimental group was determined by spirochete reisolation in BSKII medium. This analysis revealed that 60–90% of each experimental group of larvae successfully acquired *B. burgdorferi* upon feeding on infected mice. As an additional means to determine the percentage of infected larvae and to quantitate the number of spirochetes per tick, individual fed larvae were crushed and plated in solid medium for colony forming units (CFUs). Similar to the spirochete reisolation analysis, the groups of fed larvae were found by CFU analysis to be 66–100% infected. Moreover, although a broad range of spirochetes per tick was detected, there was no statistical difference between the average spirochete load per tick for each of the *B. burgdorferi* clones (Figure 4). These data suggest that all of the clones were able to colonize the ticks with the same efficiency.

Based on our quantitation of the average number of spirochetes per tick (Figure 4), we estimated that pools of 24
Fed larvae would equate to approximately $10^4$ spirochetes per sample, suggesting that the Rluc$\text{Bb}$ activity would be quantifiable by our assay (Figure 2B). Therefore, 2 weeks following the blood meal, 24 fed larvae per experimental group, in triplicate, were crushed in PBS and relative Fluc$\text{Bb}$ and Rluc$\text{Bb}$ units measured using the luciferin and coelenterazine substrates, respectively (Table 2). The remaining fed larvae were reserved and allowed to molt into nymphs. The unfed, infected nymphs were then fed to repletion on naïve mice. Approximately 2 weeks post feeding, groups of eight fed nymphs were crushed in PBS, in triplicate, and assessed for relative Fluc$\text{Bb}$ and Rluc$\text{Bb}$ units (Table 2). Under the assumption that the spirochete load per fed nymph is increased approximately 10-fold compared to that of fed larvae (Jewett et al., 2007, 2009), we estimated the average spirochete load per fed nymph to be approximately $10^4$. Therefore, a pool of eight fed nymphs was estimated to equate to approximately $8 \times 10^4$ spirochetes, which is above both the LoD and LoQ of the in vitro assay (Figure 2B). The actual LoQ for the in vivo tick assay was established using the Rluc$\text{Bb}$ units plus 10 standard deviations for tick extracts from fed ticks infected with B. burgdorferi lacking rlu$\text{cBb}$, but expressing flab$p$-fluc$\text{Bb}$, (+pJSB175), rather than PBS alone. This is due to the observation that this tick extract negative control resulted in lower background relative Rluc$\text{Bb}$ units compared to PBS alone (Table 2). In contrast, there was no observed difference in the background relative Fluc$\text{Bb}$ units between PBS and the tick samples containing B. burgdorferi with a promoterless fluc$\text{Bb}$ and expressing flab$p$-rluc$\text{Bb}$ (+pCFA701) in the luciferin assay. Therefore, the LoQ for Fluc$\text{Bb}$ in the in vivo tick assay was determined using the average relative Fluc$\text{Bb}$ units for PBS plus 10 standard deviations. Samples that fell below the LoQ threshold for either luciferase enzyme were considered no different than background (Table 2). As expected, we detected quantifiable relative Rluc$\text{Bb}$ units for all fed larvae samples, and all but two fed nymph samples (Table 2), indicating that sufficient spirochetes were present in the samples for the assay. In the pools of fed larvae only samples containing B. burgdorferi carrying flab$p$-fluc$\text{Bb}$ (+pCFA801) demonstrated quantifiable relative Fluc$\text{Bb}$ units. The activities of osp$\text{Ap}$ and osp$\text{Cp}$ were below the LoQ for Fluc$\text{Bb}$ (Table 2). In contrast, all three promoters produced quantifiable relative Fluc$\text{Bb}$ units in the fed nymphs. Although one of the extracts from the fed nymphs infected with B. burgdorferi carrying osp$\text{Cp}$-fluc$\text{Bb}$ (+pCFA803) did not result in quantifiable relative Fluc$\text{Bb}$ units, this sample also failed to achieve quantifiable relative Rluc$\text{Bb}$ units (Table 2), indicating that the number of spirochetes in the sample was insufficient for the assay. The promoter activities of the spirochetes in the fed nymph samples were analyzed by subtracting the average relative Fluc$\text{Bb}$ units of the infected tick samples, a portion of each sample from the fed infected nymphs used for Rluc$\text{Bb}$ and Fluc$\text{Bb}$ quantitation (Table 2, Figure 5A), was plated in solid BSKII medium for determination of B. burgdorferi CFUs. The average CFUs per 100 µl of tick extract, the same volume used for the dual luciferase assay, across all clones, was found to be $3.72 \times 10^3$ spirochetes. These data support our rationalization for the use of 8 fed nymphs in the assay. Raw relative Rluc$\text{Bb}$ units (Table 2) for these samples plotted against their corresponding CFU counts demonstrated a significant positive correlation (Figure 5B). Furthermore, this analysis indicated that $1.2 \times 10^3$ spirochetes are sufficient to generate relative Rluc$\text{Bb}$ units above the LoQ for the in vivo tick assay, which is similar to the sensitivity we observed for the in vitro assay. Collectively, we have described a valuable new method to determine the activity of B. burgdorferi promoters of interest under in vitro growth conditions and in infected ticks. This is the first application of a dual reporter system for B. burgdorferi and, to the best of our knowledge, the first quantification of spirochete promoter activities in the tick vector.

**DISCUSSION**

Promoter fusion reporter systems are elegant, simple, and powerful tools to quantitate bacterial promoter activities in environments of interest. Herein we have established a new
dual luciferase reporter method using the Renilla (sea pansy) and Photinus (firefly) luciferase enzymes for measurement of B. burgdorferi promoter activities in vitro and in the feeding tick during spirochete acquisition from an infected vertebrate host and transmission to a naïve vertebrate host. We demonstrate that constitutive expression of the B. burgdorferi codon-optimized Renilla luciferase gene (rluc\textsubscript{Bb}) is a specific and sensitive measurement of spirochete numbers for normalization of Photinus luciferase gene (fluc\textsubscript{Bb}) expression under the control of a promoter of interest.

Several reporter genes have been applied to B. burgdorferi including chloramphenicol acetyl transferase (cat) (Sohaskey et al., 1997), genes encoding a variety of fluorescent proteins (Eggers et al., 2002; Carroll et al., 2003; Schulze and Zucker, 2006), the Photinus pyralis luciferase gene (fluc\textsubscript{Bb}) (Blevins et al., 2007), and lacZ encoding β-galactosidase (lacZ\textsubscript{Bb}) (Hayes et al., 2010). Here we describe the first use of a dual reporter system for B. burgdorferi. The combined application of the Renilla and Photinus luciferase genes has several advantages compared to other B. burgdorferi reporter systems as well as other methods of gene expression quantitation such as RT-qPCR. No sample extraction or purification is required to achieve detectable bioluminescence signals, allowing for rapid assay read out with little sample manipulation. Our data indicate that the rluc\textsubscript{Bb} gene under the control of the strong, constitutive flaB promoter results in relative Rluc\textsubscript{Bb} units reflective of the number of live spirochetes. This allows relative Rluc\textsubscript{Bb} units to serve as the endogenous control against which the relative luciferase units of promoter fusions to fluc\textsubscript{Bb} on the same plasmid, in the same sample, can be normalized. It is even possible to measure Fluc\textsubscript{Bb} and Rluc\textsubscript{Bb} signals back-to-back in the same assay well using firefly luciferase quenching reagents, such as Stop & Glo by Promega (McNabb et al., 2005) and therefore little sample material is required. Use of optical density at 600 nm (OD\textsubscript{600}) to quantitate sample turbidity as a measure of cell number does not distinguish between live and dead cells in the sample and therefore may not accurately reflect the number of live cells that contribute to the bioluminescence signal. Furthermore, OD\textsubscript{600} cannot be used for complex biological samples such as extracts from fed ticks. We demonstrate a significant positive correlation between relative Rluc\textsubscript{Bb} units and numbers of live spirochetes both in vitro and in ticks. The B. burgdorferi clone containing pCFA802 exhibited statistically different relative Rluc\textsubscript{Bb} units in vitro when normalized to OD\textsubscript{600} compared to the other clones. However, the relative Fluc\textsubscript{Bb} units/10\textsuperscript{8} relative Rluc\textsubscript{Bb} units for this clone followed the expected pattern of ospA expression in vitro and in nymphs. Furthermore, the relative Fluc\textsubscript{Bb} units for spirochetes carrying pCFA802 correlated to the number of live spirochetes in fed nymph extracts from this clone, suggesting that the observed difference may not result in a biologically significant effect. Utilizing flaBp-fluc\textsubscript{Bb} as an endogenous constitutive control provides new opportunities for the development of novel high-throughput genetic screening approaches. DNA libraries engineered to drive expression of fluc\textsubscript{Bb} could be effectively screened for active promoters in various growth conditions of interest and relative Fluc\textsubscript{Bb} units normalized to relative Rluc\textsubscript{Bb} units. Further, the dual luciferase reporter plasmid can be manipulated to engineer Fluc\textsubscript{Bb} translational fusions to quantitate protein production and stability in growth conditions of interest. An additional important benefit of the dual luciferase

### TABLE 2 | In vivo tick dual luciferase assay.

| Tick life stage | Plasmid | Luciferase cassette(s) | Relative Rluc\textsubscript{Bb} units/biological replicate\textsuperscript{a} | Relative Fluc\textsubscript{Bb} units/biological replicate\textsuperscript{b} |
|----------------|--------|------------------------|--------------------------|--------------------------|
|                |        |                        | 1           | 2           | 3           | 1           | 2           | 3           |
| Fed Larvae\textsuperscript{c} | PBS    | n/a\textsuperscript{2} | 85.2        | 89.6        | 92.8        | 20.0        | 24.8        | 24.4        |
|                | pJSB175 | flaBp-fluc\textsubscript{Bb} | 37.6        | 36.4        | 34.4        | 137.6       | 245.6       | 138         |
|                | pCFA701 | flaBp-rluc\textsubscript{Bb} | 185.6       | 108.8       | 208.0       | 22.4*       | 24.0*       | 24.0*       |
|                | pCFA801 | flaBp-rluc\textsubscript{Bb}, flaBp-fluc\textsubscript{Bb} | 603.6       | 1,502.4     | 1,187.6     | 56.0        | 136.0       | 100.8       |
|                | pCFA802 | flaBp-rluc\textsubscript{Bb}, ospAp-fluc\textsubscript{Bb} | 144.4       | 153.2       | 176.0       | 26.0*       | 25.2*       | 24.0*       |
|                | pCFA803 | flaBp-rluc\textsubscript{Bb}, ospCp-fluc\textsubscript{Bb} | 348.8       | 341.2       | 234.8       | 22.4*       | 23.6*       | 19.6*       |
| Fed Nymph\textsuperscript{d} | PBS    | n/a\textsuperscript{2} | 82.4        | 93.2        | 83.2        | 19.6        | 20.4        | 18.8        |
|                | pJSB175 | flaBp-fluc\textsubscript{Bb} | 31.6        | 31.6        | 30.0        | 504.8       | 1,700.8     | 881.6       |
|                | pCFA701 | flaBp-rluc\textsubscript{Bb} | 41.6        | 29.6*       | 296.0       | 18.0*       | 19.6*       | 18.0*       |
|                | pCFA801 | flaBp-rluc\textsubscript{Bb}, flaBp-fluc\textsubscript{Bb} | 2,416.8     | 2,560.0     | 780.4       | 956         | 552.8       | 388         |
|                | pCFA802 | flaBp-rluc\textsubscript{Bb}, ospAp-fluc\textsubscript{Bb} | 1,001.2     | 2,257.6     | 338.4       | 107.6       | 262         | 36          |
|                | pCFA803 | flaBp-rluc\textsubscript{Bb}, ospCp-fluc\textsubscript{Bb} | 1,500.8     | 1,239.6     | 34.8*       | 21.2*       | 32.8        | 16.4*       |

\textsuperscript{a}Relative Rluc\textsubscript{Bb} units from three independent tick extracts incubated with 3.5 mM coelenterazine.

\textsuperscript{b}Relative Fluc\textsubscript{Bb} units from three independent tick extracts incubated with 700 μM luciferin.

\textsuperscript{c}Not applicable.

\textsuperscript{d}Limit of Quantification (LoQ) defined as the average background signal for each assay plus 10 standard deviations.

\textsuperscript{e}Extract from groups of 24 fed larvae crushed in PBS.

\textsuperscript{f}Extract from groups of 8 fed nymphs crushed in PBS.

\textsuperscript{g}Samples that fell below their respective LoQ.
reporter assay is the ability to quantitate the promoter activity of a transcript in a strand-specific manner. We and others have recently reported recognition of novel RNA transcripts in the B. burgdorferi genome (Arnold et al., 2016; Adams et al., 2017; Popitsch et al., 2017). Through global 5′ end mapping of the B. burgdorferi transcriptome, we have predicted promoter sequences for previously unannotated RNAs, including antisense and intragenic transcripts, and validated their activities in a variety of environments (Adams et al., 2017). Application of the dual luciferase reporter system now provides a robust means for quantitative comparative analysis of strand-specific B. burgdorferi transcription in complex regions of the genome at the tick-pathogen interface.

For the correct interpretation of molecular techniques it is important to define the lowest level of a measurement, in this case relative luciferase units, which can be reliably analyzed. The limit of detection (LoD) is the lowest amount of measurable signal above background and the limit of quantification (LoQ) signifies the lowest interpretable signal above background. Effective use of LoD and LoQ are based off the standard deviation (SD) of background readings and assume at least 95% of analyzed values are true measurements in the biological assay (Armbruster and Pry, 2008). We have stringently defined LoD as the mean-background RLUs + 3SD and LoQ as the mean-background RLUs + 10SD. Thereby LoQ should be calculated for each luciferase substrate and each independent application of the B. burgdorferi dual luciferase assay to best distinguish low but quantifiable bioluminescence signals from background. It is also important to define the appropriate background controls in the context of the assay. Indeed, our studies have demonstrated that background relative RlucBb units were ~60% decreased in fed tick extracts compared to PBS alone. Therefore, extracts from fed ticks infected with B. burgdorferi lacking rluCBb expression (+pJSB175) served as the background control to calculate the LoQ for RlucBb in ticks. Conversely, this was not observed for the background relative FlucBb units for fed tick extracts and PBS alone served as the negative control for these measurements. We hypothesize that the biological matrix of the fed tick extracts contributes, in part, to alteration of the RlucBb signal by inhibiting non-specific activation of the coelenterazine substrate.

We found that not all samples with quantifiable relative RlucBb units, also had quantifiable relative FlucBb units. In some cases, the finding that a promoter fusion has non-quantifiable relative FlucBb units may accurately reflect the weak to no biological activity of that promoter in a particular environment and/or non-quantifiable relative FlucBb units may result from low numbers of spirochetes, albeit quantifiable relative RlucBb units. These challenges may be overcome by increasing the number of spirochetes used in the assay. This is evident in the data we present for the in vivo tick assay, in which the fed larvae samples for all B. burgdorferi clones achieved quantifiable relative RlucBb units; however, the clone containing flaBp-flucBb (+pCFA801), but not the clones containing ospAp-flucBb (+pCFA802) or the ospCp-flucBb (+pCFA803), produced quantifiable relative FlucBb units. This finding was not surprising for the ospC promoter, given that the ospC transcript is known to have weak to no activity in fed larvae following B. burgdorferi acquisition from infected mice. This finding was, however, unexpected for the ospA promoter, whose transcript is known to have strong activity in this environment (Caimano et al., 2015). Yet, the average number of spirochetes in the ospAp-flucBb (+pCFA802) and ospCp-flucBb (+pCFA803) containing clone extracts, as reflected by the average relative FlucBb units (1.6 × 10^2 and 3.1 × 10^2, respectively), were approximately 10-fold and 4-fold

![Image](https://via.placeholder.com/150)
RT-qPCR does remain and few publications report exploration of coelenterazine. Successful and reliable dual luciferase for tracking transcription factors away from the promoter fusions by the endogenous promoters. However, expression of flaB, ospA, and ospC are essential for survival of B. burgdorferi throughout its infectious cycle (Samuels, 2011; Sultan et al., 2013) and thus these experiments could not be conducted in the absence of these genes.

While dual luc and rluc reporter systems have been used successfully for live imaging and quantitation of eukaryotic tumor cells in mice (Bhauimik and Gambhir, 2002), the use of Renilla luciferase and the coelenterazine substrate for live imaging of microbial infections in mice has proven challenging (Andreu et al., 2011) and few publications report exploration of the use of dual Renilla and Photinus luciferase reporters in the context of infectious disease applications. There is great interest in applying a luciferase dual reporter system to quantification of B. burgdorferi promoter activities during an active mammalian infection. We and others have demonstrated the power of the luc reporter for tracking B. burgdorferi dissemination and qualitative detection of promoter activities over time in live mice (Hyde et al., 2011; Chan et al., 2015; Adams et al., 2017). By extension we investigated the efficacy of the dual luciferase reporter system for live imaging applications with B. burgdorferi in infected mice. Exhaustive examination of available coelenterazine substrates including: h-Coelenterazine-SOL in vivo (NanoLight), Inject-A-Lume h-Coelenterazine (NanoLight), ViviRen™ in vivo Renilla Luciferase Substrate (Promega), and XenoLight RedIject Coelenterazine h (PerkinElmer) as well as various substrate concentrations, substrate injection methods and imaging times, resulted in no significant luc signals above background (data not shown). Unlike for applications for solid cancers, use of luciferase substrates for in vivo detection of microbial pathogens relies on the substrates to be available in excess, systemically throughout the animal. Luciferin has been documented to rapidly distribute throughout the mouse (Contag et al., 1997), but the bioavailability of coelenterazine may be more limited (Luker et al., 2002). In addition, we found coelenterazine to have an extraordinary high background signal. Indeed, luc signals following coelenterazine delivery were observed for mice infected with spirochetes lacking luc entirely, which were not able to be overcome in mice infected with spirochetes expressing flaB-rluc (data not shown). These findings are consistent with what has been reported for attempted in vivo imaging applications using coelenterazine and Mycobacterium smegmatis expressing Gaussia luciferase (Andreu et al., 2010). Rather, alternative methods of normalization may be used, such as determining spirochete loads of infected tissues immediately following Fluc imaging (Skare et al., 2016), in instances where quantification of promoter activity during murine infection is warranted.

B. burgdorferi has been shown to colonize Ixodes scapularis via a biphasic mode of dissemination which is believed to involve complex interactions between the pathogen and the arthropod vector (Dunham-Embs et al., 2009). We are still discovering many of the mechanisms B. burgdorferi employs to survive throughout its enzootic cycle. Additionally, the recently sequenced Ixodes scapularis genome opens new areas of study for host-pathogen interactions (Gulia-Nuss et al., 2016). Successful and reliable techniques for analysis of spirochete biology in the tick are critical to drive understanding of these interactions. The dual luciferase system presented here is a simple and powerful approach for measuring transcript expression, which can be easily modified to meet the needs of the researcher and adds to the ever growing molecular genetic toolbox for investigation of B. burgdorferi transcription and gene regulation.

AUTHOR CONTRIBUTIONS

PA and MJ conceived the study and designed experiments; PA and CF performed experiments; PA, CF, and MJ interpreted results; PA and MJ wrote the manuscript; all authors critiqued and edited the final manuscript.

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