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THE ROLE OF DKSA IN THE STRINGENT RESPONSE IN THE LYME DISEASE SPIROCHETE BORRELIA BURGDORFERI

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THE ROLE OF DKSA IN THE STRINGENT RESPONSE IN THE LYME DISEASE SPIROCHETE *BORRELIA BURGDORFERI*

By

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ABSTRACT

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THE ROLE OF DKSA IN THE STRINGENT RESPONSE IN THE LYME DISEASE SPIROCHETE *BORRELIA BURGDORFERI*

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Borrelia burgdorferi, the bacterium that causes Lyme disease, is maintained in nature through an enzootic cycle that includes a tick vector and a vertebrate host. The bacterium is acquired by an *Ixodes* tick from an infected vertebrate. The bacterium alters its gene expression to adapt to different environments of the tick and vertebrate. Between tick feedings, *B. burgdorferi* must contend with nutrient stress. The stringent response is a physiological mechanism when bacteria switch from “thriving” to “surviving” mode in response to limited nutrient resources; it is mediated by an increase in the nucleotide alarmone guanosine penta- or tetraphosphate, abbreviated as (p)ppGpp. An important target of the stringent response is transcriptional regulation, which is mediated by the RNA polymerase-binding transcription factor DksA. We hypothesized that DksA potentiates the effects of the stringent response for *B. burgdorferi* during starvation, and regulates *B. burgdorferi* genes involved in tick persistence. We have taken a reverse genetic approach in which we have generated a *dksA* null mutant and the corresponding complemented strain. *In vitro* experiments indicate that DksA may play a role in growth, cell viability during starvation, and cell morphology. We also provide evidence that the presence of glycerol during starvation affects cell viability and morphology of *B. burgdorferi*.

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I. Background and Significance

Lyme disease is a bacterial infection that affects humans worldwide, with high prevalence in northeastern and upper Midwestern states of the US (1–3). The Centers for Disease Control and Prevention estimate about 300,000 cases annually, making Lyme disease the most common vector-borne illness in the US (3). The bacterium that causes Lyme disease is *Borrelia burgdorferi* (4). This bacterium is a spirochete with an unusual genome consisting of both linear and circular DNA (5). The symptoms of Lyme disease begin with flu-like symptoms often accompanied by a bull's-eye-like rash called an erythema migrans. These symptoms usually begin to occur a few days after exposure to *B. burgdorferi* through a tick bite. As the disease progresses, the symptoms advance to include arthritic pain in the joints and neurological symptoms (6,7).

B. burgdorferi persists in nature in an enzootic cycle (Fig. 1) (6,8). The naïve tick larvae feed on a small, infected mammal to acquire the bacteria. After the blood meal, the larvae then molt into nymphs. These nymphs then take their second blood meal before molting into adults. The adults will take a third and final blood meal and lay their eggs. Infected adults do not pass the bacteria to their eggs; therefore, the larvae that hatch will begin as naïve larvae and must acquire the bacteria from an infected host (6,8).

During the life cycle, *B. burgdorferi* regulates expression of its genes, as the environments of the mammals and ticks require different genes for survival (6,9). In addition, *B. burgdorferi* experiences periods of starvation within the tick. Most bacteria have adapted to regulate gene expression during periods of starvation to switch to a nutrient-saving survival state. This process is known as the stringent response (10,11).

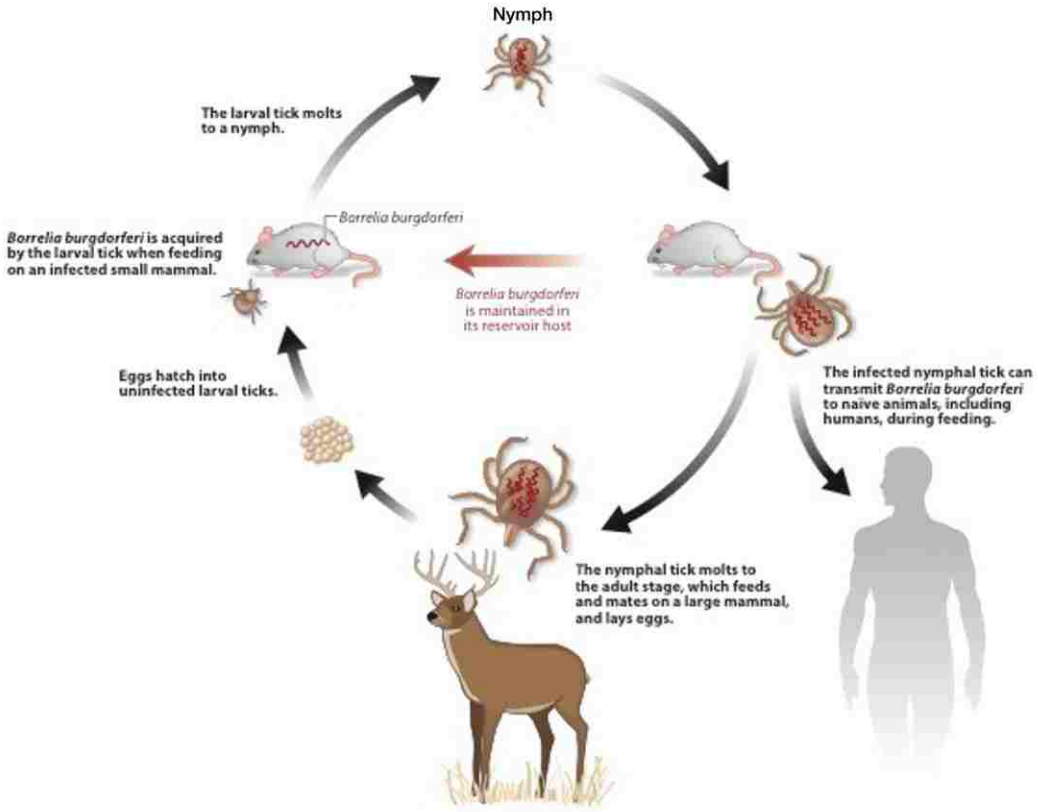

Figure 1- Enzootic cycle of *Borrelia burgdorferi*.
B. burgdorferi (Bb) is maintained in an enzootic cycle. The *Ixodes* ticks hatch as naïve larvae and take their first blood meal, typically on a small mammal. The tick can acquire Bb at this point. The larvae will then molt into nymphs and take their second blood meal. Infected ticks can transmit Bb to a host, such as a small mammal or a human. The nymphs will molt into adults, mate, take their final blood meal, and females will lay eggs. (Figure from (8).)

During the stringent response, bacteria allocate resources by turning cell growth genes down and stress response genes up (11). Guanosine tetraphosphate and pentaphosphate ((p)ppGpp) serve as an alarmone: their levels increase when bacteria are faced with nutritional stress (10–12). Levels of (p)ppGpp are regulated by the synthase RelA and the synthase/hydrolase SpoT (Fig. 2) (10,11). In some bacteria, such as *Escherichia coli*, RelA and SpoT are separate molecules, but in many others, like *B. burgdorferi*, both enzymatic activities are carried on one protein, which is called Rel_{Bbu} in *B. burgdorferi* (13,14). (p)ppGpp works together with DksA (DnaK suppressor) to directly affect RNA polymerase (RNAP) and increase or decrease transcription of certain genes to execute the stringent response. Although DksA and (p)ppGpp have been shown to work together to carry out the stringent response, they have also been shown to have independent effects as well (10,15). Furthermore, DksA and (p)ppGpp may affect virulence in *Haemophilus ducreyi*, *Staphylococcus aureus*, *Myxococcus xanthus*, and other bacteria (16,17).

dksA was originally named in other bacteria for its function in restoring thermotolerance in *dnaK* mutants (18). The structure of DksA, in other bacteria, is most similar to GreA and GreB, which act as transcription factors (19). These transcription factors directly bind RNAP to alter transcription (19). DksA has zinc finger domains that facilitate binding to RNAP as well as sensing oxidative stress (20).

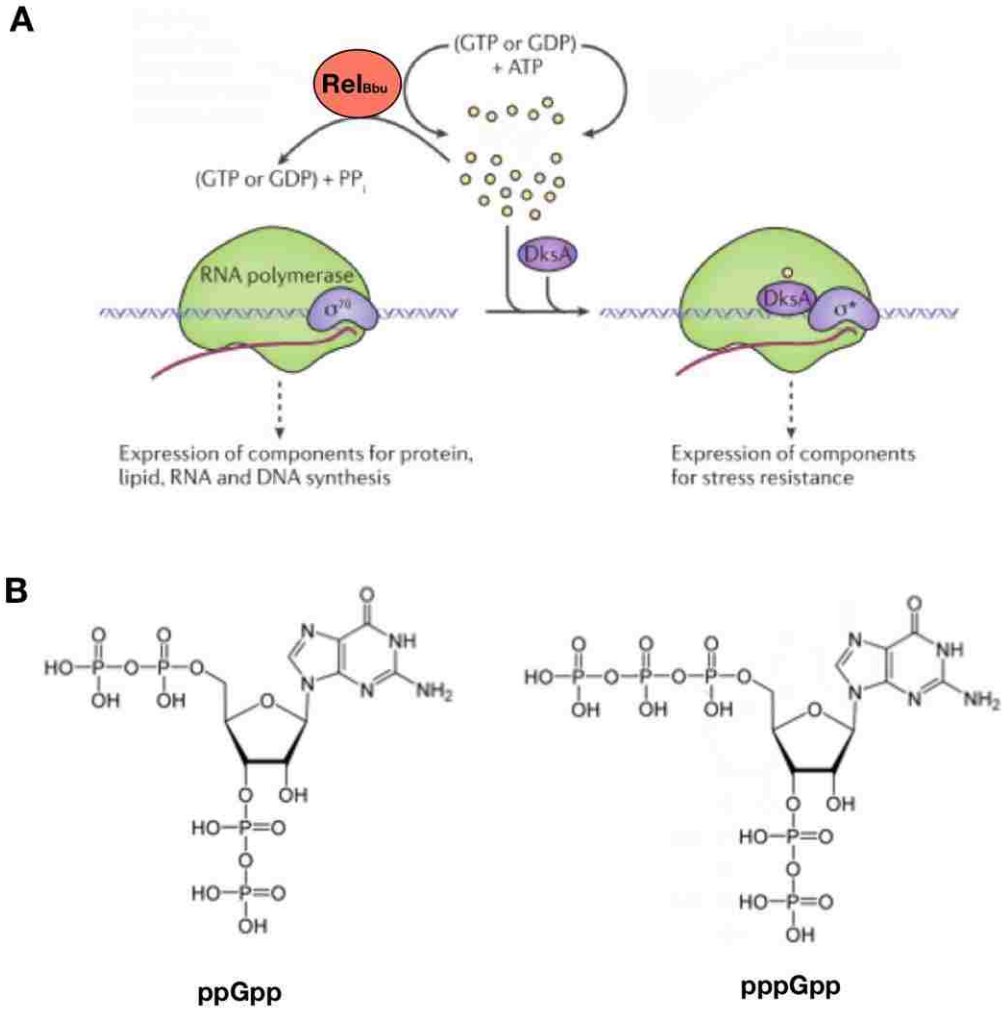

Figure 2- Stringent response.
(A) When *B. burgdorferi* is faced with nutrient stress, Rel_{Bbu} synthesizes and hydrolyzes (p)ppGpp, which serves as an alarmone. (p)ppGpp works with DksA to regulate transcription by interacting with RNAP. (B) Structures of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp). (A modified from (11); B modified from https://en.wikipedia.org/wiki/Guanosine_pentaphosphate)

After the tick has consumed its blood meal, the low-nutrient environment induces the stringent response in *B. burgdorferi* (21). The stringent response is crucial for *B. burgdorferi* to persist in the tick and rel_{Bbu} mutants are defective in their ability to survive between the larval and nymph blood meals (21). In addition, Rel_{Bbu} influences the morphological change that *B. burgdorferi* undergoes in limited nutrient mediums and in the tick midgut from the typical spirochete shape to a cyst-like morphology called a round body (RB) (21). Neither the function nor the mechanism of the morphological change to an RB is known; however, the morphology is reversible upon addition of nutrients, producing viable spirochetes (22–26). *B. burgdorferi* has been hypothesized to use glycerol as an alternative sugar source in the tick (6,27,28). The *glp* operon, encoding three genes required for glycerol utilization, has been shown to be involved in tick persistence in *B. burgdorferi* (6,27,29). Two previous studies using independent rel_{Bbu} mutants have demonstrated that transcription of the *glp* operon is increased upon induction of the stringent response (21,29).

Comparing the results from experiments with the extant rel_{Bbu} mutant (21) and the *dksA* mutant described in this thesis will provide information about the mechanism of gene regulation during the stringent response in *B. burgdorferi*. Until now, no studies on *dksA* have been performed in *B. burgdorferi* and the function of *dksA* in *B. burgdorferi* was not known. Therefore, we hypothesize that DksA potentiates the effects of the stringent response during nutrient stress allowing *B. burgdorferi* to persist in its tick vector. To test this, a null mutant and corresponding complemented strain were constructed. *In vitro* experiments were performed and show that glycerol starvation

affects cell survival and cell morphology of *B. burgdorferi*. Our results also support a role for *dksA* in growth and proper RB formation.

II. Materials and Methods

2.1 Culture Conditions and Quantification

All strains were maintained in Barbour-Stoenner-Kelly II (BSK) liquid medium with a pH of 7.5 and addition of 6% rabbit serum (RS) (Pel-Freez Biologicals) (30). Cultures were inoculated at with 1×10^3 or 1×10^4 cells and grown at 35°C until reaching late log or stationary phase. To measure cell density, a Petroff-Hausser counting chamber was used. To mimic the tick environment during molting, cells were grown in nutrient rich medium, BSK+RS until stationary phase and then pelleted at 7,000 RPM for 10 min. After which cells were then resuspended in nutrient poor medium, Roswell Park Memorial Institute medium (RPMI). RPMI 1640 without L-glutamine (Mediatech, Inc.) and without RS, or in RPMI 1640 without L-glutamine and without glucose (Biological Industries) were used. To replace glucose with glycerol, 2000 mg/L of glycerol was added to RPMI 1640 without L-glutamine and glucose (Biological Industries). RPMI cultures were grown at 35°C for the designated time points described for each experiment.

To measure cell viability, cells were grown to stationary phase in BSK+RS and then transferred to the two RPMI media described above. Cells were then grown in these media for the indicated time points before being plated in a semi-solid BSK+RS medium. Plates were incubated in a 35°C CO_2 incubator for 2 weeks. Colonies were then counted and the average was calculated for at least three independent experiments.

2.2 Constructing the Mutant and Complement

To generate a *B. burgdorferi dksA* mutant, KOD polymerase (Novagen) was used to amplify the upstream (primers *dksA*_U988F and *dksA*_3R+AatII+AgeI, Table 1) and downstream (primers *dksA*_362F+AatII and *dksA*_D1455R+AgeI, Table 1) regions of *dksA* on the chromosome. These PCR products were cloned into pCR®2.1-TOPO (Invitrogen) and sent to the University of Montana Murdock Sequencing Facility to verify that the sequences were correct. The upstream (pTA-dksA-up) and downstream (pTA-dksA-down) plasmids were then digested with AatII and AgeI and the downstream fragment was ligated into the upstream plasmid, which yields an artificial AatII site instead of the *dksA* open reading frame. This AatII site was used to introduce a streptomycin/spectinomycin resistance cassette driven by the *B. burgdorferi flgB* promoter (31). The plasmid containing the upstream and downstream fragments as well as the antibiotic resistance cassette was then linearized with AdhI and ethanol precipitated. The *B. burgdorferi* competent cells (B31-5A4 bbe02-lacI-aacCI) that were used carry a kanamycin resistance marker on the infectivity plasmid lp54 as well as the *E. coli lacI* gene (32). B31-5A4 bbe02-aacCI cells were made competent and electroporated with 10 µg of the linearized plasmid (33). Cells were then added to BSK-II medium containing 50 µg/ml of streptomycin and plated on 96-well plates. The plates were then incubated at 35°C in a CO_2 incubator. Positive colonies were grown in 5 mL cultures and total genomic DNA was purified using a phenol/chloroform extraction. PCR amplification of the genomic DNA (primers *dksA*_U75F+AatII and *dksA*_382R+AatII, Table 1) confirmed the absence of the *dksA* open reading frame and its replacement with the antibiotic resistance cassette.

To develop a *cis*-complemented strain, the *dksA* upstream plasmid (pTA-*dksA*-up) as previously described was used. KOD polymerase (Novagen) was used to amplify

Table 1. Oligonucleotides used in this study.

Name	**Sequence (5′ to 3′)**
*dksA*_U988F	CAGGACTTTGGTTAAATAGA
*dksA*_3R+AatII+AgeI	ACCGGTGCTGACGTCATGATACCTCCCTATAAATT
*dksA*_362F+AatII	GACGTCAAAAGAACAAAAGATGATACA
*dksA*_D1455R+AgeI	ACCGGTAGATTAGAAGGCACAGATA
*dksA*_U75F+AatII	GACGTCTTTTGTTTACATTATAAGAAATTG
*dksA*_382R+AatII	GACGTCTGTATCATCTTTTGTTCTTTT
*dksA*_1F+NedI+AatII	GACGTCGGGAGGTCATATGCAAAAAGCTGTTTCTGA
*dksA*_265F	GGTAAGTGTTTGGCTTGTGAAAGA
*dksA*_341R	CTAATACACACAAAAAAGCATAAGGAATAGCT
dksA probe	6-FAM-ATTGCTAGGGAGAGACT-TAMRA
flaB 423F	TTCTCAAAATGTAAGAACAGCTGAAGA
flaB 542R	TGGTTTGTCCAACATGAACTC
flaB probe	6-FAM-TCACTTTCAGGGTCTCAAGCGTCTTGGAC-TAMRA
glpF 166F	GGATTGGGTGTAACGTTTGGTATT
glpF 256R	CAACACTTGCTAATCCTATGCTAACAG
glpF probe	6-FAM-CAGCAAGAATGAGCGGAGCACACCTAAAC-TAMRA

the *dksA* gene as well as the downstream region (using primers *dksA*_1F+NedI+AatII and *dksA*_D1455R+AgeI, Table 1) and cloned into pCR®2.1-TOPO (Invitrogen) and sent to the University of Montana Murdock Sequencing Facility to verify that the sequences were correct. The inducible *flac* promoter was introduced into the dksA-downstream plasmid through digestion with AatII and NdeI followed by ligation (pTA-flacp-*dksA*-down). The upstream plasmid (pTA-*dksA*-up) and the plasmid described above (pTA-flacp-*dksA*-down) were digested with KpnI and AatII and ligated together to create a plasmid with the upstream piece, downstream piece and *dksA* fused to the *flac* promoter (pTA-up-flacp-*dksA*-down). The gentamicin resistance cassette (*flgBp-aacCI*) was then inserted into the AatII site. The resulting plasmid (pTA-dksA *cis* aacCI) was linearized with AdhI and ethanol precipitated. Competent cells were made from the *dksA* mutant described above and electroporated with 10 µg of the linearized plasmid. Electroporated cells were plated and positive colonies were isolated for genomic DNA purification and PCR (primers *dksA*_U75F+AatII and *dksA*_382R+AatII, Table 1) was used to confirm the presence of *dksA* fused to the inducible promoter linked to the antibiotic resistance cassette (*flgBp-aacCI*).

2.3 RNA Isolation and qRT-PCR Analysis

Cells were grown to late log or stationary phase as described above. RNA was isolated using TRIzol (Invitrogen) from 40 mL cultures grown at 35°C under media conditions indicated in each experiment and described above. RNA samples were treated with Turbo DNase (Ambion) to remove any contaminating DNA. RNA from the DNase-treated samples was extracted through phenol/chloroform extraction. To confirm no DNA remained in our DNase-treated RNA samples, PCR (using primers *flaB* 423F and *flaB* 542R, Table 1) was performed before cDNA was synthesized. Following confirmation of

no DNA contamination, 1 µg of the RNA samples were converted to cDNA using the SuperScript®III kit (Invitrogen) by following the manufacturer's instructions.

Primer Express® 3.0 (Applied Biosystems) or MacVector (MacVector, Inc.) were used to design primers and FAM-TAMRA-labeled probes. TaqMan Universal PCR Master mix (Applied Biosystems) was used in 96-well plates to perform TaqMan qRT-PCR. Applied Biosystems StepOnePlus™ real time PCR machine was used (cycling conditions: 50°C 2 min; 95°C 10 min; 95°C 15 sec and 60°C 1 min for 40 cycles). Transcript amounts of *dksA* (primers used *dksA*_265F and *dksA*_341R, Table 1) and *flaB* (using primers *flaB* 423F and *flaB* 542R, Table 1) were calculated using a standard curve generated using known amounts of the *dksA* ORF (nucleotides 1-382) or a portion of the *flaB* ORF (nucleotides 278-551) cloned into pCR®2.1-TOPO. *B. burgdorferi* genomic DNA was used to generate standard curves for *glpF* transcript levels (using primers *glpF*_166F and *glpF* 256_R) . Transcript copy number of the gene of interest in each sample was determined using the standard curve and then normalized to the number of *flaB* copies. Each set of samples was performed in triplicate from 3 independent experiments and the mean ± the SEM were calculated.

2.4 Staining and Fluorescence Microscopy

For cell viability experiments and cell morphology, *B. burgdorferi* cultures were stained with propidium iodide (PI) to identify dead cells. Cultures of 150 to 500 µL were collected in a tabletop centrifuge at 10,600 x g for 5 min. The cells were then resuspended in 10 µL of 0.85% sodium chloride solution and 1.5 µM of PI were added to cells. Cells were incubated in the dark for 15 minutes before being wet mounted on a slide and covered with a glass coverslip. Slides were examined using an Olympus BX51 fluorescence microscope with 100x/0.75 NA objectives. Fluorescent images were

overlaid over DIC images, the live and dead cells were counted, and percentages were calculated. Images were processed using Pixelmator (Pixelmator Team, Ltd).

III. Results

3.1 *dksA* Mutagenesis

A *dksA* null mutant was generated by replacing most of the open reading frame with the streptomycin/spectinomycin resistance cassette (Fig. 3A) (31). The *dksA* mutant was complemented by genetic reconstitution in *cis* with the open reading frame fused to an inducible promoter (*flacp*) and linked to the gentamicin resistance cassette (Fig. 3A) (32,34). To verify these genetic manipulations, genomic DNA was isolated from the *dksA* mutant and complemented strains. PCR analyses was performed using the primers dksA U75F and dksA 382R (Table 1). The PCR products were resolved by 1% agarose gel electrophoresis; the difference in size of the PCR products confirmed the constructs (Fig. 3B). The *flacp-dksA* fusion was induced in the complemented strain using varying amounts of IPTG. *dksA* transcript levels were assayed by qRT-PCR and compared to wild type (Fig. 3C). The amount of *dksA* mRNA does not reach wild-type levels at the highest IPTG dose (5 mM), although the levels are partially restored.

3.2 Growth of the *dksA* Mutant

We hypothesized that DksA, which regulates transcription of genes involved in the stringent response, is required for normal growth. Wild type, *dksA* mutant and

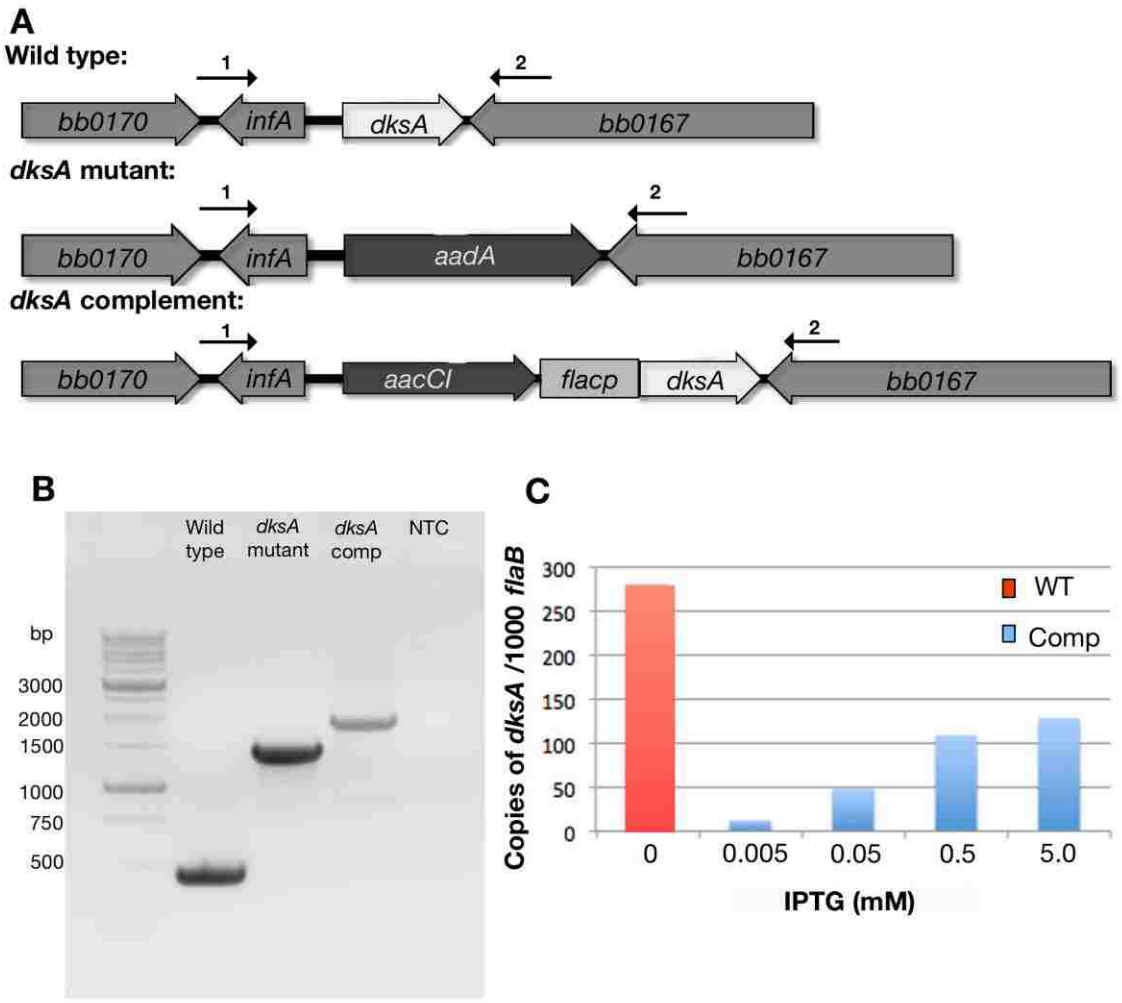

Figure 3- Construction of the *dksA* mutant and *cis*-complemented strains.
(A) Graphical depiction of the *dksA* chromosomal loci from wild-type, *dksA* mutant, and *dksA* complemented strains. Arrows indicate positions of the oligonucleotide primers used in PCR analyses in (B). *aadA* encodes the spectinomycin/streptomycin resistance cassette, *aacCI* encodes the gentamicin resistance cassette, and *flacp* is the inducible *flac* promoter. (B) PCR analyses of genomic DNA from wild-type, *dksA* mutant, and complemented (*dksA* comp) strains using primers dksA U75F (1) and dksA 382R (2). A no-template control (NTC) was used as a negative control for the PCR. (C) IPTG induction of *dksA* transcript levels in the complemented strain compared to *dksA* transcript levels in wild type. Wild-type cultures were grown in BSK + RS (red bar) and complemented cultures were grown in BSK + RS containing different concentrations of IPTG (blue bars). RNA was isolated, *dksA* transcript levels were measured using qRT-PCR and normalized to *flaB* transcript levels.

complemented strains were grown in normal growth medium, Barbour-Stoenner-Kelly II medium containing 6% rabbit serum (BSK+RS) (30). Cultures were inoculated at 5 x 10^3 cells mL^{-1} and grown at 35°C and cell density was quantified (Fig. 4) (33). Four independent replicates were performed and the mean and standard error were calculated. The *dksA* mutant demonstrated a growth phenotype, with statistically significant differences in growth found between wild type and the *dksA* mutant on days 4, 6 and 7. The complemented strain partially restored growth to wild-type levels, which is most likely due to the lower *dksA* transcript levels than in wild type.

3.3 *dksA* Transcript Levels

To determine the effect of nutrient starvation on wild-type levels of *dksA* transcription, wild-type cells were grown to stationary phase in BSK + RS. Cells were then shifted to a starvation medium, RPMI with glucose (21). RNA was harvested at 0, 6 and 24-h time points. qRT-PCR was used to determine the number of *dksA* transcripts and was normalized to *flaB* transcript levels (Fig. 5). The transcript levels of *dksA* decrease at 6 h, although the change is not statistically significant, likely due to the variation between assays. The *dksA* transcript levels are restored at 24 h.

3.4 Cell Viability

Viability of the *dksA* mutant, wild-type, *dksA* mutant and complemented cells was assayed after growth in BSK + RS and starvation in RPMI with glucose. Cells were incubated with propidium iodide (PI), which does not enter live cells, at 0, 24 and 72 h, and imaged by fluorescence microscopy; fluorescent images were overlaid with differential interference contrast (DIC) images to visualize all cells (Fig. 6A). The

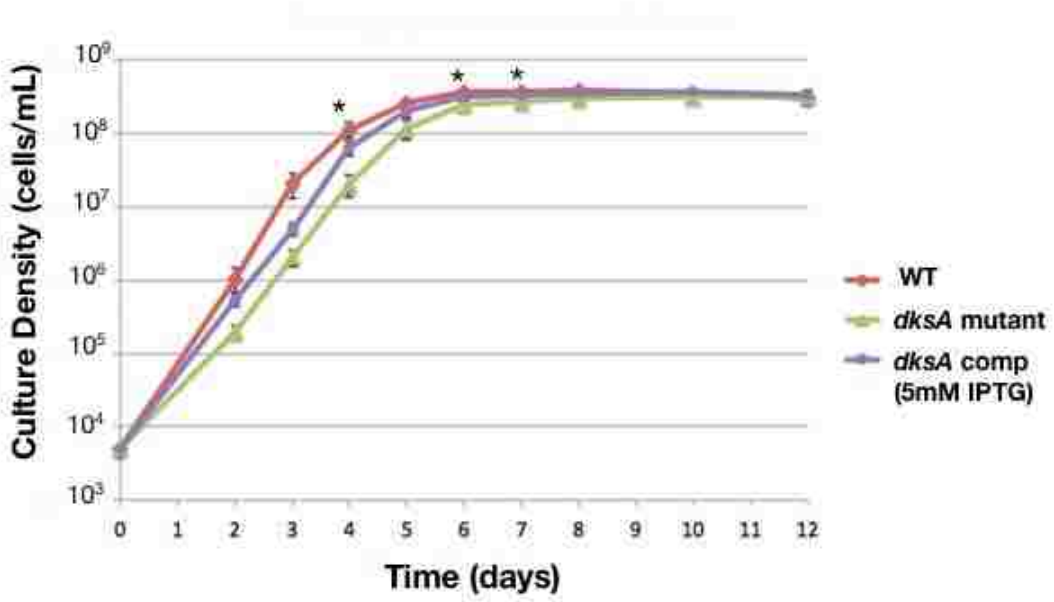

Figure 4- Growth of the *dksA* mutant.
Wild-type, *dksA* mutant and complemented cells were inoculated at a density of 5 x 10^3 cells ml^{-1} in BSK + RS and grown at 35°C. Cell density was quantified using a Petroff-Hausser cell counter. Each data point represents the average of four independent replicates and error bars are the standard error of the mean. Asterisks indicate statistically significant differences between the wild type and *dksA* mutant.

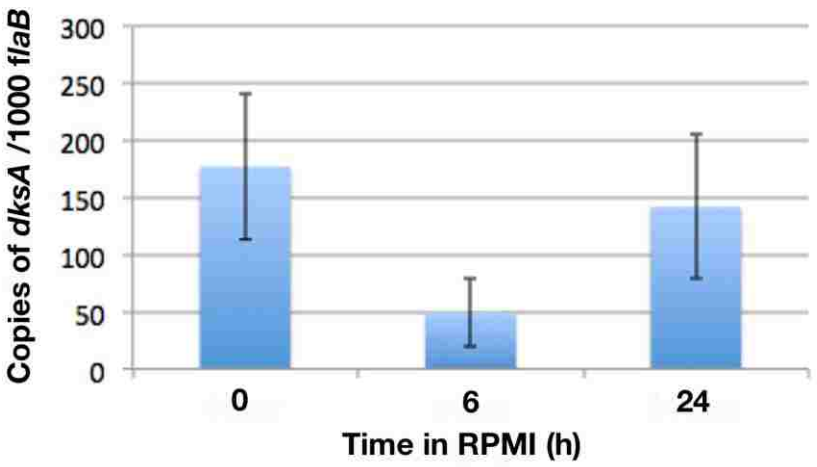

Figure 5- *dksA* transcript levels during starvation.
Wild-type cells were grown in BSK + RS before starving in RPMI. RNA was harvested before shifting to RPMI (0 h), and after 6 h and 24 h in RPMI. qRT-PCR was used to measure *dksA* transcript levels. *dksA* transcript levels were normalized to *flaB* transcript levels. Each data point represents the average of three independent experiments and error bars represent the standard error of the mean.

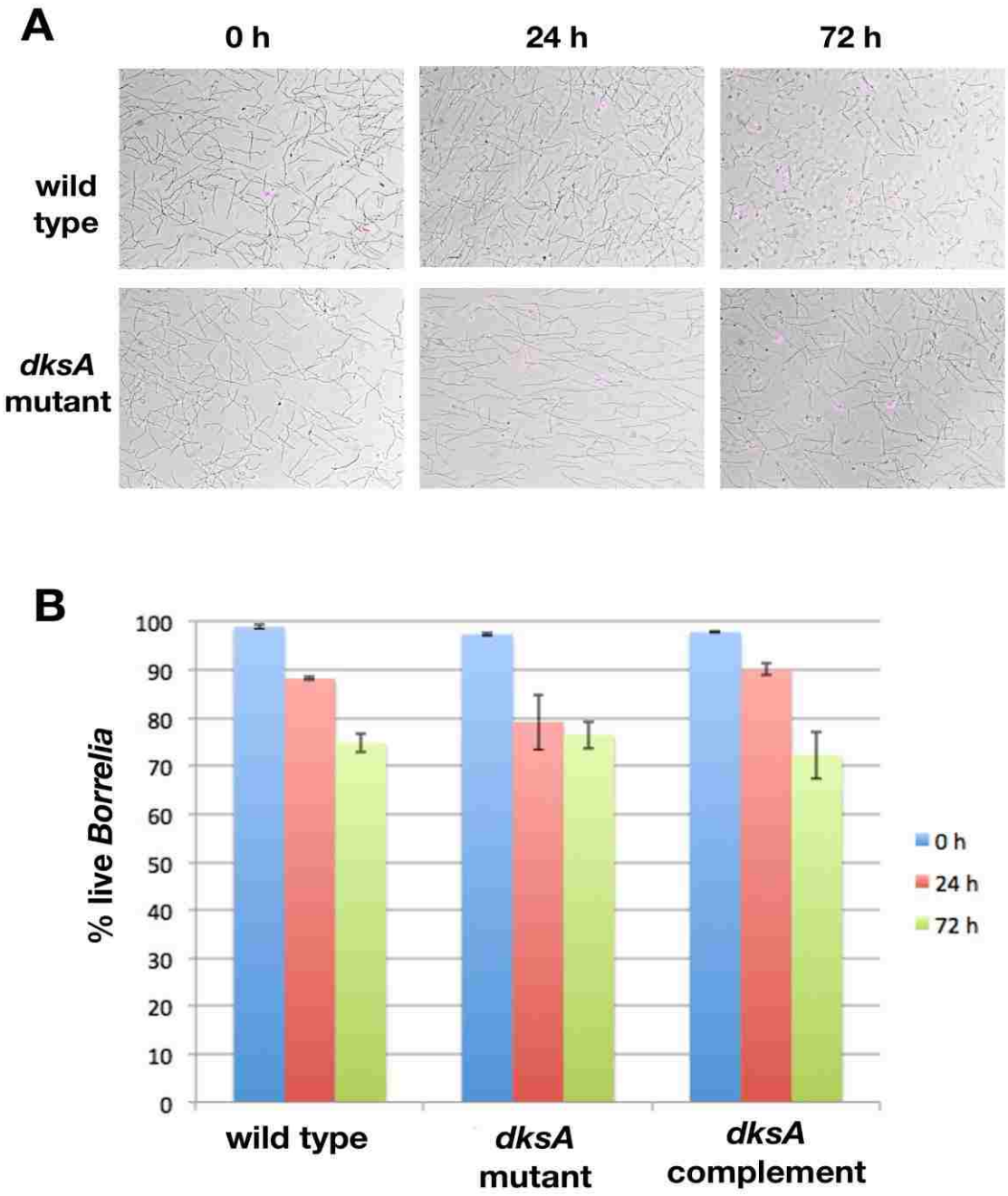

Figure 6- *dksA* cell viability during starvation.
(A) Wild-type, *dksA* mutant and complemented cells were grown in BSK + RS before starvation in RPMI. After 24 and 72 h in RPMI, cells were incubated with propidium iodide (PI) to identify dead cells. Fluorescent images were overlaid with DIC images. (B) The numbers of live and dead cells were counted, and the percentage of live cells was calculated. Each data point represents the average from three independent experiments and error bars represent the standard error of the mean.

numbers of live and dead cells were quantified (Fig. 6B). Although there were no significant differences between the three strains at each of the three time points, the viability trended toward decreasing over 24 and 72 h of starvation in all three strains. These results suggest that *dksA* does not have an essential role in survival during starvation in RPMI.

To analyze the effect of *dksA* on the utilization of different sugar sources during nutrient limitation, wild type, *dksA* mutant and complemented cells were grown in BSK + RS and shifted to one of three starvation media: RPMI with glucose (Glc), RPMI with glycerol (Gly) or RPMI with no sugar. We choose to use glycerol because *B. burgdorferi* can utilize glycerol and glycerol is hypothesized to be the major sugar source during nutrient limitation in the tick mid-gut (21,27–29). Cells were incubated in the starvation media for 0, 24 and 72 h and then assayed for viability with PI as described above. At 0 h, 98% of all three strains are alive (data not shown). There is no significant change in cell viability between the three strains at 24 h (Fig. 7A). However, the viability of the complemented cells at 24h is decreased in the glycerol medium when compared to the other two media; wild type has the same trend, but the decrease is not statistically significant. Interestingly, the cell viability for all three strains in both the glucose and no sugar media is similar at both 24h and 72h. There is also no difference in the survival of the different strains at 72 h (Fig. 7B). However, cell viability is significantly reduced at 72 hours for the *dksA* mutant in the glycerol medium compared the glucose medium.

3.5 Cell Morphology

When evaluating cell viability, we noticed differences in cell morphology in cells grown in the different sugar sources. To further investigate this observation, wild type, *dksA* mutant and complemented cells were grown in BSK + RS and shifted to one of

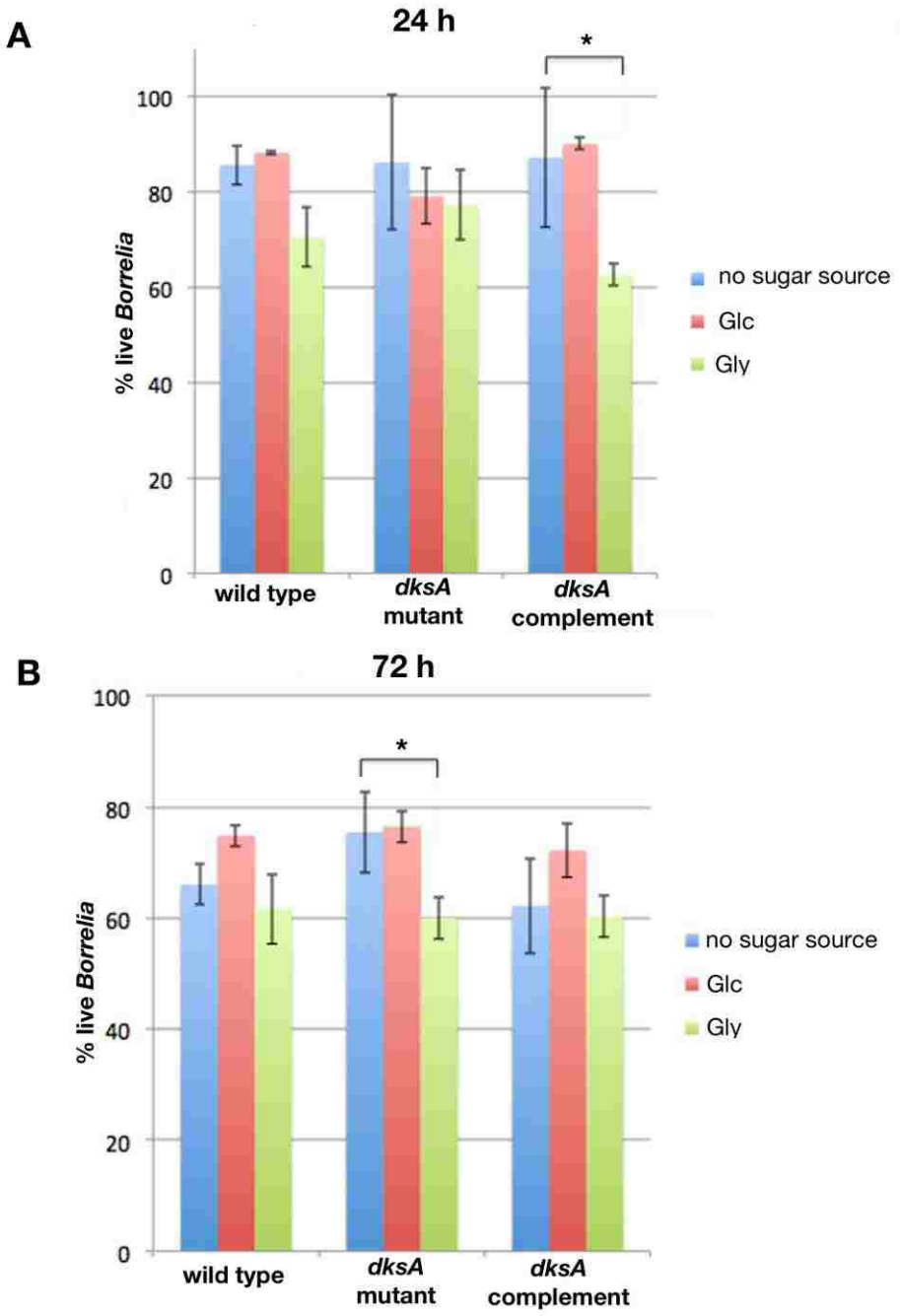

Figure 7- Cell viability of the *dksA* mutant with different sugar sources.
Wild-type, *dksA* mutant and complemented cells were grown in BSK + RS before starving in RPMI media with glucose (Glc), glycerol (Gly), or no sugar. Following starvation for (A) 24 h and (B) 72 h, cells were stained with propidium iodide (PI). Fluorescent images were overlaid with DIC images, live and dead cells were quantified and the percentage of live cells calculated. Each data point represents the average from three independent experiments and error bars represent the standard error.

three starvation media as previously described (no sugar, glucose added, or glycerol added). DIC microscopy was performed at 0, 24 and 72 h for all three strains. Figure 8 shows wild type at 0 h (A) and at 24 and 72 h in each of the media, no sugar source (B,C), glucose (D,E) and glycerol (F, G). As can be seen in the images, the cells change their morphology during starvation from the typical long, thin spirochete shape to a condensed, spherical shape called a round body (RB) (21–23,25). The amount of round bodies increases over time in all three media. However, more RBs are apparent in the no sugar and glycerol media. To determine if this difference is significant, the numbers of RBs were quantified. Less than 2% of cells were RBs at 0 h in all three strains (data not shown). There is an increase in the number of RBs in all three starvation media at 24 h (Fig. 9A). All three strains have significantly more RBs in the glycerol medium compared to the glucose medium. The *dksA* mutant and complement also have significantly more RBs in the glycerol and the no sugar media. In addition, the *dksA* mutant has significantly more RBs in the no sugar medium than the glucose medium. After 72 h, the number of RBs continues to increase in all strains (Fig. 9B). The number of RBs in the glycerol and no sugar media become comparable for the wild-type and complemented strains. However, there are still significantly more RBs in the *dksA* mutant in glycerol medium compared to the no sugar medium. In all three strains, there is a trend toward slightly fewer RBs in the glucose medium compared to the other two media; however, this is not a significant difference. These results demonstrate that after 72 h of starvation, RB formation is increased regardless of the sugar source. The data also suggest a role for *dksA* in the formation of RBs.

We observed two different types of RBs while imaging cell morphology. One type appeared to be tighter and more compact, possibly partially formed, which we call

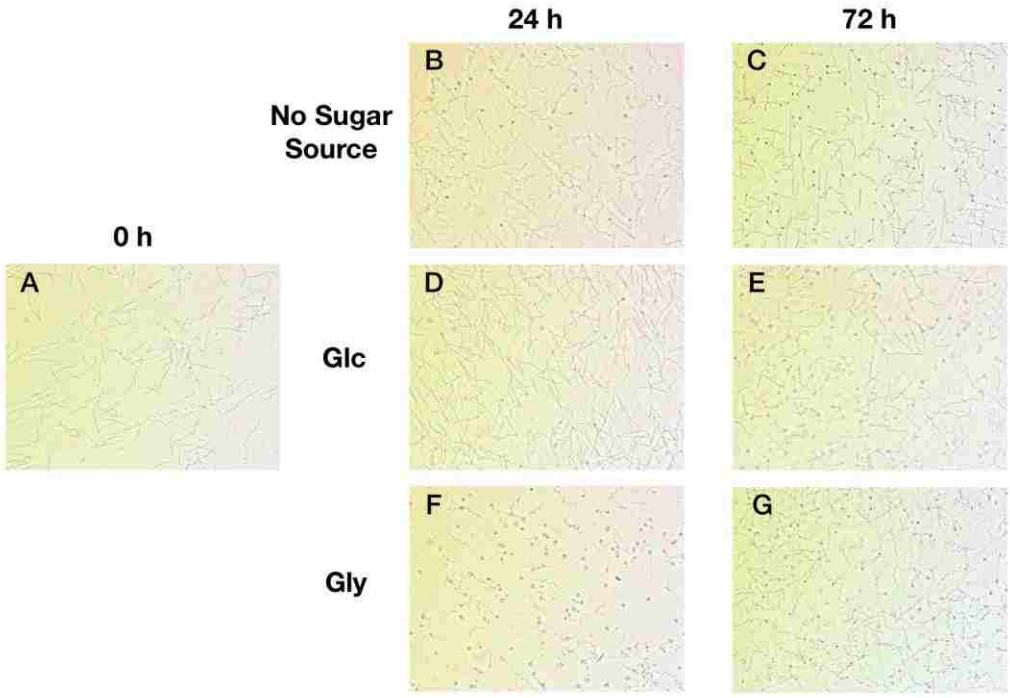

Figure 8- Cell morphology in different sugar sources.
Wild-type cells were grown in BSK+RS until they reached (A) stationary phase and then starved in RPMI media with no sugar (B and C), with glucose (Glc) (D and E), or with glycerol (Glc) (F and G). Cells were imaged using DIC microscopy at 0 h (A), 24 h (B, D and F) and 72 h (C, E and G) of starvation.

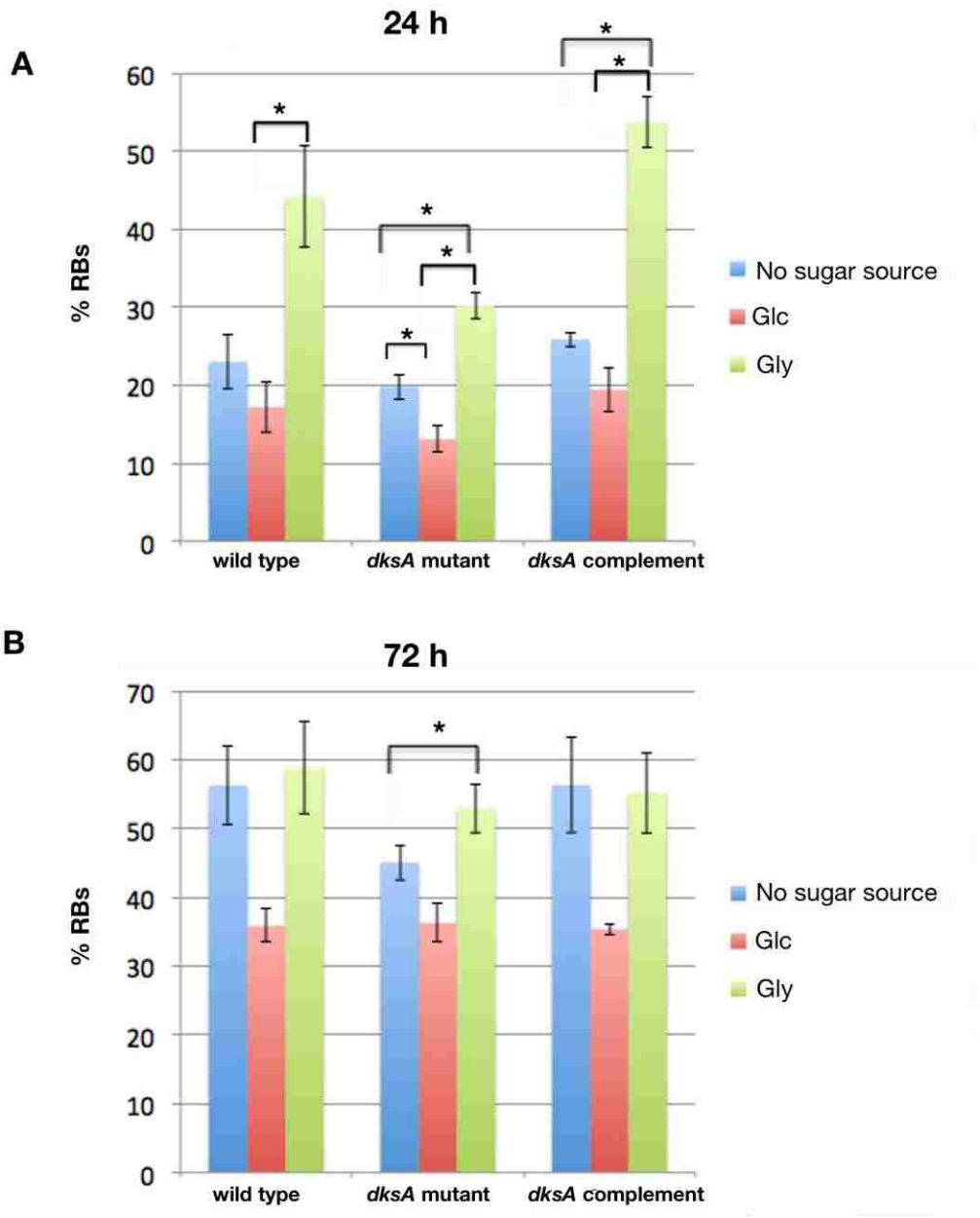

Figure 9- Quantification of RBs formed in different sugar sources.
Wild-type, *dksA* mutant and complemented cells were grown in BSK+RS until they reached stationary phase and then starved in RPMI media with no sugar, with glucose (Glc), or with glycerol (Glc). Cells were imaged by DIC microscopy at (A) 24 h and (B) 72 h of starvation and the number of RBs were counted and expressed a percentage of total cells. Values are the averages from at least three independent experiments and error bars are the standard error of the mean. Asterisks indicated significant ($P < 0.05$) differences as determined by a one-way ANOVA with a Tukey's post-hoc test.

small RBs (sRBs) (Fig. 10A, blue arrows). The second type are what has been previously observed, which we will refer to as RBs (Fig. 10A, black arrowheads). To determine if there is a difference between the number of sRBs in the different starvation media, the 24-h images obtained and quantified in Figures 8 and 9 were used to quantify the relative number of sRBs. The percentages of total RBs that are sRBs were calculated (Fig. 10B). Both wild type and complement have significantly more sRBs in the no sugar medium and significantly fewer sRBs in the glycerol medium. In all three strains, there is no difference in the percentage of sRBs or RBs between glucose and glycerol media. The *dksA* mutant had no significant differences for the type of RB in any of the three media. These observations further support a role for *dksA* in RB formation.

3.6 Ribosomal Degradation

Our results (Fig. 7-10) suggest that glycerol affects cell viability and morphology. To further test the effect of glycerol, ribosomal RNA (rRNA) quality was observed to assess the molecular physiology of the cells. Wild-type and *dksA* mutant cells were grown in BSK+RS until stationary phase and then shifted to starvation media with either glucose or glycerol. RNA was harvested at 0, 6 and 24-h time points. rRNA was separated by size on a 2% agarose gel and the 5S, 16S and 23S rRNAs were visualized (Fig. 11). At 0, 6 and 24 h of starvation in glucose medium, the rRNA is intact and not affected by starvation. After 6 h of starvation in glycerol medium, both the wild-type and *dksA* mutant rRNA appeared in good condition (Fig. 11 and data not shown). However, the rRNA was degraded, indicating ribosome disassembly, after 24 h of starvation in glycerol medium in both wild type and the *dksA* mutant. The degradation of rRNA at 24h suggests that cells starved in glycerol may be shifting into a quiescent state.

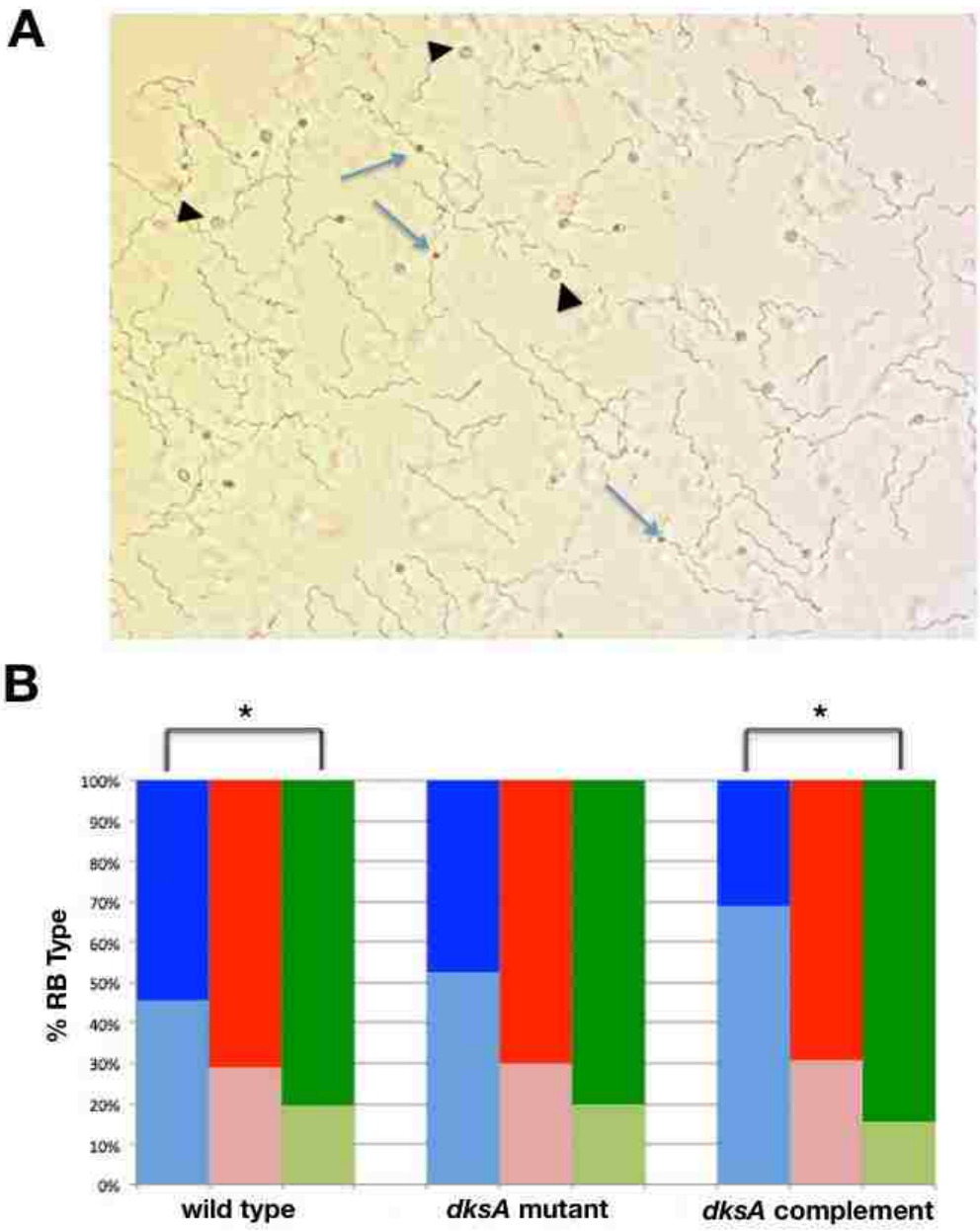

Figure 10- Quantification of small RBs in different sugar sources.
Wild-type, *dksA* mutant and complemented cells were grown in BSK+RS until they reached stationary phase and then starved in RPMI medium with no sugar, with glucose (Glc) or with glycerol (Glc). (A) DIC image of wild-type cells starved for 24 h in RPMI with no sugar showing RBs (black arrowhead) and small RBs (sRBs) (blue arrow). (B) The number of RBs and sRBs were counted and the percentage of all RBs that are sRBs ((sRBs/ RBs + sRBs) x 100) was graphed. Light shade represents sRBs and dark shade represents RBs, in the three starvation conditions: no sugar (Blue), with glucose (Red) or with glycerol (Green). Values are the averages from at least three independent experiments and error bars are the standard error of the mean. Asterisks indicated significant ($P < 0.05$) differences as determined by a one-way ANOVA with a Tukey's post-hoc test.

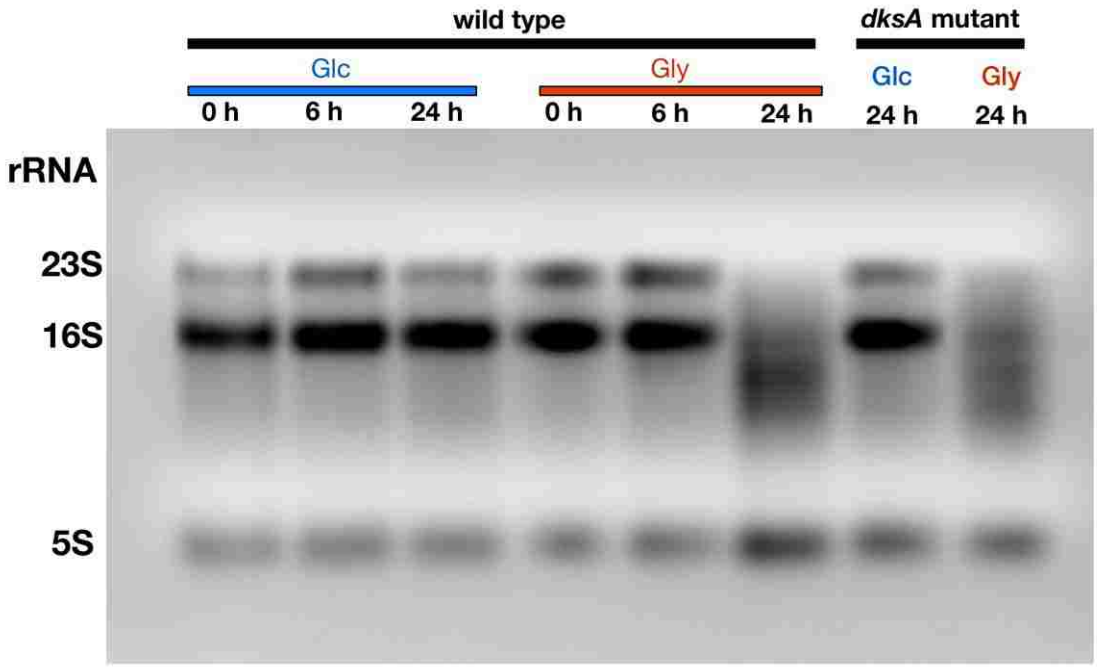

Figure 11- rRNA during starvation.
Wild-type and *dksA* mutant cells were grown in BSK+RS to stationary phase before starving in RPMI containing glucose (Glc) or glycerol (Gly). RNA was harvested at 0, 6 and 24 h after starvation. One µg of total RNA from each sample was separated on a 2% agarose gel and visualized with ethidium bromide.

3.7 Regulation of *glpF* Transcription

Glycerol has been hypothesized to serve as an important sugar source during starvation in the tick midgut (21, 27–29). *glpF* is one of three annotated genes in the glycerol utilization operon and encodes a glycerol transporter. To investigate the expression of *glpF* in response to starvation with either glucose or glycerol, wild type, *dksA* mutant and complemented cells were grown in BSK + RS. Cells were then starved in either glucose medium or glycerol medium. rRNA quality indicate that at 24 h of starvation in glycerol, RNA begins to degrade (Fig 11) and, therefore, we decided to harvest RNA at 0 and 6 h. qRT-PCR was then used to measure *glpF* transcript levels, which were then normalized to transcript levels of *flaB*. Normalized *glpF* levels were then normalized to the 0-h time point, so values are shown as a proportion of 1.0 (Fig. 12). The amount of *glpF* transcript in wild type decreased more after 6 h of starvation in glucose medium than in glycerol medium. Transcript levels of *glpF* in the *dksA* mutant during both glucose and glycerol starvation remain fairly equal and do not deviate far from 1.0. Additionally, there are significantly fewer *glpF* transcripts in wild type compared to *dksA* mutant during starvation with glucose. *glpF* levels trended toward an increase in the complemented strain in glycerol medium, but there was a considerable amount of variation and the difference was not significant between strains and conditions. These data suggest that *dksA* has a role in the regulation of *glpF* in the presence of glycerol during starvation.

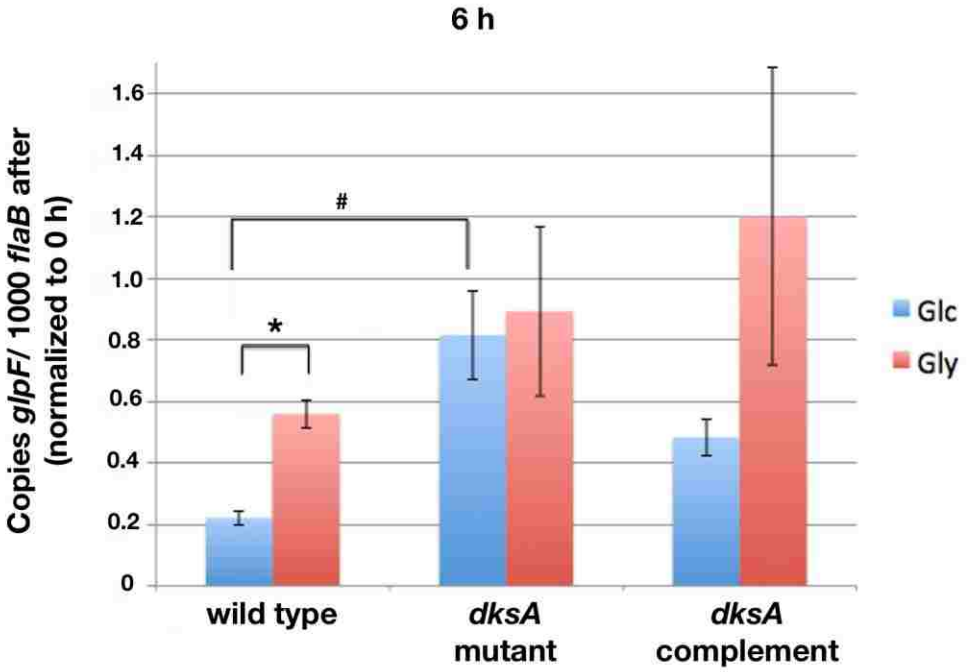

Figure 12- *glpF* transcript levels during starvation.
Wild-type, *dksA* mutant and complemented cells were grown in BSK+RS to stationary phase before starving in RPMI containing glucose (Glc) or glycerol (Gly). RNA was harvested at 0 and 6 h after starvation. qRT-PCR was used to measure *glpF* transcript levels, which were normalized to *flaB* levels. Values are the average of three independent experiments where *glpF* levels at 6 h were normalized to 0 h (set to 1) and error bars represent the standard error of the mean. Asterisks (*) indicate significant ($P < 0.05$) differences between conditions, as determined by two-tailed t-test. Pound signs (#) indicate significant ($P < 0.05$) differences between strains, as determined by a one-way ANOVA with a Tukey's post-hoc test.

IV. Discussion

The tick provides a harsh environment with limited nutrients and *Borrelia burgdorferi* must adapt to persist in its tick vector and complete its enzootic cycle. Bacteria invoke the stringent response when faced with limited nutrients, which directly affects transcription of certain genes to create a nutrient-saving mode.

During the stringent response, resources are conserved and growth is curtailed. We hypothesized that DksA would decrease transcription of genes required for growth to contend with the harsh conditions in the tick. We found that the *dksA* mutant strain grows significantly slower than wild type and normal growth rates are restored in the complemented strain (Fig. 4). These results support a role for *dksA* in the regulation of growth and are consistent with the phenotype of a rel_{Bbu} mutant, which also exhibits slow growth (21).

Drecktrah *et al.* show, using both PI staining and semi-solid plating, that (p)ppGpp plays an important role in cell viability during the stringent response (21). Therefore, we expected that *dksA* would affect cell viability during starvation. However, PI staining showed that *dksA* did not affect cell survival in starvation conditions (Fig. 6). The results of the *dksA* mutant were unable to be confirmed by semi-solid plating due to issues with reproducibility and technical errors.

Glycerol has been suggested to have an important role in tick persistence and several laboratories have shown that glycerol can replace glucose to support growth in wild-type cells *in vitro* (6,27–29). As the blood meal is consumed in the tick, *B. burgdorferi* can take advantage of the glycerol found in the tick hemolymph to survive and persist in the tick (6,27). However, the mechanism has not yet been determined. To investigate this, we evaluated the cell viability of the *dksA* mutant and complement grown

under nutrient-limited conditions in the presence of glycerol. After 24 h of incubation, cell viability was significantly decreased in complemented cells incubated in glycerol-containing starvation medium. Furthermore, after 72 h, cell viability was significantly decreased in our *dksA* mutant incubated in glycerol medium. Our data suggest that *B. burgdorferi* is unable to solely utilize glycerol and must take advantage of other sugar sources. Differences between our results and previous work may be due to the use of different media that contained other sugar sources, such as BSK+RS and BSK-Lite (27–29). Resolving these discrepancies will require further investigation.

In vitro cultures of *B. burgdorferi* have been shown to change their cell morphology from the typical spirochete form to round, cyst-like structures, called round bodies (RBs), under stressful conditions, including limited nutrients (22–26). Although the purpose of this morphological change is not yet known and not well studied, a recent report has shown that RBs can form within the tick midgut and that this morphology is reversible, producing viable cells (25). Previous experiments showed that the rel_{Bbu} mutant had increased RBs when starved in RPMI (21). We found there was an increase in the number of RBs in all three strains, with no effect of DksA, under the same conditions (glucose-containing starvation medium) (Fig. 8 and 9). Cell morphology of the three strains was further investigated in different sugar sources. In all three strains, we found a significant increase in the number of RBs after cells were starved for 24 h with glycerol compared to glucose. We saw a significant increase in the number of RBs incubated in glycerol medium compared to medium with no sugar source in the *dksA* mutant and complement after 24 h. These results suggest that glycerol may serve as a signal to induce RBs. The *dksA* mutant has slightly fewer RBs in all three starvation conditions after 24 h. In addition, there are significantly fewer RBs in starvation with glucose compared with

no sugar source after 24 h. These data suggest that DksA may be involved in RB formation.

When investigating cell morphology in different sugar sources, we noticed that there appeared to be two different types of RBs. The first type of RB is the typical one; however, a smaller, more compact type of RB was also observed (Fig. 10A). We hypothesize that these smaller, more compact RBs may be immature or not properly formed. Both the wild-type and complemented strain had significantly more small RBs when starved without a sugar source (blue bars, Fig. 10B), as well as significantly more typical RBs when starved with glycerol (green bars, Fig. 10B). The *dksA* mutant, however, did not have a significant difference. These data support a function for DksA in proper RB formation. Further experiments may also elucidate the difference between the two types of RBs seen in these experiments.

Previous work has suggested that the glycerol utilization operon is necessary for tick persistence (6,27–29). The presence of glycerol was shown to increase *glpF* and *glpD* transcript levels (27,28). This is consistent with our results as the transcript levels of *glpF* were significantly higher in wild type after 6 h of starvation in glycerol compared to glucose. In the *dksA* mutant, transcript levels of *glpF* in glucose and glycerol starvation media remain similar and do not appreciably change from the levels at 0 h. These data suggest that under starvation conditions, DksA may play a role regulating *glpF* transcription. Additionally, there is significantly fewer *glpF* transcripts in wild type compared to *dksA* mutant during starvation with glucose. These data suggest that DksA is involved in the regulation of *glpF* expression in the presence of glycerol. Experiments with rel_{Bbu} mutants have shown an increase in *glpF* and *glpD* transcript levels upon starvation (21,29). These data suggest that both (p)ppGpp and DksA regulate glycerol

utilization genes. These data also suggest that (p)ppGpp and DksA have overlapping roles in *B. burgdorferi*.

As mentioned previously, *B. burgdorferi* has been thought to be able to utilize glycerol as an alternative sugar source (6,27). Furthermore, *in vitro* work has shown that cells can grow when glycerol replaces glucose in some media, such as BSK and BSK-Lite (27–29). Our results show that rRNA was intact after 24 h of starvation in the presence of glucose in all three strains (Fig. 11 and data not shown). However, rRNA from cells starved in glycerol were viable after 6 h, but the rRNA had started to degrade after 24 h. The degradation appeared to result in several distinct smaller bands, which suggests that the cleavage of rRNA is occurring at specific sites. Similar rRNA degradation has been seen during nutrient stress (35,36). These data suggest that cells starved in glycerol after 24 h are preparing to slow growth by disassembling ribosomes. Whether these changes are part of the stringent response or some other cellular processes is unclear at present. Future experiments could examine this phenotype and determine the molecular mechanisms during glycerol starvation.

Overall, we see several overlapping roles for DksA and (p)ppGpp in regulating *glpF* and cell morphology as well as distinct roles in cell viability (27,33, this work). Until now, the function of *dksA* had not been characterized in *B. burgdorferi.* We have provided data to begin to understand the function of this important gene in this unique bacterium. More experimental evidence is needed to support a role for DksA in the stringent response of *B. burgdorferi*. However, our results refuted the previous hypothesis that glycerol can serve as an alternative sugar source for *B. burgdorferi* and further studies are warranted to decipher this discrepancy.

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