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RESEARCH ARTICLE

Borrelia burgdorferi SpoVG DNA- and RNA-Binding Protein Modulates the Physiology of the Lyme Disease Spirochete

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ABSTRACT The SpoVG protein of *Borrelia burgdorferi*, the Lyme disease spirochete, binds to specific sites of DNA and RNA. The bacterium regulates transcription of *spoVG* during the natural tick-mammal infectious cycle and in response to some changes in culture conditions. Bacterial levels of *spoVG* mRNA and SpoVG protein did not necessarily correlate, suggesting that posttranscriptional mechanisms also control protein levels. Consistent with this, SpoVG binds to its own mRNA, adjacent to the ribosome-binding site. SpoVG also binds to two DNA sites in the *glpFKD* operon and to two RNA sites in *glpFKD* mRNA; that operon encodes genes necessary for glycerol catabolism and is important for colonization in ticks. In addition, spirochetes engineered to dysregulate *spoVG* exhibited physiological alterations.

IMPORTANCE *B. burgdorferi* persists in nature by cycling between ticks and vertebrates. Little is known about how the bacterium senses and adapts to each niche of the cycle. The present studies indicate that *B. burgdorferi* controls production of SpoVG and that this protein binds to specific sites of DNA and RNA in the genome and transcriptome, respectively. Altered expression of *spoVG* exerts effects on bacterial replication and other aspects of the spirochete's physiology.

KEYWORDS *Borrelia*, DNA-binding proteins, RNA-binding proteins, regulation

Borrelia burgdorferi has evolved to survive within a defined enzootic cycle, alternately colonizing and transmitting between tick vectors and vertebrate hosts (1, 2). These two animal types represent drastically different environments, requiring *B. burgdorferi* to sense specific cues and to adapt its cellular programs in response to those cues (3). Appropriately executing such adaptations is critical to the success of *B. burgdorferi* as a pathogen. Very little is known, however, about the mechanisms by which *B. burgdorferi* accomplishes these feats.

The SpoVG protein was originally named for its role as a negative regulator of asymmetric division during *Bacillus* sp. sporulation (4). It has since been recognized as a highly conserved protein that is produced by many eubacteria, including *B. burgdorferi*. We previously reported that the SpoVG proteins of *B. burgdorferi*, *Listeria monocytogenes*, and *Staphylococcus aureus* are site-specific DNA-binding proteins (5). *L. monocytogenes* SpoVG has since been shown to also bind RNA (6).

When in a feeding tick or within a vertebrate, *B. burgdorferi* grows and divides rapidly, fed by the glucose and other nutrients in blood and serum. After a tick has digested the blood meal, however, nutrients available to colonizing *B. burgdorferi* are severely limited (2, 7–9). In response, the bacteria reduce or cease processes that

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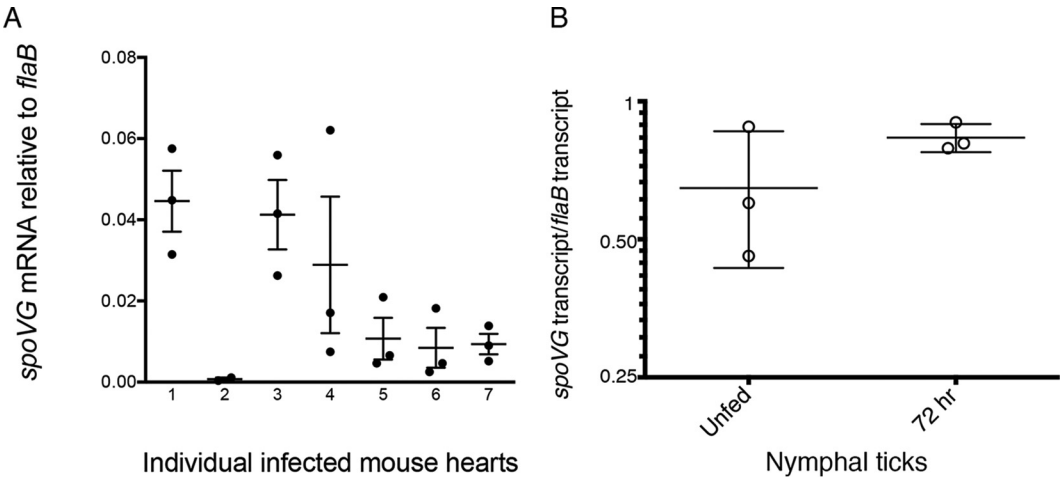

FIG 1 Relative levels of *spoVG* expression by *Borrelia burgdorferi* in mice and ticks. (A) Cohorts of mice were infected with *B. burgdorferi* on two separate occasions. RNA was harvested from the heart of each mouse and analyzed by qRT-PCR. Shown are technical triplicates for each mouse heart. (B) Pools of 10 to 20 nymphal ticks were analyzed by qRT-PCR. Levels of *spoVG* transcripts were quantified and are depicted as a percentage of *flaB* transcript levels. Shown are biological replicates of tick pools for each group.

require substantial energy, such as growth and division (2, 10, 11). Cells of postfeeding ticks secrete glycerol as a mechanism to survive cold stress (8). That glycerol serves as an important energy source for *B. burgdorferi* within unfed ticks. Mutants of the *glpFKD* operon are unable to use glycerol as a carbon source and are significantly impaired in their abilities to survive in ticks (12). Appropriate regulation of the *glpFKD* operon is necessary in order to traverse the enzootic cycle successfully (12, 13).

Herein we present characterization of *spoVG* expression throughout the mouse-tick infectious cycle. Those studies, and investigations of cultured *B. burgdorferi*, indicate that bacteria regulate *spoVG* transcript and SpoVG protein levels by both transcriptional and posttranscriptional mechanisms and *spoVG* affects the physiology of *B. burgdorferi*.

RESULTS

***Borrelia burgdorferi* controls expression of *spoVG* mRNA.** Having demonstrated previously that the *B. burgdorferi* SpoVG protein is a site-specific DNA-binding protein (5), we hypothesized that it acts as a regulatory factor, which would require that SpoVG itself is regulated. To that end, we quantified *spoVG* expression levels during colonization of mice and ticks.

A cohort of seven mice were infected with wild-type *B. burgdorferi*. After 4 weeks, hearts were harvested, RNA extracted, and transcript levels analyzed by quantitative reverse transcription-PCR (qRT-PCR). Levels of *spoVG* transcripts were determined relative to those of the constitutively expressed *flaB* mRNA (14). Detectable levels of *spoVG* transcripts, ranging from 6% to <1% of those of *flaB*, were present for all of the mice (Fig. 1A).

Ticks that were colonized with *B. burgdorferi* were similarly examined for *spoVG* mRNA levels. RNA was extracted from unfed tick nymphs and from nymphs that had attached to mice for 72 h (midway through the feeding process). Under both conditions, *spoVG* transcripts were nearly as abundant as *flaB* transcripts (Fig. 1B). The difference in *spoVG* transcript levels between unfed and feeding ticks was not significant. Adding further evidence that *spoVG* is abundantly expressed during tick colonization, an array-based study published while this manuscript was in preparation reported that *spoVG* was one of the 20 most abundant *B. burgdorferi* transcripts expressed in fed tick nymphs and larvae (15). From these results, it is apparent that *B. burgdorferi* naturally regulates *spoVG* transcript levels.

SpoVG expression in cultured *Borrelia burgdorferi*. To gain insight into the mechanisms controlling SpoVG expression, transcript and protein levels were assessed

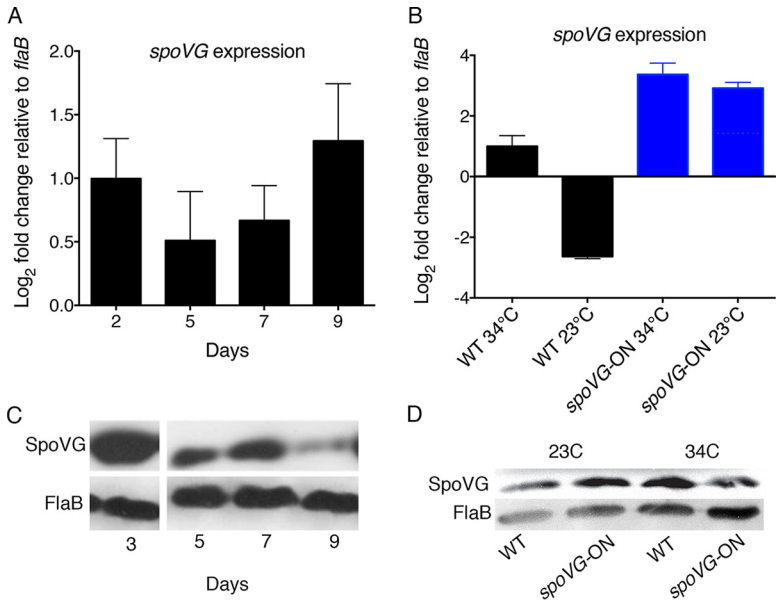

FIG 2 SpoVG transcript and protein expression in culture. (A) RNA extracted from cultured *B. burgdorferi* harvested on days 2, 5, 7, and 9 was analyzed by qRT-PCR. *spoVG* transcript levels were determined, relative to *flaB* transcripts, by the $\Delta\Delta C_T$ method. (B) RNAs extracted from cultured wild-type (WT) and *spoVG*-ON *B. burgdorferi* grown at 34°C and 23°C were analyzed by qRT-PCR. Relative *spoVG* transcript abundance was normalized to *flaB* transcript levels by the $\Delta\Delta C_T$ method. (C) Lysates from cultured *B. burgdorferi* harvested on days 3, 5, 7, and 9 were analyzed for SpoVG protein production. FlaB protein levels were used as a loading control. (D) Lysates from wild-type and *spoVG*-ON *B. burgdorferi* were harvested on day 3 from cultures grown at 34°C and on day 10 from cultures grown at 23°C and were analyzed by Western blotting. SpoVG abundance is shown with FlaB as the loading control.

in cultured bacteria throughout different growth phases. Wild-type *B. burgdorferi* was cultured at 34°C and then harvested at either day 2 or day 3 and at days 5, 7, and 9. RNA was extracted and analyzed by qRT-PCR, and *spoVG* abundance was normalized to *flaB* transcript levels (Fig. 2A). There were no significant changes in *spoVG* transcript levels through the different growth stages. This experiment, using triplicate cultures, was repeated twice with similar results. Lysates for Western blotting were prepared from 34°C cultures harvested on days 3, 5, 7, and 9. SpoVG protein levels were readily detectable on day 3 but were nearly undetectable on day 9 (Fig. 2C). These disparities between protein and mRNA expression levels suggest that SpoVG protein levels are controlled through a posttranscriptional mechanism.

B. burgdorferi differentially expresses numerous genes and proteins in response to changing culture temperatures (3, 16–21). Levels of *spoVG* mRNA were found to be 12 times greater in wild-type cultures grown at a constant 34°C than they were in bacteria maintained at a constant 23°C (Fig. 2B). Conversely, levels of SpoVG protein were not significantly different in the 34°C cultures than in the 23°C cultures, providing further evidence of posttranscriptional regulation (Fig. 2D).

Additional insight regarding borrelial control of SpoVG protein levels was serendipitously obtained from bacteria that were engineered to ectopically transcribe elevated levels of *spoVG*. We produced a plasmid that constitutively expressed *spoVG* from the borrelial *flgB* promoter, with the intent of producing greater amounts of the protein. This plasmid, named pCRS5, was transformed into *B. burgdorferi* on three separate occasions. Essentially identical results were obtained for all three transformants for all experiments; therefore, we collectively refer to the three mutant strains as *spoVG*-ON. Analyses of the naturally occurring small DNAs (plasmids) of the wild-type strain and two *spoVG*-ON strains revealed that the wild-type strain and one mutant had identical plasmid contents, while one of the mutants was lacking lp56. The identical results for the *spoVG*-ON strains and the identical plasmid contents of the wild-type strain and the

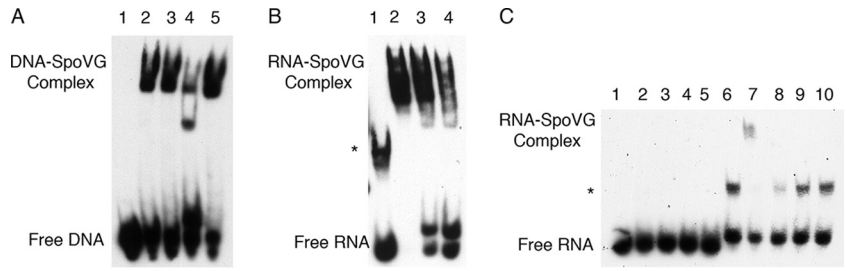

FIG 3 SpoVG binding of its own DNA and RNA. EMSAs were performed using either labeled DNA or RNA derived from the sequence 5′ of the *spoVG* translational start site or RNA derived from the sequence 5′ of the unrelated *erpAB* operon (23). The *spoVG* RNA probe formed a secondary structure in the absence of added protein (indicated by an asterisk). (A) Lane 1, 1 ng DNA probe; lane 2, 1 ng DNA probe with 2.4 μM rSpoVG; lane 3, 1 ng DNA probe with 4.8 μM rSpoVG; lane 4, 1 ng DNA probe with 4.8 μM rSpoVG and 100 ng unlabeled *spoVG* DNA; lane 5, 1 ng DNA probe with 4.8 μM rSpoVG and 100 ng unlabeled EMSA-pCR2.1 DNA. (B) Lane 1, 1 ng RNA probe (asterisk indicates a secondary structure of the RNA); lane 2, 1 ng RNA probe with 2.4 μM rSpoVG; lane 3, 1 ng RNA probe with 2.4 μM rSpoVG and 100 ng unlabeled *spoVG* DNA; lane 4, 1 ng RNA probe with 2.4 μM rSpoVG and 1,000 ng unlabeled *spoVG* DNA. (C) Lanes 1 to 5, 1 ng *erp* RNA; lanes 6 to 10, 1 ng *spoVG* RNA; lanes 1 and 6, no protein; lanes 2 and 7, 1.6 μM r SpoVG; lanes 3 and 8, 160 nM rSpoVG; lanes 4 and 9, 16 nM rSpoVG; lanes 5 and 10, 1.6 nM rSpoVG.

mutant indicate that lp56 does not play a significant role in the effects observed in these studies.

Levels of *spoVG* mRNA were determined for the *spoVG*-ON and wild-type strains. Transcript levels were 5 times greater in the *spoVG*-ON strains than in the wild-type strain during cultivation at 34°C and were nearly 47 times greater during cultivation at 23°C (Fig. 2C). Both of those differences were statistically significant. However, increased expression of *spoVG* transcripts did not result in significantly elevated levels of SpoVG protein in the *spoVG*-ON strains at 34°C or 23°C (Fig. 2D). The pCR5 constructs were reisolated from the *spoVG*-ON strains and were found to have sequences that were identical to that of the initial plasmid, indicating that no mutations had occurred within *B. burgdorferi*. A control plasmid that produced elevated levels of *revA* mRNA yielded elevated levels of the RevA protein, indicating that transcripts could be translated from such constructs (22).

SpoVG binds its own DNA and RNA. As reported previously, SpoVG is a site-specific DNA-binding protein (5). To gain insight into whether SpoVG influences its own expression, we queried whether the protein binds to its DNA and RNA. Electrophoretic mobility assays (EMSAs) using recombinant SpoVG found specific binding to a 34-bp DNA region upstream of the translational start codon (Fig. 3A and 4A). The shifted band was partially ablated by addition of 100× specific unlabeled DNA but not by addition of 100× nonspecific DNA, indicating that the interaction is specific (Fig. 3A). As this region is 3′ of the transcriptional promoter and includes the ribosome-binding site, we examined whether SpoVG binds to RNA of the same sequence. EMSAs demonstrated that SpoVG bound to that RNA (Fig. 3B). Addition of 1,000× unlabeled DNA could not entirely compete away the shifted RNA, indicating that SpoVG binds to this RNA with a higher affinity than that for the template DNA sequence.

The specificity of SpoVG binding to RNA was assessed by an EMSA using a sequence based on the operator region of the *erpAB* operon (23). When used at the same concentrations as used to generate the findings in Fig. 3B, recombinant SpoVG protein did not bind to the *erpAB*-derived RNA sequence (Fig. 3C).

Influence of *spoVG* mRNA on *B. burgdorferi* growth and division. Several analyses of cultured *spoVG*-ON bacteria were undertaken prior to the recognition that *spoVG* mRNA levels did not necessarily correspond directly to SpoVG protein levels. The following results indicate that substantial effects on *B. burgdorferi* physiology can be caused by changes in *spoVG* mRNA levels, even in the absence of changes in SpoVG protein levels. A possible explanation for these observations is that elevated levels of

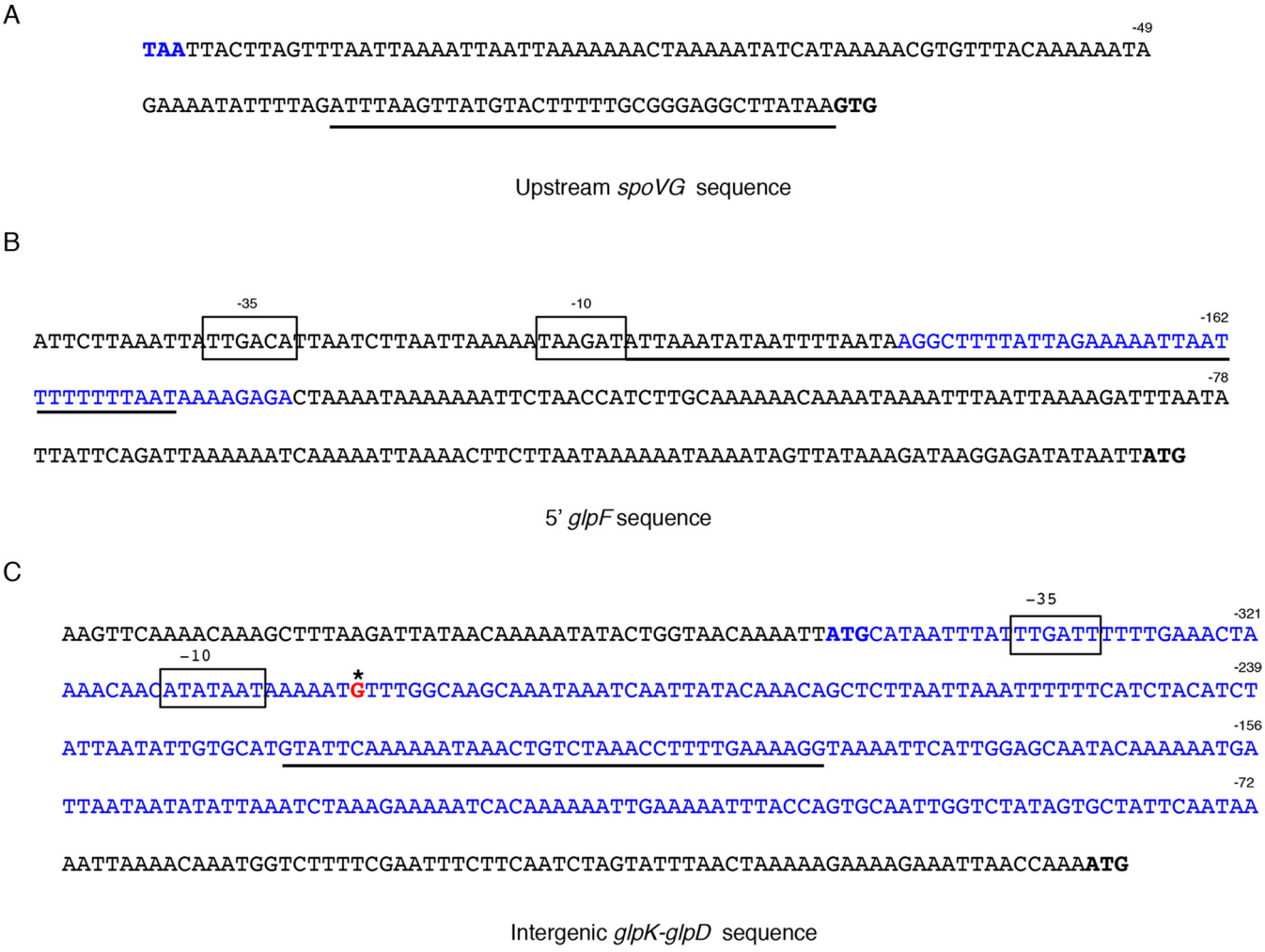

FIG 4 Nucleic acid sequences that contain SpoVG binding sites. The sequences are shown as DNA, although SpoVG also bound to all of these sites in single-stranded RNA. The DNA/RNA target regions used for EMSAs are underlined. (A) Intergenic region between the start of the *spoVG* open reading frame (ORF) and the gene located immediately upstream, *rsf*. The transcriptional start site of the *spoVG* ORF is highlighted in bold black type, and the stop codon of the *rsf* gene is highlighted in bold blue type. (B) Sequence 5′ of the *glpFKD* operon. The −35 and −10 sequences of the probable transcriptional promoter are boxed, and the translational initiation codon of *glpF* is in bold black type. The maximal sequence bound by SpoVG is underlined. The region previously identified as having a stimulatory effect on transcript levels is shown in blue type (28). (C) Sequence of the intergenic region between *glpK* and *glpD*. The maximal sequence bound by SpoVG is underlined. The −35 and −10 sequences of the predicted transcriptional promoter are boxed. The transcription start site mapped by Adams et al. (34) is indicated in red and marked with an asterisk. The translational initiation codon of *glpD* is in bold type. A small ORF of unknown function, *bb0242*, is indicated in blue.

spoVG mRNA titrate SpoVG protein away from its other RNA and DNA targets, thereby altering transcription or translation of those targets.

Growth curves were determined for the wild-type and *spoVG*-ON *B. burgdorferi* strains cultured at either 34°C or 23°C. There were no differences between the two strains in growth rates at 34°C (Fig. 5A). As reported previously, wild-type *B. burgdorferi* grew more slowly at 23°C than at 34°C (17, 24). The *spoVG*-ON strains, however, consistently grew even more slowly at 23°C than did the wild-type strain. The exponential growth phase (days 0 to 20) for each strain was analyzed for exponential growth by nonlinear regression. The rate constant for the wild-type strain was calculated to be 0.2878 day^{-1}, while that for the *spoVG*-ON strains was 0.1388 day^{-1}; these values were significantly different, with *P* of 0.0001. During this period, the doubling time of the wild-type strain was 2.4 days, while the doubling time of the *spoVG*-ON strains was 5 days (Fig. 5B). This indicates that enhanced production of *spoVG* mRNA does not affect the growth rate at 34°C but *spoVG* mRNA in combination with some factor (or factors) that is present or absent in 23°C cultures has an appreciable impact on the growth rate of *B. burgdorferi*.

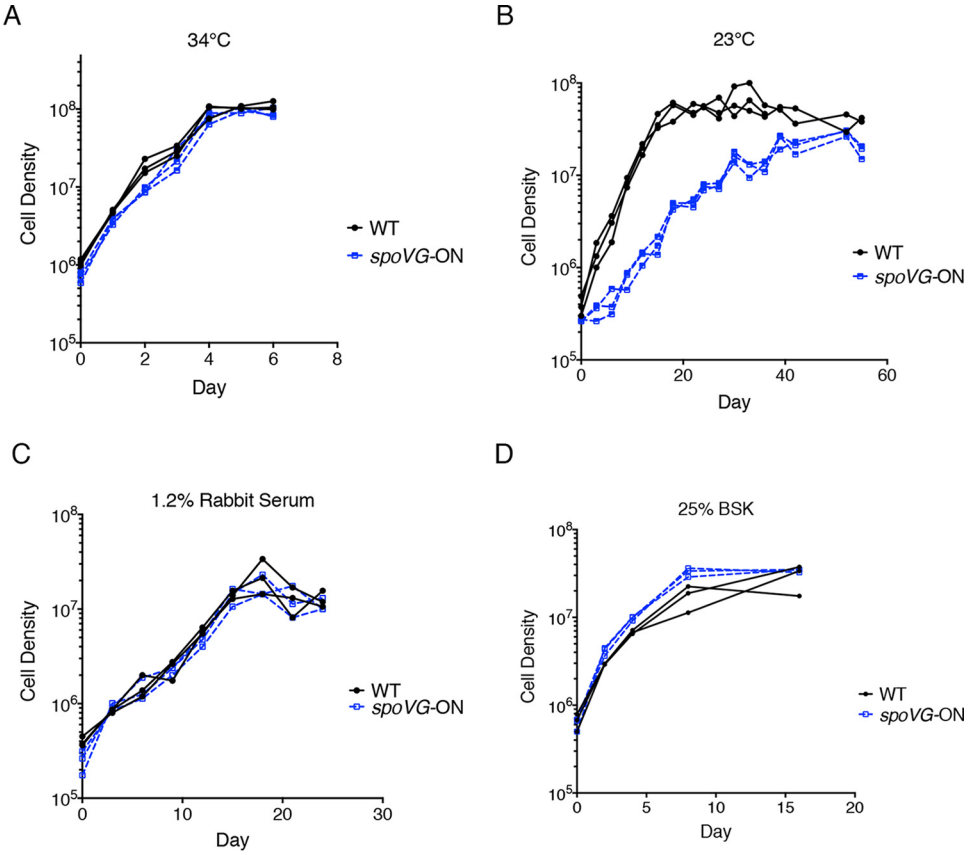

FIG 5 Effects of *spoVG* transcript levels on *B. burgdorferi* growth rates. (A and B) Cultured wild-type (WT) and *spoVG*-ON *B. burgdorferi* strains were grown in complete medium at 34°C and counted every 24 h (A) or were grown in complete medium at 23°C and counted every 3 days (B). (C and D) Cultured wild-type and *spoVG*-ON *B. burgdorferi* strains were also grown at 34°C in one of two deficient media, i.e., grown in 1.2% rabbit serum medium and counted every 3 days (C) or grown in 25% BSK medium and counted on days 2, 4, 8, and 16 (D).

An additional effect of enhanced *spoVG* mRNA and cultivation at 23°C was on bacterial survival. The 23°C cultures were left in incubators for 48 days after initial inoculation. At that time, each culture was diluted 1:100 into fresh Barbour-Stoenner-Kelly (BSK) medium and immediately observed with a dark-field microscope. At least 50 cells per culture were examined. In that time frame, none of the wild-type bacteria exhibited any form of motility. In contrast, at least 20% of the *spoVG*-ON cells had begun to move. The initial cultures were then returned to the incubator for an additional 8 days. On day 56, each culture was diluted 1:100 into fresh BSK medium and incubated at 34°C (final densities of approximately 1×10^6 bacteria per ml). By day 9, none of the wild-type cultures showed any evidence of bacterial growth, whereas all of the *spoVG*-ON cultures had reached densities of at least 1×10^8 bacteria per ml.

As noted above, *B. burgdorferi* normally grows more slowly at 23°C than 34°C. We previously demonstrated several instances in which the effects of those culture temperatures on gene expression were due to changes in borrelial growth rates and not to differences in temperature *per se* (24). Noting that culture of *spoVG*-ON strains at 23°C resulted in rates of division that were significantly lower than that of wild-type bacteria but *spoVG*-ON did not affect division rates at 34°C, we tested whether the effect of 23°C was due to temperature or growth rate. To that end, *spoVG*-ON and wild-type strains were cultured in one of two deficient media that were formulated to reduce the growth rate of wild-type *B. burgdorferi* by approximately 4-fold at 34°C (24). There were no differences in growth rates between wild-type and *spoVG*-ON *B. burgdorferi* strains cultured in either of the deficient media (Fig. 5C and D). This finding suggests that the

growth-inhibiting effect of enhanced *spoVG* transcription at 23°C may be directly linked to the environmental temperature.

SpoVG binds DNA and RNA of the *glpFKD* operon. A preliminary transcriptome sequencing (RNA-Seq) analysis was performed early during these investigations, comparing a *spoVG*-ON strain with its wild-type parent. Analysis of the preliminary data suggested an impact on the *glpFKD* operon. Those genes encode three proteins that import glycerol into the bacterial cell and perform the first two enzymatic steps of its metabolism (12, 25). Subsequent qRT-PCR analyses of *glpFKD* transcript levels in either wild-type or SpoVG-ON strains yielded inconsistent results. During the time these studies were in progress, other research indicated that *glpFKD* transcript levels are affected by several factors, including the alternative sigma protein RpoS, cyclic di-GMP, and ppGpp (13, 26–28). Those factors are produced in response to growth rate and pH, an unknown external signal, and nutrient limitation, respectively, conditions that are difficult to reproduce accurately between experiments, which likely accounted for the inconsistent results (24, 29–32). More detailed RNA-Seq and qRT-PCR analyses will be performed in the future, after the posttranscriptional regulatory mechanisms that control SpoVG protein production have been elucidated. Nonetheless, because glycerol is a critical energy source for *B. burgdorferi* in unfed ticks (12) and because *spoVG* is highly transcribed during that stage, studies were undertaken to determine whether SpoVG could play a direct role in controlling *glpFKD* expression.

The first gene of the operon, *glpF*, is preceded by an approximately 200-bp 5′ untranslated transcript (28, 33, 34) (Fig. 4B). Previous work determined that a 42-bp sequence directly 3′ of the promoter had a positive effect on transcript levels (28). EMSAs with recombinant SpoVG indicated that this protein bound specifically to a 52-bp region of DNA that overlaps the start of transcription and the previously reported activator region (Fig. 4B and 6A). SpoVG also bound to RNA that contained the same sequence. As with the binding site in the *spoVG* locus, 1,000× unlabeled DNA of the same sequence was not sufficient to entirely compete away the protein-RNA complex, indicating a higher affinity of SpoVG for *glpF* 5′ untranslated region (UTR) RNA, relative to the corresponding DNA (Fig. 6B).

There is a 400-bp gap between the termination codon of *glpK* and the initiation codon of *glpD* (25) (Fig. 4C). Our *in silico* analysis of this intergenic region revealed a potential transcriptional promoter. Other studies demonstrated that transcripts do originate from this region, providing further evidence that this is a functional promoter (27, 34). EMSAs revealed that SpoVG protein bound specifically to a 38-bp sequence that is located 3′ of this putative promoter (Fig. 4B and 6C). Further analysis revealed that SpoVG also bound to this RNA sequence. As with other investigated sites, 1,000× unlabeled DNA with that sequence was unable to compete away the SpoVG-RNA complex (Fig. 6D).

DISCUSSION

One important consequence of these studies is the demonstration that *B. burgdorferi* SpoVG protein levels do not necessarily correlate directly with *spoVG* mRNA levels, indicating that bacterial protein levels are, in part, controlled through a posttranscriptional mechanism (or mechanisms). A likely contributor is SpoVG itself, since it binds to *spoVG* mRNA at a sequence near the ribosome-binding site. Such a feedback mechanism could prevent translation when levels of SpoVG reach a certain point. A similar autoregulatory mechanism appears to control bacterial levels of another *B. burgdorferi* site-specific RNA-binding protein, BpuR (35, 36). SpoVG also binds to its operon's DNA, at a site located 3′ of the transcriptional promoter; therefore, the protein could affect levels of its own transcription. It is also possible that additional factors influence *spoVG* transcription and/or translation or interact with SpoVG protein to modulate its nucleic acid-binding activities. The interplay between SpoVG protein, the *spoVG* transcript and gene, and other bacterial factors is an ongoing focus of our research. Noting the effects on *B. burgdorferi* physiology we observed, we predict that precise regulation of SpoVG protein and *spoVG* mRNA is critical to the Lyme disease spirochete. These findings also

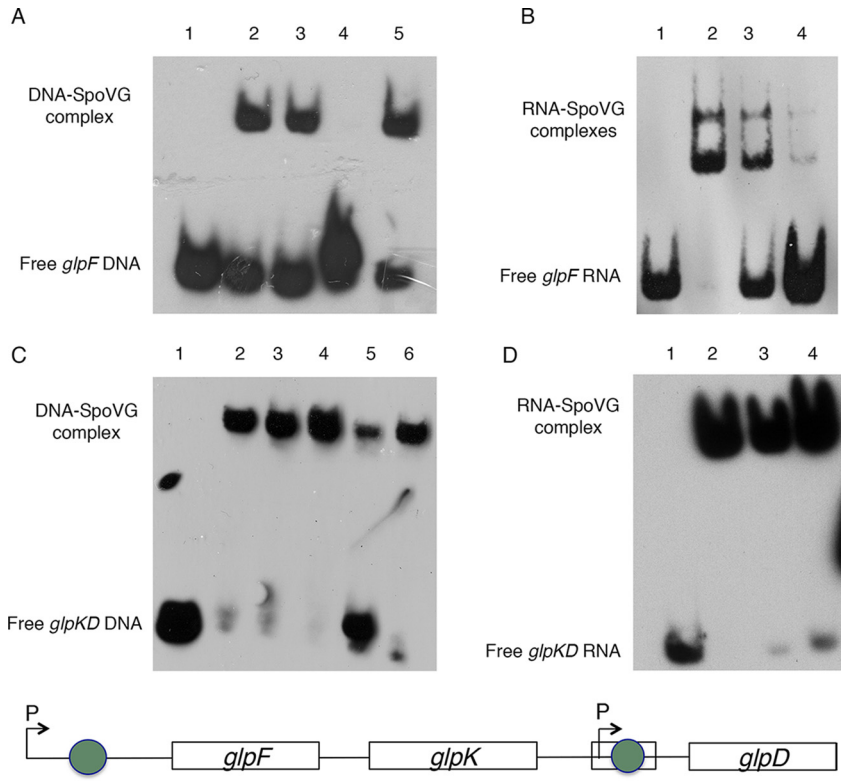

FIG 6 SpoVG binding to DNA and RNA at two sites in the *glpFKD* operon. (A and B) Sequence 5′ of *glpF*. (C and D) Region between *glpK* and *glpD*. EMSAs were performed using either labeled DNA or RNA. (A) Lane 1, 1 ng *glpF* DNA; lane 2, 1 ng *glpF* DNA with 2.4 μM rSpoVG; lane 3, 1 ng *glpF* DNA with 4.8 μM rSpoVG; lane 4, 1 ng *glpF* DNA with 4.8 μM rSpoVG and 100 ng unlabeled *glpF* DNA; lane 5, 1 ng *glpF* DNA with 4.8 μM rSpoVG and 100 ng unlabeled EMSA-pCR2.1 DNA. (B) Lane 1, 1 ng *glpF* RNA; lane 2, 1 ng *glpF* RNA with 2.4 μM rSpoVG; lane 3, 1 ng *glpF* RNA with 2.4 μM rSpoVG and 100 ng unlabeled *glpF* DNA; lane 4, 1 ng *glpF* RNA with 2.4 μM rSpoVG and 1,000 ng unlabeled *glpF* DNA. (C) Lane 1, 1 ng *glpKD* DNA; lane 2, 1 ng *glpKD* DNA with 0.73 μM rSpoVG; lane 3, 1 ng *glpKD* DNA with 1.47 μM rSpoVG; lane 4, 1 ng *glpKD* DNA with 3 μM rSpoVG; lane 5, 1 ng *glpKD* DNA with 3 μM rSpoVG and 100 ng unlabeled *glpKD* DNA; lane 6, 1 ng *glpKD* DNA with 3 μM rSpoVG and 100 ng unlabeled EMSA-pCR2.1 DNA. (D) Lane 1, 1 ng *glpKD* RNA; lane 2, 1 ng *glpKD* RNA with 2.4 μM rSpoVG; lane 3, 1 ng *glpKD* RNA with 2.4 μM rSpoVG and 100 ng unlabeled *glpKD* DNA; lane 4, 1 ng *glpKD* RNA with 2.4 μM rSpoVG and 1,000 ng unlabeled *glpKD* DNA. A graphic representation of SpoVG-binding sites and promoters, relative to the *glpFKD* operon, is shown at the bottom.

raise the possibility that other bacterial species control levels of their SpoVG proteins through similar means.

Another important finding is that situations were observed in which elevating the *spoVG* transcript levels resulted in phenotypic changes, even when there were no detectable changes in SpoVG protein levels. We observed that SpoVG binds not only its own mRNA but also two sites within the *glpFKD* mRNA and two DNA sites in the *glpFKD* operon. These findings suggest that there are likely to be additional SpoVG-binding sites in the borrelial transcriptome and genome. Analysis of the known SpoVG-binding sites has not revealed any obvious consensus sequence; therefore, we hypothesize that SpoVG may interact with certain nucleic acid structural motifs rather than a particular nucleotide sequence. A plausible explanation for how changes in *spoVG* transcript levels cause changes in bacterial physiology is that elevated *spoVG* message levels pull SpoVG protein away from other binding sites, thereby preventing SpoVG effects on those targets. This would be similar to the mechanism by which some bacteria control cellular levels of their CsrA RNA-binding proteins by producing regulatory noncoding RNAs that sequester the protein (37). If additional regulatory proteins bind to *spoVG* mRNA, then their sequestration could have further effects. It is also possible that *spoVG* mRNA can hybridize with other mRNAs or noncoding RNAs and thereby alter their activities.

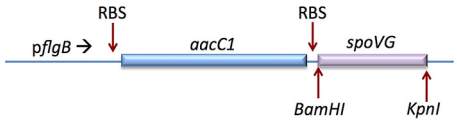

FIG 7 pCRS5 (*spoVG*-ON) plasmid construction. *spoVG* was cloned into the BamHI and KpnI sites of our previously described pBLS715 plasmid (22). The parental construct was created to contain a consensus ribosome-binding site (RBS) appropriately placed 5′ of the insert. The constitutively expressed borrelial *flgB* promoter drives expression of both the gentamicin resistance gene (*aacC1*) and *spoVG*. The identically produced pBLS715*revA* produces elevated levels of both *revA* mRNA and RevA protein (22).

The observed results could have effects far beyond *B. burgdorferi*. The observed posttranscriptional regulation of SpoVG protein levels indicates that it cannot be assumed that changes in bacterial mRNA levels necessarily equal changes in protein levels. Furthermore, changes in transcript levels can have effects on bacteria without altering levels of the encoded protein. These caveats need to be addressed in all transcriptomic studies, in *B. burgdorferi* and other organisms.

SpoVG bound to two sites within the *glpFKD* operon and to two sites in that operon's mRNA, suggesting that SpoVG could affect production of the GlpF transporter and/or GlpK and GlpD enzymes. At least three other factors influence levels of the glycerol catabolic proteins, i.e., RpoS, cyclic di-GMP, and ppGpp (13, 26–28). A concerted effort that focuses on all four of the known *glp* regulatory factors is needed to accurately address the mechanism by which *B. burgdorferi* controls glycerol import and catabolism.

Altogether, the current studies suggest a role for SpoVG in adaptation to different environments. The affinity of SpoVG for *glpFKD* DNA and mRNA suggest that it may affect that operon's transcription and/or translation and also may influence additional operons by such means. Since the expression of *spoVG* is not entirely bimodal between ticks and mice, it is conceivable that SpoVG functions as a modulator of information flow throughout the infectious cycle and helps to enact the appropriate responses to specific environmental cues.

MATERIALS AND METHODS

Bacterial strains. All studies used infectious clones of *B. burgdorferi* strain B31-MI, a derivative of the type strain, for which the complete genome sequence has been determined (25, 38). Mouse infection studies were performed using clone B31-MI-16 (39), and all other studies were performed using clone B31-A3 (40). Both clones are fully virulent, and they are genetically identical except for the absence of the small native plasmid cp9 in B31-A3.

Strains of *B. burgdorferi* that constitutively transcribe elevated levels of *spoVG* were generated as follows. The *spoVG* gene was cloned into the previously described plasmid pBLS715 (22), directly 3′ of the gentamicin resistance gene, to create pCRS5 (Fig. 7). The plasmid insert was sequenced to ensure that *spoVG* was cloned in-frame without mutations. The *spoVG* ribosomal binding site was modified to AGGAGG to ensure efficient translation of SpoVG protein (22). *B. burgdorferi* B31-A3 was transformed with pCRS5 on three separate occasions. To ensure that no mutations had arisen in *B. burgdorferi*, pCRS5 was purified from each transformant and the insert was sequenced. Bacterial contents of native plasmids were analyzed by multiplex PCR, as described by Bunikis et al. (41). As described above, growth curve, Western blot, and qRT-PCR analyses were performed for all three strains, with similar results being obtained for all strains; therefore, the strains are collectively referred to as *spoVG*-ON strains.

Culture conditions. *B. burgdorferi* was cultured at 34°C in Barbour-Stoenner-Kelly II (BSK-II) medium (which contains 6% rabbit serum), unless otherwise noted (42). The deficient culture medium termed 25% BSK medium includes the components of BSK diluted 1:4 with phosphate-buffered saline (PBS), plus the standard 6% rabbit serum. The deficient culture medium termed 1.2% rabbit serum medium includes standard concentrations of BSK components plus 1.2% rabbit serum (24).

Bacterial growth curves were initiated by diluting mid-exponential-phase cultures of *B. burgdorferi* (approximately 10^7 bacteria/ml) 1:100 into fresh medium. Bacterial numbers were counted by use of a Petroff-Hausser counting chamber with dark-field microscopy. Cultures grown at 34°C in BSK-II medium were counted once every 24 h, and cultures grown at 23°C or in 25% BSK medium or 1.2% rabbit serum medium were counted every 48 h. Viability was assessed by counting motile cells and by passaging cultures into fresh medium and determining the growth rate. Differences in growth rates between strains and conditions during exponential-phase growth were calculated using Prism statistics. Graphs were analyzed by nonlinear regression for exponential growth, with the null hypothesis that the rate constants for the two strains were not statistically different (GraphPad Software, La Jolla, CA).

Quantitative reverse transcription-PCR analyses of mRNA expression. Relative *spoVG* expression levels during tick colonization and murine infection were determined by qRT-PCR using the oligonucle-

TABLE 1 Oligonucleotides used in this study

Purpose and name[a]	Sequence (5′ to 3′)
EMSA probes and competitors	
EMSA-*spoVG*	ATTTAAGTTATGTACTTTTTGCGGGAGGCTTATAA
EMSA-*glpF*	ATTAAATATAATTTTAATAAGGCTTTTATTAGAAAAATTAATTTTTTTTAAT
EMSA-*glpKD*	GTATTCAAAAAATAAACTGTCTAAACCTTTTGAAAAGG
EMSA-pCR2.1	CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCA GTGTGCTGGAATTCGGCTTAGCCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTGGAGCA TGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTAC
M13-F	GTAAAACGACGGCCAG
M13-R	CAGGAAACAGCTATGAC
qRT-PCR analyses of infected mice and cultured bacteria	
fla3	GGGTCTCAAGCGTCTTGG
fla4	GAACCGGTGCAGCCTGAG
spo1	GCGATGCCTAACAGAAGAAC
spo2	CAAGTTCAAGATCGGCTGG
TaqMan primers and probes for analyses of infected ticks	
flaB For	TCTTTTCTCTGGTGAGGGAGCT
flaB Rev	TCCTTCCTGTTGAACACCCTCT
flaB probe	AAACTGCTCAGGCTGCACCGGTTC
spoVG For	GATGCCTAACAGAAGAAC
spoVG Rev	GAAGTTTGCAAAGCTTT
spoVG probe	AAAGACATTGTACATCCTATTAGTCAG

[a]The EMSA probes and competitors were produced as either double-stranded DNA or single-stranded RNA. EMSA probes were modified with a biotin moiety on the 5′ end; competitors were not labeled. All other oligonucleotides were single-stranded DNAs.

otide primers described in Table 1. Analyses of gene expression levels in cultured *B. burgdorferi* were performed as described previously (43), using oligonucleotide PCR primers specific for each gene being examined. Results were compared with those for the constitutively expressed flagellar gene *flaB* (14). Biological triplicates were assayed for all strains and conditions, all qRT-PCR assays were performed with technical triplicates, and runs were performed at least twice. Following each run, primer specificity and amplicon purity were assayed via melting curve analysis (43). qRT-PCR data on transcripts in culture were analyzed by one-way analysis of variance (ANOVA) (GraphPad Software).

For analyses of transcript levels during tick colonization, uninfected *Ixodes scapularis* larvae were infected by feeding to repletion on B31-A3-infected mice. Ticks were allowed to molt to nymphs and then were placed on naive mice for feeding. Ticks were collected at specific time points, snap-frozen in liquid nitrogen, and stored at −80°C until use. RNA was isolated from pooled ticks, with 10 to 20 nymphs per pool. Pooled frozen ticks were crushed in 100 μl PBS using disposable mortars and pestles. RNA was isolated using the Nucleospin kit (Macherey-Nagel Co., Bethlehem, PA), according to the manufacturer's recommendations, or TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was treated with DNase and converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), as described previously (44). Three biological replicates (independent pools of ticks), with triplicate technical replicates, were assessed for gene expression by qRT-PCR, as described previously (45). A standard curve was generated with genomic DNA representing a range of 10^6 to 10 cells (in 10-fold serial dilutions), using the threshold cycle (C_T) values from the *flaB* primer-probe set. This standard curve was then used to interpolate *spoVG* transcript copy numbers from the C_T values generated with the *spoVG* primer-probe set. The *spoVG* transcript copy numbers were normalized to *flaB* transcript copy numbers. Negative controls lacking reverse transcriptase confirmed that all genomic DNA had been removed and did not contribute to the signal.

For analyses of transcript levels during mammalian infection, seven female, 4- to 6-week-old, C3H/HEN mice were infected by subcutaneous injection of 1×10^6 B31-MI-16 bacteria from a mid-exponential-phase culture. Two weeks postinfection, mouse blood was drawn from the saphenous vein and processed to serum, and the presence of *B. burgdorferi*-specific antibodies was determined with an enzyme-linked immunosorbent assay (ELISA), as described previously (46). Mice were euthanized after 4 weeks of infection, and hearts were collected, flash frozen in liquid nitrogen, and stored at −80°C until use. Hearts were chosen because they are colonized by *B. burgdorferi* and are substantial, uniformly sized organs (47). Frozen hearts were ground with mortars and pestles, followed by homogenization (PRO Scientific) in TRIzol reagent on ice. RNA was resuspended in RNAsecure reagent (Ambion) and treated with DNase I (Ambion) to remove contaminating DNA. The DNase was inactivated using DNase inactivation reagent (Ambion). A 1-μg aliquot of each DNA-free RNA preparation was reverse transcribed using first-strand cDNA synthesis kits (Life Technologies) with random hexamers. Controls containing all components except reverse transcriptase were prepared and treated similarly. Quantitative PCR was performed as described above. All cDNA samples were analyzed in triplicate. Every qRT-PCR series included negative-control samples of RNA processed without reverse transcriptase (see above), to test for

DNA contamination, and samples that lacked a template, to test for DNA contamination of reagents. Tenfold serial dilutions of B31-MI-16 genomic DNA (100 ng to 100 fg) were included in every assay for each primer set. This enabled the generation of standard curves, from which the amount of transcript present in each cDNA sample could be calculated by using Bio-Rad myIQ software. Average expression values obtained from triplicate runs of each cDNA sample for all of the genes of interest were calculated relative to the average triplicate value for the constitutively expressed *B. burgdorferi flaB* transcript from the same cDNA preparation (43). All animal studies were performed with approval from and under the supervision of the institutional animal care and use committees of the University of Kentucky, the University of North Dakota, and Rocky Mountain Laboratories, NIH.

Protein analysis by Western blotting. Western blotting was performed as described previously (17). Polyclonal rabbit antisera specific for SpoVG were produced by NEO Group Inc. (Cambridge, MA). Unless otherwise noted, mid-exponential-phase *B. burgdorferi* cultures were passaged 1:100 into fresh BSK medium and harvested by centrifugation after 3 days. Cell pellets were frozen overnight at −80°C and resuspended in SDS loading buffer. Lysates were separated by SDS-PAGE, and proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk, incubated with primary antibody, then incubated with protein A-conjugated horseradish peroxidase. Blots were imaged with SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher).

Purification of recombinant SpoVG. SpoVG was purified as described previously (5). pBLJ132 containing *spoVG* cloned into pET101 was transformed into Rosetta II *Escherichia coli*, and expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). SpoVG was purified using MagneHis nickel particles (Promega, WI) and dialyzed into buffer containing 100 nM dithiothreitol, 50 mM Tris-HCl, 25 mM KCl, 10% glycerol (vol/vol), 0.01% Tween 20, and 1 mM phenylmethanesulfonyl fluoride. Protein purity and concentrations were assessed by SDS-PAGE and the bicinchoninic acid (BCA) assay (Thermo Fisher), respectively.

Electrophoretic mobility shift assays. EMSAs were conducted as described previously (48). Biotinylated DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA) were annealed in order to generate specific probes. Biotinylated RNA oligonucleotides (IDT) contained the same nucleotide sequences as the sense strands of the DNA probes. Specific competitors were made from the same nucleic acid sequences, without biotin. Nonspecific competitor was made from pCR2.1 DNA amplified with M13-F and M13-R primers. Protein-binding sites were identified by the combined use of differently sized labeled probes and unlabeled competitors. Reaction mixtures containing EMSA buffer, 40 μg/ml poly (dI-dC), 1 nM DNA with the indicated amounts of protein, and unlabeled DNA were incubated for 20 min at room temperature. Reaction mixtures were separated by 6% native PAGE and transferred to positively charged nylon membranes. Blots were blocked, incubated with streptavidin-horseradish peroxidase, and imaged using the chemiluminescent nucleic acid detection module kit (Thermo Fisher).

***In silico* promoter analysis.** The putative promoter between *glpK* and *glpD* was mapped using BPROM from SoftBerry (49).

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