DksA-dependent regulation of RpoS contributes to *Borrelia burgdorferi* tick-borne transmission and mammalian infectivity

William K. Boyle[1], Crystal L. Richards[2], Daniel P. Dulebohn[2], Amanda K. Zalud[1], Jeff A. Shaw[1], Sándor Lovas[3], Frank C. Gherardini[2], Travis J. Bourret[1]*

Affiliations: [1]Department of Medical Microbiology and Immunology, Creighton University, Omaha, NE 68105, [2]Laboratory of Bacteriology, Gene Regulation Section, Division of Intramural Research, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840; [3]Department of Biomedical Sciences, Creighton University, Omaha, NE 68105

Author contributions:
Conceptualization: William K. Boyle, Crystal L. Richards, Frank C. Gherardini, Travis J. Bourret
Data curation: William K. Boyle, Crystal L. Richards, Daniel P. Dulebohn
Formal analysis: William K. Boyle, Crystal L. Richards
Funding acquisition: Frank C. Gherardini, Travis J. Bourret
Investigation: William K. Boyle, Crystal L. Richards, Daniel P. Dulebohn, Amanda K. Zalud, Jeff A. Shaw
Methodology: William K. Boyle, Crystal L. Richards, Sandor Lovas
Project administration: William K. Boyle, Frank C. Gherardini, Travis J. Bourret
Resources: Sándor Lovas, Frank C. Gherardini, Travis J. Bourret
Software: William K. Boyle
Supervision: Crystal L. Richards, Daniel P. Dulebohn, Sandor Lovas, Frank C. Gherardini, Travis J. Bourret
Validation: William K. Boyle, Crystal L. Richards, Amanda K. Zalud, Jeff A. Shaw
Visualization: William K. Boyle
Writing – original draft preparation: William K. Boyle
Writing – review & editing: William K. Boyle, Crystal L. Richards, Daniel P. Dulebohn, Amanda K. Zalud, Sándor Lovas, Frank C. Gherardini, Travis J. Bourret

Competing interests: The authors declare that they have no competing interests with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

45

ABSTRACT

Throughout its enzootic cycle, the Lyme disease spirochete *Borreliella* (*Borrelia*) *burgdorferi*, senses and responds to changes in its environment by using a small repertoire of transcription factors which coordinate the expression of genes required for infection of *Ixodes* ticks and various mammalian hosts. Among these transcription factors, the DnaK suppressor protein (DksA) plays a pivotal role in regulating gene expression in *B. burgdorferi* during periods of nutrient limitation and is required for mammalian infectivity. In many pathogenic bacteria, the gene regulatory activity of DksA along with the alarmone guanosine penta- and tetra-phosphate ((p)ppGpp) coordinates the stringent response to various environmental stresses including nutrient limitation. In this study, we sought to characterize the role of DksA in regulating the transcriptional activity of RNA polymerase and in the regulation of RpoS-dependent gene expression required for *B. burgdorferi* infectivity. Using *in vitro* transcription assays, we observed recombinant DksA inhibits RpoD-dependent transcription by *B. burgdorferi* RNA polymerase independent of ppGpp Additionally, we determined the pH-inducible expression of RpoS-dependent genes relies on DksA, but is independent of (p)ppGpp produced by Rel$_{bbu}$. Subsequent transcriptomic and western blot assays indicated DksA regulates the expression of BBD18, a protein previously implicated in the post-transcriptional regulation of RpoS. Moreover, we observed DksA was required for infection of mice following intraperitoneal inoculation or for transmission of *B. burgdorferi* by *Ixodes scapularis* nymphs. Together, these data suggest DksA plays a central role in coordinating transcriptional responses of *B. burgdorferi* required for infectivity through its interactions with RNA polymerase and post-transcriptional control of RpoS.

Author Summary

Lyme disease, caused by the spirochetal bacteria *Borrelia burgdorferi*, is the most common vector-borne illness in North America. The ability of *B. burgdorferi* to establish infection is predicated by its ability to coordinate the expression of virulence factors in response to diverse environmental stimuli encountered within *Ixodes* ticks and mammalian hosts. Previous studies have shown an essential role for the alternative sigma factor RpoS in regulating the expression of genes required for the successful transmission of *B. burgdorferi* by *Ixodes* ticks and infection of mammalian hosts. The DnaK suppressor protein (DksA) is a global gene regulator in *B. burgdorferi* that also contributes to the expression of RpoS-dependent genes. In this study, we determined DksA exerts its gene regulatory function through direct interactions with the *B. burgdorferi* RNA polymerase using *in vitro* transcription assays and controls the expression of RpoS-dependent genes required for mammalian infection by post-transcriptionally regulating cellular levels of RpoS. Our results demonstrate the utility of *in vitro* transcription assays to determine how gene regulatory proteins like DksA control gene expression in *B. burgdorferi*, and reveal a novel role for DksA in the infectious cycle of *B. burgdorferi*.

88

89

INTRODUCTION

91 *Borrelia burgdorferi*, a causative agent of Lyme disease, is the most predominant
92 vector-borne pathogen in the United States (1, 2). *B. burgdorferi* is an extracellular
93 pathogen that traverses diverse environments within its tick vector, *Ixodes scapularis*,
94 and mammalian hosts. During blood meal acquisition, *B. burgdorferi* residing within *I.*
95 *scapularis* midguts are subjected to changes in temperature, osmolarity, pH, and host-
96 derived reactive oxygen and nitrogen species (3-5). In addition to these potential
97 environmental stressors, the midgut concentrates components of mammalian blood,
98 including nutrients, organic acids, host derived-antibodies and immune complement
99 components during and following tick-feeding (6-8). *B. burgdorferi* adapts to this
100 dynamic environment within feeding ticks by shifting gene expression and replicating
101 rapidly prior to transiting from the tick midgut to mammalian hosts.
102
103 The DnaK suppressor protein (DksA) and the metabolite guanosine penta- and
104 tetra phosphate ((p)ppGpp) produced by Rel$_{bbu}$ coordinate the stringent response of *B.*
105 *burgdorferi* by regulating the transcription of genes in response to nutrient limitation (9-
106 11). Furthermore, there is an extensive overlap in genes under the control of DksA and
107 (p)ppGpp in *B. burgdorferi* (11). DksA and (p)ppGpp both control genes involved in
108 protein synthesis, nutrient uptake pathways, as well as several lipoproteins. Additionally,
109 DksA and (p)ppGpp control the expression of genes encoding products that facilitate
110 glycerol and chitobiose utilization, along with genes encoding oligopeptide transporters,
111 which are essential for the adaptation of *B. burgdorferi* during infection of *I. scapularis*
112 (12-14). These observations suggest DksA and (p)ppGpp work synergistically to
113 regulate transcription by RNA polymerase in *B. burgdorferi* (11) and are consistent with
114 the generation of a stringent response due to the overlapping regulatory roles of DksA
115 and (p)ppGpp in other organisms (15-18) where these regulators control the response
116 to stationary phase growth and nutrient limiting conditions (19, 20). DksA derived from
117 other bacteria such as *Escherichia coli* interacts with (p)ppGpp *in vitro* which can
118 fundamentally change the direction and magnitude of gene regulation from specific
119 gene promoters in *E. coli* using *in vitro* transcription reactions (21-24).
120
121 The nature of the stringent response in *B. burgdorferi* is not fully understood. *B.*
122 *burgdorferi* requires a complex, nutrient-rich medium for growth and what constitutes
123 nutrient limitation in *B. burgdorferi* is not well-defined. Previous attempts to study the
124 stringent response were carried out by altering multiple environmental and metabolic
125 factors simultaneously, making it difficult to interpret which key factors and metabolites
126 played a role in the generation of a stringent response by *B. burgdorferi* (9, 11, 25-27).
127 While limitation of nutrients including fatty acids, carbohydrates, or amino acids can
128 activate the stringent response in bacteria, responses involving DksA and (p)ppGpp are
129 also triggered by environmental changes, such as low pH and the presence of reactive
130 nitrogen species (28-30). For example, in *E. coli*, the adaptation to an acidic
131 extracellular environment is aided by the regulation of the alternative sigma factor RpoS
132 by DksA and DksA appears to be a key component of the response of *E. coli* to acid
133 stress (31, 32). We recently observed *dksA*-deficient *B. burgdorferi* strains showed
134 reduced expression of genes under the control of the alternative sigma factor RpoS
135 (11). RpoS plays a gatekeeper function in the infectious cycle of *B. burgdorferi* by

136 coordinating the expression of a substantial portion of genes during the transmission of
137 *B. burgdorferi* from ticks to mammalian hosts.

138 During *in vitro* growth, RpoS is robustly expressed by *B. burgdorferi* in Barbour-
139 Stoenner-Kelly II (BSKII) growth media under mildly acidic conditions designed to mimic
140 the pH and organic acid stresses encountered within the *I. scapularis* midgut (5, 33, 34).
141 The *I. scapularis* midgut lumen is slightly acidic (pH 6.8) and contains millimolar
142 concentrations of organic acids following blood meal acquisition by ticks from
143 mammalian hosts (5, 35). Although the mechanisms underlying the activation of the
144 stringent response in *B. burgdorferi* remain unclear, the role DksA plays in the
145 regulation of RpoS may yield answers to what environmental conditions are important
146 for unlocking DksA-dependent gene regulation.

148 RpoS regulates the expression of genes encoding lipoproteins, which contribute
149 to mammalian infection, such as *bba66*, decorin binding protein (*dbpA*), and outer
150 surface protein C (*ospC*) (36-38). Each of these genes are highly expressed by *B.*
151 *burgdorferi* residing within the *I. scapularis* midgut during transmission, and RpoS-
152 dependent expression of these genes is required by *B. burgdorferi* to adapt and
153 transition to the mammalian host environment (39-41). RpoS itself is subject to both
154 transcriptional and post-transcriptional regulation in *B. burgdorferi* (42, 43). The
155 alternative sigma factor RpoN, drives the expression of a short *rpoS* transcript, which
156 leads to the accumulation of RpoS (44, 45). The RpoN-dependent expression of *rpoS*
157 relies on additional transcription factors, including the response regulator Rrp2 and the
158 ferric uptake regulator homolog, BosR. Additionally, *rpoS* expression is inhibited by the
159 *Borrelia* adaptation protein (BadR) (45-52). Alternatively, accumulation of RpoS can be
160 driven by the expression of a long transcript through a RpoN-independent mechanism
161 (53). Post-transcriptional control of *rpoS* is regulated by small RNA-dependent
162 mechanisms and by BBD18, a protein that contributes to RpoS degradation (54-57).
163 However, the mechanisms underlying the ability of *B. burgdorferi* to sense shifts in pH
164 or organic acid stress to regulate *rpoS*/RpoS remain unclear.

166 Further characterization of DksA-dependent gene regulation is required to
167 reconcile the nature of the stringent response and the possible role of DksA in
168 controlling RpoS. In this study, we used both biochemical and genetic approaches to
169 dissect the gene regulatory role played by DksA and its relationship with (p)ppGpp in *B.*
170 *burgdorferi*. In our genetic approach, we assessed pH- and acetate-dependent
171 responses of *B. burgdorferi* in the presence and absence of DksA and the (p)ppGpp
172 synthase (Rel$_{bbu}$) because previous studies suggested DksA plays a much larger role in
173 regulating RpoS (11, 25). To test the hypothesis that a synergy exists between DksA
174 (p)ppGpp in regulating gene transcription, we used a newly developed *in vitro*
175 transcription assay system using the *B. burgdorferi* RNA polymerase (58). Due to the
176 important regulatory role of DksA in controlling factors required for the transmission of
177 *B. burgdorferi*, we determined the contribution of DksA to tick-borne transmission and
178 mammalian infectivity. We show that DksA is required for murine infection following
179 intraperitoneal inoculation as well as transmission by artificially-infected *I. scapularis*
180 nymphs to mice. Additionally, we found DksA and (p)ppGpp play independent roles in
181 controlling RpoS levels in *B. burgdorferi*. DksA contributes to the post-transcriptional

182   regulation of RpoS, possibly through its control of BBD18, and the pH-dependent
183   expression of RpoS-regulated genes. We observed (p)ppGpp is produced at high levels
184   in response to growth of *B. burgdorferi* under acidic conditions but is not required for
185   RpoS expression. Collectively, the results of this study provide evidence that weak
186   acidic environments activate the *B. burgdorferi* stringent response and that DksA is an
187   indispensable transcriptional regulator coordinating the expression of genes required for
188   mammalian infection.
189
190

191   **RESULTS**

192

193   **DksA acts as a transcriptional repressor**

194

195   Previous work by our laboratory characterized the role of DksA in the *B.*
196   *burgdorferi* stringent response (11) and found DksA plays an important global regulatory
197   role that partially overlapped with (p)ppGpp-dependent gene regulation in nutrient
198   limited conditions. To further dissect the role of DksA, ppGpp, the interaction of these
199   two regulatory factors and define the molecular basis of transcriptional regulation
200   enacted by these regulators we analyzed structural and functional aspects of DksA in
201   vitro. Similar to other DksA homologs, *B. burgdorferi* DksA is predicted to be an α-helix
202   rich peptide with a carboxy-terminal zinc-finger motif (11). To better understand the
203   molecular characteristics of the *B. burgdorferi* DksA, we carried out experiments to
204   determine alpha helicity and zinc binding using recombinant *B. burgdorferi* DksA
205   protein. DksA was purified to apparent homogeneity using a maltose affinity purification
206   system (Fig. 1A), and the α-helicity of the protein was measured by circular dichroism
207   (Fig. 1B). Recombinant DksA had a high positive molar ellipticity maximum at 195 nm
208   and negative molar ellipticity maxima at 208 and 222 nm wavelengths, indicating *B.*
209   *burgdorferi* DksA is an α-helix rich protein (Fig. 1C). The putative zinc finger domain of
210   DksA is comprised of four cysteinyl residues (C91, C94, C112, and C115), suggesting
211   *B. burgdorferi* DksA falls within a redox-active group of DksA proteins (59, 60). The
212   release of Zn^{2+} from DksA following exposure to increasing concentrations of H_2O_2 was
213   tested using the Zn^{2+}-sensitive dye 4-(2-pyridylazo)resorcinol (PAR) (Fig. 1D).
214   Incubation of 100 μmol DksA with 1.25 mM H_2O_2 for 1.5 hours resulted in the release of
215   40 μmol Zn^{2+}, presumably due to the release of Zn^{2+} following the oxidation of the Cys
216   residues comprising the zinc finger. Together, these results indicate recombinant *B.*
217   *burgdorferi* DksA is an α-helix rich, Zn^{2+}-containing polypeptide, similar to other
218   characterized DksA proteins.

219

220   Two strong RpoD-dependent promoters (*flgBp* and *rplUp*) and five promoters
221   regulated by the stringent response (11, 25) (*clpCp*, *glpFp*, *groLp*, *napAp*, and *nagAp*)
222   were selected to determine the impact of recombinant *B. burgdorferi* DksA or ppGpp on
223   RNA polymerase activity from these promoters. Varying levels of ppGpp (0, 20, and 200
224   μM) and DksA (0, 50, and 500 nM) were added to *in vitro* transcription reactions to test
225   for their effects on transcriptional initiation by RNA polymerase as shown in previous
226   investigations of other *in vitro* transcription systems (29, 61, 62). *In vitro* transcription
227   reactions with RNA polymerase supplemented with recombinant RpoD along with

228 varying concentrations of DksA and/or ppGpp were prepared in parallel. Reactions were
229 then initiated by the addition of double-stranded linear DNA templates encoding the
230 promoters of interest and the reaction proceeded for five minutes at 37 °C. Following
231 termination, RNA products were separated by gel electrophoresis and the relative
232 incorporation of α-^{32}P-ATP was detected by phosphor imaging (Fig. 2A). The addition of
233 500 nM of DksA (roughly 24 molecules DksA: 1 molecule RNA polymerase) led to a
234 significant reduction of RNA polymerase activity from all of the tested promoters (Fig.
235 2B). In contrast, the addition of ppGpp did not lead to significant changes in RNA
236 polymerase activity from any of the promoters tested (Fig 2C), indicating ppGpp had no
237 direct effects on *B. burgdorferi* RNA polymerase activity *in vitro*.

239   The promoters used in this study displayed varying levels of transcription in the
240 absence of DksA, as illustrated by the higher levels of transcription from the *flgBp*
241 compared to *napAp.* However, transcription from *flgBp* and *napAp* appeared similar in
242 the presence of 500 nM DksA. To test the promoter-dependent effects of DksA on
243 transcription, a broader range of DksA concentrations were tested for *in vitro*
244 transcription reactions initiated on *flgBp* or *napAp* (Fig. 3). DksA affected both
245 promoters in a concentration-dependent manner, demonstrated by reduced
246 transcription from each promoter with the addition of increasing concentrations of DksA
247 in the reaction. The addition of only 250 nM DksA (roughly 12 molecules DksA: 1
248 molecule RNA polymerase) resulted in a 2 – 4 fold reduction of RNA polymerase activity
249 from both promoters (Fig. 3A). Together, these data support a role for DksA as a
250 transcriptional regulator that directly affects RNA polymerase activity from various *B.*
251 *burgdorferi* promoters.

DksA contributes to post-transcriptional regulation of RpoS

255   Previous studies have demonstrated *B. burgdorferi* strains with mutations of
256 *dksA* show a low levels of expression of genes under the control of the Rrp2-RpoN-
257 RpoS regulatory cascade (e.g. *dbpA*, *ospC*, *bba66*) compared to wild-type controls (11,
258 63). The expression of genes under the control of the Rrp2-RpoN-RpoS pathway are
259 known to increase during growth in mildly acidic BSKII, pH 6.7 medium and with the
260 addition of organic acids such as acetate (5, 33, 64). To determine whether DksA
261 contributes to the pH-dependent expression of RpoS, western blot analyses were
262 carried out using whole cell lysates collected from mid-log phase cultures of wild-type,
263 Δrel_{bbu}, and $\Delta dksA$ *B. burgdorferi* strains, along with a chromosomally complemented
264 strain ($\Delta dksA$ cDksA), grown in either BSKII, pH 7.6 or BSKII, pH 6.7 + 20 mM acetate.
265 Consistent with previous studies (49, 65), the expression of RpoS was higher in cultures
266 of wild-type *B. burgdorferi* grown in pH 6.7 + 20 mM acetate compared to cultures
267 grown in BSKII, pH 7.6 (Fig. 4A). In contrast, RpoS was undetectable in the $\Delta dksA$
268 strain, while patterns of RpoS expression in the Δrel_{bbu} and $\Delta dksA$ cDksA strains were
269 comparable to wild-type controls. Similarly, the expression of OspC was increased in all
270 but the $\Delta dksA$ strain in response to growth in pH 6.7 + 20 mM acetate (Fig. 4B).

272   Next, we investigated whether DksA contributed to the transcription of *rpoS* and
273 the RpoS-regulated gene *ospC* in response to growth in mildly acidic conditions. All

274 three strains, wild-type, Δ*rel*bbu, and Δ*dksA B. burgdorferi*, showed increased expression
275 of *rpoS* when grown in BSKII, pH 6.7 + 20 mM acetate compared to cultures grown in
276 BSKII, pH 7.6 (Fig. 5A), suggesting DksA is not required for the pH-dependent
277 increases in *rpoS* transcription. We observed pH-dependent increases in *ospC* in both
278 wild-type and Δ*rel*bbu strains, while the Δ*dksA* strain was unable to induce the
279 expression of *ospC* in response to growth under acidic conditions, consistent with the
280 patterns of OspC protein levels observed in each strain. The observation that pH-
281 induced expression of *rpoS* remains intact in the Δ*dksA* strain was further supported by
282 results from experiments where RNA was collected from mid-logarithmic and stationary
283 phase cultures to detect the increase of *rpoS* expression at stationary phase
284 (supplemental Fig. 1). At stationary phase, wild-type and Δ*dksA* cultures showed
285 increased *rpoS* expression, while the Δ*dksA* strain did not express RpoS-regulated
286 genes *dbpA*, *ospC*, and *bba66* at levels observed in wild-type controls as determined by
287 a multiple comparisons test (*p*-value < 0.05). These results support a role for DksA in
288 the post-transcriptional regulation of *rpoS*/RpoS that is independent of Relbbu.
289
290 In a recent study by Mason, et al., *B. burgdorferi* DksA was implicated in the
291 post-transcriptional regulation of *rpoS,* possibly through the transcriptional regulation of
292 the sRNA chaperone encoded by *hfq* and along with the protease *clpX*/*clpP*/*clpP2*. In
293 this study we assayed another known post-transcriptional regulator of RpoS in *B.
294 burgdorferi*, BBD18. Increased levels of BBD18 are correlated with reduced levels of
295 RpoS (56). Our previous transcriptomic study indicated increased expression of *bbd18*
296 in the Δ*dksA* strain (11). We quantified the relative levels of BBD18 by western blot to
297 determine if elevated levels of BBD18 correspond with a reduction in RpoS. BBD18
298 expression levels were lower in response to growth in BSK II pH 6.7 + 20 mM acetate in
299 all strains except the Δ*dksA* strain (Fig. 6). Results indicate the Δ*dksA* strain is unable
300 to downregulate the expression of BBD18 in response to mildly acidic growth
301 conditions, which corresponds with the reduced levels of RpoS shown in Fig. 1.
302 Additionally, the alteration of the growth rate of *B. burgdorferi* has been proposed as an
303 alternative mechanism for regulating cellular levels of OspC based on protein
304 accumulation (66). To determine if growth rates are significantly altered in the *B.
305 burgdorferi* Δ*dksA*- and Δ*rel*bbu mutant strains, BSKII containing 0, 30, and 100 mM
306 acetate, at pH 7.6, 6.1, and 4.6 respectively, were inoculated with 10^6 spirochetes / ml
307 and monitored for cell density over time (Fig. 7). Similar growth rates were observed for
308 each strain in BSKII + 30 mM acetate, indicating differences in OspC expression could
309 not be explained by differences in the growth of these strains. These results suggest
310 DksA controls BBD18-dependent post-transcriptional regulation of *rpoS*/RpoS,
311 independent of the growth rate of *B. burgdorferi*.
312
313 **DksA is required for mammalian infection**
314 A recent study by Mason, et. al. performed concurrently with our own
315 investigation showed DksA is required for the infection of mice following intradermal
316 inoculation with *B. burgdorferi* (63). Consistent with the data shown in our study, the
317 requirement of DksA for mammalian infection was linked with dysregulation of the Rrp2-
318 RpoN-RpoS pathway. Therefore, we independently assessed the role of DksA in
319 mammalian infection by intraperitoneal inoculation of naïve RML mice with 5 x 10^7 *B.

7

320   *burgdorferi* 297 wild-type, Δ*dksA*, or Δ*dksA* cDksA *B. burgdorferi* strains. At 23 days
321   post inoculation, the ear, joint, and bladder tissues were collected from mice and
322   transferred to BSKII medium to detect the presence of *Borrelia*. Viable spirochetes were
323   isolated from all cultured tissues of the mice injected with wild-type and Δ*dksA* cDksA
324   strains; however, none of the tissues from mice inoculated with the Δ*dksA* strain
325   showed spirochete outgrowth (Table 1). Additionally, seroconversion of mice infected
326   with the *B. burgdorferi* strains was tested against wild-type *B. burgdorferi* cell lysates by
327   western blot using serum collected from mice 23 days post infection. Mice infected with
328   wild-type and Δ*dksA* cDksA strains showed seroconversion, while mice infected with the
329   Δ*dksA* strain did not (Supplemental Fig. 2). Collectively, these data indicate DksA is
330   required for *B. burgdorferi* to establish infection in mice.
331
332   Our previous transcriptomic study showed DksA controls the expression of a
333   wide variety of genes that have previously been shown to contribute to colonization and
334   transmission by *I. scapularis*. To more robustly test the role of DksA in host and vector
335   infection and colonization, we artificially infected *I. scapularis* larvae by submersion in
336   wild-type, Δ*dksA*, or Δ*dksA* cDksA cultures grown to a density of 5 x 10^7 spirochetes ·
337   ml^{-1} in BSKII and tested the ability of the larvae to transmit the three strains to naïve
338   mice. Approximately 200 artificially infected larval stage ticks with either the wild-type,
339   Δ*dksA*, or Δ*dksA* cDksA strains were allowed to feed on mice. Spirochetes in larval ticks
340   were quantified, 3 – 5 days following the larval bloodmeal, by plating a subset of
341   homogenized ticks on semi solid BSK media. An additional subset of ticks were
342   analyzed following the molt of larvae to nymphs roughly 6 weeks after feeding
343   (Supplemental Fig. 3). *I. scapularis* larvae and nymphs colonized by the Δ*dksA* or
344   Δ*dksA* cDksA strains showed lower spirochete numbers compared to wild-type controls,
345   however, statistical analysis by one-way ANOVA and multiple comparisons test
346   indicated the differences in spirochete numbers were not statistically significant
347   (ANOVA *p*-value = 0.270, Tukey's multiple comparisons test of Δd*ksA* fed larvae vs.
348   Δ*dksA* colonized nymphs, *p*-value > 0.999). Cohorts of 5 – 10 *I. scapularis* nymphs
349   infected with either the wild-type, Δ*dksA*, or Δ*dksA* cDksA strains fed upon a total of
350   three mice. Three weeks after the *I. scapularis* bloodmeal, mice were euthanized. The
351   ear, joint, and bladder tissues dissected from euthanized mice were collected and
352   placed into BSK II media. Outgrowth was detected in the tissues collected from mice fed
353   upon by nymphs infected with the wild-type and Δ*dksA* cDksA strains, but not the
354   Δ*dksA* strain (Table 2). Seroconversion was only observed in blood collected from mice
355   infected with wild-type and Δ*dksA* cDksA *B. burgdorferi* strains whose collected tissues
356   showed detectable *B. burgdorferi* outgrowth following culture in BSKII media
357   (Supplemental Fig. 4). These data provide additional evidence that DksA is either
358   required for transmission from *I. scapularis* to the host or, that the Δ*dksA* strain is readily
359   cleared by the host immune system, prior to the generation of a strong antibody
360   response, similar to what is seen for mutants unable to synthesize OspC (Ref).
361
362   **Activation of the *B. burgdorferi* stringent response by changes in pH**
363
364   The stringent response regulator DksA and Rel$_{bbu}$ synthesized (p)ppGpp control
365   the expression of many genes associated with nutrient uptake and peptide

366 synthesis/protease functions (11, 25). In this study, we assessed the role of DksA and
367 Rel$_{bbu}$ on the expression of RpoS during growth in an acidic medium. To better
368 understand the potential involvement of the stringent response during growth in mildly
369 acidic conditions, we directly analyzed the levels of DksA and (p)ppGpp in *B.*
370 *burgdorferi*. The relative levels of DksA in wild-type, Δ*rel*$_{bbu}$, Δ*dksA*, Δ*dksA* cDksA
371 strains were analyzed during mid-logarithmic growth in BSKII pH 7.6 and BSKII pH 6.7
372 + 20 mM acetate by western blot (Fig. 8). DksA protein levels decreased by roughly
373 one-half during growth under the mildly acidic conditions in wild-type cells. DksA
374 expression levels were not significantly different between wild-type, Δ*rel*$_{bbu}$ and the
375 Δ*dksA* cDksA strains. These results suggest the expression of DksA is independent of
376 Rel$_{bbu}$ and chromosomal complementation of the Δ*dksA* strain restored wild-type
377 patterns of DksA expression when strains were grown to mid-logarithmic phase in BSKII
378 pH 7.6 and BSKII pH 6.7 + 20 mM acetate.
379
380   Previously, (p)ppGpp levels were shown to be constitutively elevated in the
381 Δ*dksA* mutant compared to the wild-type strain (11). Since the absence of DksA leads
382 to elevated cellular levels of (p)ppGpp in *B. burgdorferi*, we reasoned the DksA and
383 (p)ppGpp levels may have an inverse relationship. (p)ppGpp is a product of GTP
384 modification and formation of (p)ppGpp can sequester GTP from other cellular
385 processes. To characterize the levels of GTP and (p)ppGpp in *B. burgdorferi* GTP and
386 (p)ppGpp levels were quantified from cell lysates by HPLC. Spirochetes were grown
387 under mildly acidic conditions, wild-type and Δ*dksA* strains were cultured to 1 – 2 x 10^7
388 spirochetes · ml^{-1} in either BSK II pH 7.6 or BSK II pH 6.7 with and without 20 mM
389 acetate. Cultures of wild-type and Δ*dksA* strains grown at pH 6.7 had roughly 2-fold
390 lower levels of GTP compared to cultures grown at pH 7.6 (Fig. 9A). Concurrently, both
391 wild-type and Δ*dksA* strains had nearly 10-fold higher levels of (p)ppGpp when cultured
392 at pH 6.7 compared to levels of (p)ppGpp in wild-type spirochetes grown at pH 7.6. GTP
393 levels decrease as (p)ppGpp levels increase in wild-type cells. Additionally, regardless
394 of growth condition, the Δ*dksA* strain had (p)ppGpp levels that were 10-fold higher than
395 wild-type spirochetes grown at pH 7.6, consistent with our previously published
396 observations (11). The ratio of GTP to (p)ppGpp was roughly 70:1 in wild-type cells
397 during growth at pH 7.6, while the GTP to (p)ppGpp ratio was near 2:1 during growth at
398 pH 6.7, meaning a greatly increased molar concentration of (p)ppGpp relative to GTP
399 occurred in cells during growth at pH 6.7. Cellular levels of (p)ppGpp are canonically
400 associated with stationary phase growth and the arrest of cell replication (67). The
401 levels of (p)ppGpp in wild-type *B. burgdorferi* grown to logarithmic or stationary phase in
402 BSKII pH 6.7 or pH 6.7 + 20 mM acetate were also measured by HPLC to determine if
403 increases in (p)ppGpp are associated with growth to stationary phase (Fig. 9C). Results
404 indicate the levels of (p)ppGpp in wild-type cells grown to stationary phase are identical
405 to log phase cells in BSKII pH 6.7 or pH 6.7 + 20 mM acetate. Together these results
406 illustrate *B. burgdorferi* robustly regulates the stringent response in an acidic
407 environment and suggests a relationship between pH and nutrient stress responses in
408 *B. burgdorferi*.
409
410 **DISCUSSION**
411

9

412   In this study, we established the role of DksA in the infectious cycle of *B.*
413 *burgdorferi* and determined the regulatory relationship between DksA and RpoS, a
414 transcriptional regulator required for murine infection. Mice inoculated with the Δ*dksA B.*
415 *burgdorferi* strain via intraperitoneal inoculation had no culturable spirochetes 23 days
416 post-infection and failed to develop serum antibodies against *B. burgdorferi*, suggesting
417 the mice cleared the mutant strain prior to the development of an immune response.
418 The clearance of the Δ*dksA B. burgdorferi* strain is likely not due to defects in motility
419 (11). It is more likely due to the inability of this strain to express host-associated
420 lipoproteins regulated by RpoS, such as OspC, which are essential to subvert host
421 immune clearance. These observations are consistent with a recent report by Mason C.
422 et. al. that showed DksA was required for the expression of RpoS-regulated genes and
423 for infection of mice following intradermal inoculation, however, the seroconversion of
424 mice infected with *dksA*-deficient *B. burgdorferi* were not reported in that study (63).
425 While DksA is required for mammalian infection by *B. burgdorferi* following intradermal
426 or intraperitoneal inoculation, it is not essential for colonization of *I. scapularis*. We
427 observed the persistence of Δ*dksA B. burgdorferi* in *I. scapularis* for over 6 weeks
428 following artificial infection of ticks by immersion, suggesting DksA is not required for the
429 long-term survival of *B. burgdorferi* within its tick vector. The absence of DksA does not
430 lead to a defect in stationary-phase-like metabolism when *B. burgdorferi* are nutrient
431 constrained and do not replicate. *I. scapularis* are the natural vectors of *B. burgdorferi*
432 and immunomodulatory effects of *I. scapularis* saliva aid in *B. burgdorferi* transmission
433 (68). Despite the successful colonization and transstadial survival of Δ*dksA B.*
434 *burgdorferi* in *I. scapularis*, these spirochetes are unable to be successfully transmitted
435 by ticks to naïve mice. These observations demonstrate the essential role of DksA in
436 the natural infectious cycle, extend observations by Mason, et. al. and provide further
437 evidence that DksA plays a critical role in gene regulation during *B. burgdorferi*
438 transmission.
439

440   Regulation of RpoS plays a crucial role in infectivity of *B. burgdorferi* (45, 50) with
441 multiple transcriptional regulators (Rrp2, RpoN, BosR, BadR) modulating the
442 transcription of *rpoS* (REFS). In this study, we observed differences in RpoS but did not
443 observe differences in the levels of *rpoS* transcription between our wild-type, Δ*dksA*,
444 and Δ*dksA* cDksA strains. This suggests that the control of RpoS levels by DksA is
445 post-transcriptional and is consistent with the recent report by Mason C. et. al. (63),
446 which suggested the small RNA regulator DsrA and the ClpXP protease system are
447 involved in DksA-dependent RpoS regulation. However, whether ClpXP plays a role
448 controlling RpoS levels is currently unknown. Conversely, our study provides evidence
449 that BBD18 likely contributes to the DksA-dependent regulation of RpoS. BBD18 is a
450 post-transcriptional regulator of RpoS in *B. burgdorferi*, and elevated levels of BBD18
451 correspond to reduced cellular levels of RpoS. In our previous transcriptomic study and
452 in data provided here, we observed elevated levels of *bbd18* transcripts and BBD18 in
453 Δ*dksA B. burgdorferi* strains, suggesting DksA contributes to the regulation of *bbd18*.
454 Collectively, these data suggest DksA indirectly mediates the regulation of RpoS and
455 RpoS-dependent lipoproteins required for infection by controlling the cellular levels of
456 BBD18. Further studies will be needed to decipher the mechanisms underlying DksA-

457 dependent transcriptional regulation of *bbd18* and other potential post-transcriptional
458 regulators of RpoS.
459
460   DksA from *Escherichia coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa*
461 are all transcriptional repressors (22, 59, 60, 69). Moreover, our previous study
462 indicated DksA represses the transcription of a wide variety of genes in *B. burgdorferi*
463 during *in vitro* culture under nutrient-limiting conditions (11). In this study, we
464 determined the molecular basis of DksA function using our recently established *in vitro*
465 transcription assay. The *in vitro* transcription reactions initiated from seven RpoD-
466 dependent promoters all resulted in lower levels of RNA produced by RNA polymerase
467 with the addition of DksA. The relative quantities of DksA required to observe repression
468 of RNA polymerase activity in the *B. burgdorferi in vitro* transcription assay were similar
469 to those observed in *E. coli* and *S. enterica* RNA-polymerase based assays, where the
470 addition of 2 - 5 µM DksA led to reduced transcription from target promoters (21, 30).
471 The addition of 1 µM *E. coli* DksA to *in vitro* transcription reactions reduced the
472 transcription of *rrnB* P1 by 10-fold, given an acidic assay environment (29). This DksA-
473 dependent change in transcriptional activity is similar to the magnitude of transcriptional
474 changes observed in our *B. burgdorferi in vitro* transcription system with the addition of
475 1 µM DksA in reactions initiated from *napAp* and *flgBp* dsDNA templates. These
476 observations are consistent with our previously published transcriptomic study
477 comparing wild-type and Δ*dksA B. burgdorferi* strains, indicating DksA constrains global
478 transcription during periods of nutrient limitation.
479
480   DksA and (p)ppGpp cooperate in regulating gene expression in other organisms
481 (20, 28). We tested whether both ppGpp and DksA are required for regulating
482 transcription by RNA polymerase from five promoters (*clpCp*, *glpFp*, *groLp*, *napAp*, and
483 *nagAp*) using our *in vitro* transcription system which previous studies suggested were
484 regulated by both Rel$_{bbu}$ and DksA (9, 11, 25). While the impact of DksA on *in vitro*
485 transcription from these promoters was apparent at concentrations as low as 250 nM of
486 DksA, we failed to detect a role for ppGpp in regulating RNA polymerase activity at
487 concentrations as high as 200 µM of ppGpp. While ppGpp did not modulate RNA
488 polymerase activity in this assay, we cannot rule out a role for (p)ppGpp due to the
489 absence of other RNA polymerase binding factors, such as the omega subunit (70, 71).
490 These data indicate DksA is capable of independently regulating RNA polymerase
491 activity in *B. burgdorferi*. Further work is necessary to clarify the role of (p)ppGpp in
492 regulating RNA polymerase function, along with its interaction with DksA.
493
494   Present data suggest the role of DksA is divergent from (p)ppGpp in *B.
495 burgdorferi*. Previous studies have shown (p)ppGpp is essential for survival in and
496 transmission from *I. scapularis*, but it is not required for murine infection by needle
497 inoculation. In this study, we showed the Δ*rel*$_{bbu}$ *B. burgdorferi* strain expresses OspC
498 when cultured in BSKII pH 6.8 + 20 mM acetate, while the Δ*dksA* strain does not.
499 Therefore, DksA appears to play a stronger role in controlling the expression of
500 lipoproteins required for mammalian infection than (p)ppGpp. In *E. coli*, (p)ppGpp levels
501 regulate *dksA* transcription (72). However, in *B. burgdorferi*, the regulation of DksA
502 expression under low pH was independent of (p)ppGpp (Fig. 8), indicating the

503   regulatory relationship between (p)ppGpp and DksA in *B. burgdorferi* is not synonymous
504   with *E. coli*. Rather, low levels of DksA correspond closely with elevated cellular levels
505   of (p)ppGpp, and the Δ*dksA B. burgdorferi* strain displayed elevated concentrations of
506   (p)ppGpp regardless of growth condition. The regulatory schemes of DksA and
507   (p)ppGpp do not appear reciprocal, and the roles of DksA and (p)ppGpp as regulators
508   of RpoS are potentially divergent.

509

510   DksA and (p)ppGpp are canonically considered regulators of stationary phase
511   growth and have been shown to regulate the transcriptional responses of bacteria to
512   nutritional stress (20). However, in *B. burgdorferi*, DksA and (p)ppGpp are regulated
513   dramatically in response to growth in a slightly acidic environment. While the growth
514   rate of all strains in BSKII pH 7.6 and BSKII pH 6.8 + 20 mM acetate are similar, growth
515   in acidic medium reduced the expression levels of DksA by one-half. Additionally,
516   growth of wild-type cells in BSKII pH 6.8 + 20 mM acetate, led to a drastic shift in
517   intracellular GTP:(p)ppGpp ratios from 50:1 to 2:1. A previous study showed growth of
518   *B. burgdorferi* in BSKII pH 6.8 + 20 mM acetate leads to acidification of the cytosol (5). It
519   is possible this slightly acidic environment is nutritionally stressful for *B. burgdorferi*. For
520   example, maintenance of proton transport during growth in acidic environments is likely
521   linked with nutrient transport in *B. burgdorferi* (REF book chapter). The *B. burgdorferi*
522   genome lacks a recognizable electron transport chain to translocate H^+ ions from the
523   intracellular space to the periplasm (73). Instead, *B. burgdorferi* is thought to rely on an
524   ATP-dependent proton transport through the V-type ATPase to generate and maintain a
525   proton motive force (5). *B. burgdorferi* also lacks the capacity for amino acid
526   biosynthesis and relies largely on ABC-type oligopeptide transporters for growth (14).
527   ATP is required for both ABC-type transporters and the V-type ATPase, indicating these
528   two processes may compete if ATP pools are limited. Previous transcriptomic studies
529   do not support a role for Rel$_{bbu}$ or DksA in the regulation V-type ATPase genes.
530   However, both Rel$_{bbu}$ and DksA contribute to the expression of ABC-type oligopeptide
531   transporter genes (11, 25). Oligopeptide transporters have also been linked to
532   expression of virulence genes like OspC (74, 75). However, the interplay between the
533   pH environment, ATP levels, oligopeptide transporters, DksA and (p)ppGpp requires
534   further investigation.

535

536   Our work establishes DksA as a regulator of *B. burgdorferi* RpoS and uncovers
537   a previously unexplored pH-dependent regulation of (p)ppGpp and DksA.
538   Environmental sensing mechanisms feeding into *B. burgdorferi* virulence gene
539   regulation are poorly understood. In other organisms, Rel and DksA most likely act as
540   sensors of the intracellular environment (16, 29, 60). For example, Rel proteins directly
541   trigger the accumulation of (p)ppGpp in many organisms in response amino acid-
542   starved environments (76), and DksA was recently proposed to be an intracellular
543   sensor of pH (29, 77). Additionally, this study demonstrated the utility of our recently
544   developed *in vitro* transcription assay system to determine the impact of DksA on RNA
545   polymerase-dependent transcription and will facilitate future studies on the mechanisms
546   underlying regulatory activity of DksA and other *B. burgdorferi* transcription factors.

547
548

METHODS

Recombinant DksA expression

Oligonucleotides encoding an *E. coli* codon-optimized version of *B. burgdorferi* *dksA* was commercially synthesized (GenScript, Piscataway, NJ, United States). The *dksA* gene was PCR amplified using primers listed in supplemental table 1 and cloned into the NheI site of the pMAL-C5X plasmid expression vector using a Gibson assembly kit (New England Biosciences, Ipswich, MA, United States). Top Shot BL21 (DE3) pLysS Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA, United States) were transformed with the resulting expression vector. For protein expression, overnight *E. coli* cultures passaged 1:200 in Lysogeny broth (LB)-lennox broth containing 2 g · L^{-1} glucose and 100 µg · ml^{-1} ampicillin at 37 °C were grown until the cell density reached OD = 0.5. Cultures were then incubated for an additional 2 hours in the presence of 0.3 mM Isopropyl β-D-1-thiogalactopyranoside. Proteins were purified as described in the pMAL-C5X protein expression system protocols (New England Biosciences, Ipswich, MA, United States). To cleave the MBP-tag from the extracted DksA protein, 2 mM CaCl$_2$ was added to the protein elution fractions. The mixture was incubated overnight with Factor Xa protease at a weight ratio of 1:200 of recombinant protein to protease. To perform cation exchange chromatography, the mixture containing DksA was pushed through a HiTrap SP column (GE Healthcare, Chicago, IL, United States) to bind the DksA protein and subsequently eluted with 50 mM – 1M gradient of sodium chloride. DksA protein was purified to apparent homogeneity when proteins in the elution mixtures where separated by SDS-PAGE and visualized following incubation with Coomassie stain. DksA was stored in 50% glycerol, 1 mM 2-mercaptoethanol, and 25 mM Tris buffer pH 7.5.

Circular dichroism (CD) spectropolarimetry

For CD analysis, DksA was exchanged into a buffer containing 15 mM sodium phosphate and 100 mM NaCl (pH 7) over a PD-10 Sephadex column (GE Healthcare Bio-Sciences, Pittsburg, PA). DksA protein solutions (12.5 µM, 25 µM, 50 µM, 100 µM and 200 µM) were loaded into a 0.01 cm pathlength quartz cell, and CD spectra were scanned at 0.1 nm increments for wavelengths 180 – 260 nm on the Jasco J-810 polarimeter. CD spectra accumulated over 30 passes at pH 7.0 and 22 °C were averaged and data was visualized in GraphPad Prism software (La Jolla, CA, United States).

Zn^{2+} release assay

Zn^{2+} release from DksA was measured using the metal chelator 4-(2-pyridylazo)resorcinol (PAR) (Sigma-Aldrich, St. Louis, MO, United States) (59). A standard curve consisting of various concentrations of ZnCl$_2$ was incubated with PAR, and absorbance at 500 nm was measured using the Cytation 5 multimode plate reader in order to determine a linear detectable range for the zinc ion release assay. To assay Zn^{2+} release from DksA protein samples, 25 µL of DksA at a 100 µM concentration was exposed to 0 – 1.25 mM H$_2$O$_2$ in a 96-well assay plate, and free Zn^{2+} was measured following the addition of 25 µL of 2 mM PAR after 0, 30, 60, and 90 minutes of exposure.

In vitro transcription

The *in vitro* transcription reaction was carried out as described previously (58). The reaction contained 0.8 U Ribolock RNase inhibitor (Invitrogen, Carlsbad, CA, United States), 21 nM RNA polymerase, 500 nM RpoD, 2 µCi ATP [α-32P] (PerkinElmer, Waltham, MA, United States), 20 µM ATP, 200 µM GTP, 200 µM CTP, and 200 µM UTP. A preliminary mixture containing reaction buffer, RNA polymerase, and RpoD were incubated for 10 minutes on ice prior to the addition of subsequent components. Transcription was initiated with the addition of double-stranded linear template DNA generated by PCR amplification of genomic DNA segments encoding the transcriptional start site using primers previously described and found on supplemental table 1.The double-stranded DNA templates were added to the *in vitro* transcription reaction to a concentration of 10 nM, and reactions were allowed to proceed for 5 minutes at 37 °C. Transcription was terminated by adjusting the reaction mixture to contain 48% formamide and then incubating the reaction at 65 ºC for 5 minute. RNA products were separated by gel electrophoresis in 10% TBE-Urea gels (Invitrogen, Carlsbad, CA, United States) at 180 V for 45 minutes. Gels were placed on a Phosphor Screen (GE Healthcare, Chicago, IL, United States) overnight (16 hours) to detect beta decay from ATP [α-32P] incorporated into the RNA, and the resulting signal was developed into an image using the Typhoon FLA 9500 (GE Healthcare, Chicago, IL, United States). Densitometry measurements were determined with Image Lab 6.0.1. Software (Bio-Rad, Hercules, CA, United States).

Bacterial strains and culture conditions

A low-passage *B. burgdorferi* 297 strain and respective mutants (Supplemental Table 1) were maintained under microaerobic conditions (5% CO_2, 3% O_2) in BSK II liquid medium pH 7.6 at 34°C. Cultures started from frozen stocks were passaged twice before performing assays. To assess *B. burgdorferi* responses to environments mimicking the pH of the tick midgut, spirochetes were cultured in pH-adjusted BSK II medium. Mid logarithmic phase (5×10^7 spirochetes · ml^{-1}) cultures were passaged by 1:100 dilution directly to pH 6.8 medium with or without various levels of acetate and allowed to reach mid-logarithmic phase.

Genetic transformation

To assess the phenotype of a Δ*dksA* strain complemented *in cis* with a chromosomal copy of *dksA*, a homologous recombination vector was prepared using a Gibson assembly approach for introducing *dksA* into the 297 Δ*dksA* strain used in a previous study (11, 42, 78). To re-introduce *dksA* to its original genomic location, a 3 kB size fragment surrounding the *dksA* mutagenesis site, including an *aph* gene conferring resistance to kanamycin, was amplified by the primers AG-*bb0168*-F1-5′ and AG-*bb0168*-F2-3′ (Supplemental Table 1). The amplified fragment was cloned into the Zero Blunt TOPO vector (Invitrogen, Carlsbad, CA, United States) and transformed into Top10 *E. coli* with kanamycin selection. The TOPO vector containing the 3 kB fragment was amplified by PCR to generate a 6.5 kB linear fragment using the primers AG-F2R2 and AG-F2F2 to prepare for a three-fragment Gibson assembly using the TOPO-vector as the backbone. A roughly 500 bp long fragment encoding a wild-type copy of *dksA* was amplified by PCR from the wild-type *B. burgdorferi* 297 strain by PCR using the

14

641 primers *dksA*-gibson-F- F2R2 and *dksA*-gibson-R-*aadA* and a roughly 1 kB fragment
642 encoding a streptomycin resistance cassette (*aadA*) driven by the *flgB* promoter was
643 amplified from the pKFSS1 plasmid by PCR using the *aadA*-gibson-F-*dksA* and *aadA*-
644 gibson-R-*kan* (79). Overlapping oligonucleotides for Gibson cloning (Supplemental
645 Table 1) were encoded on primers and were designed to produce the arrangement
646 *bb0167-dksA-kan-aadA-bb0169* (supplemental Fig. 5). The Gibson assembly was
647 performed per manufacturer instructions with a reaction containing 10-fold molar excess
648 of *dksA* and *aadA* insertion fragments to the 6.5 kB backbone fragment. Top10 *E. coli*
649 was transformed with the Gibson assembly reaction mixture with 50 µg · ml^{-1}
650 spectinomycin selection. Plasmids isolated from Top10 *E. coli* clones were sequenced
651 using primers used during the cloning process. The resulting plasmid, TOPO-297-comp-
652 2B, was electroporated into *B. burgdorferi* 297 Δ*dksA*, and *B. burgdorferi* were plated on
653 semi-solid BSK with 50 µg · ml^{-1} streptomycin for selection. PCR was used to assess
654 the presence of the *dksA* gene, the *aadA* gene, and the plasmid content of transformed
655 *B. burgdorferi* (42, 80). The resulting 297 *dksA* cDksA strain plasmid content was
656 identical to wild-type and Δ*dksA* strains and harbors a wild-type *dksA* allele on the
657 chromosome.
658
659 **Mouse model of infection**
660 A mouse model of infection was initiated to test the ability of *B. burgdorferi*
661 strains to cause infection. An established outbred strain of Swiss Webster mice at RML,
662 Rocky Mountain Laboratories (RML) mice, were chosen as the model organism. RML
663 mice are susceptible to *B. burgdorferi* infection, approximating the reservoir host with
664 high practicality due to the well-established animal husbandry protocols. Female 6 – 8-
665 week-old mice were inoculated with *B. burgdorferi* via intraperitoneal injection, as
666 previously described (3, 25). *B. burgdorferi* were enumerated by dark-field microscopy
667 on a Petroff-Hauser Counter prior to inoculation and mice were injected with an
668 inoculum containing 10^5 *B. burgdorferi*. At 23 days post inoculation, mice were
669 anesthetized, and blood samples were obtained by cardiac puncture during euthanasia.
670 The *B. burgdorferi* infection in mice were evaluated by incubating tissues from the ear,
671 joint, and bladder in BSK II medium containing *Borrelia* antibiotics (2.5 µg / mL
672 Amphotericin, 20 µg / mL Phosphomycin, 50 µg / mL Rifampicin). BSK II cultures were
673 incubated at 34 °C in microaerobic conditions and observed continuously for 4 weeks by
674 dark-field microscopy to detect outgrowth.
675
676 **Arthropod transmission model**
677 To test the ability of *B. burgdorferi* strains to transmit from vector to host, *I.*
678 *scapularis* harboring *B. burgdorferi* were allowed to feed upon mice. To prepare *I.*
679 *scapularis* colonized with various *B. burgdorferi* strains, two hundred *I. scapularis* larvae
680 were artificially infected with *B. burgdorferi*. *I. scapularis* larvae and nymphs originating
681 from an uninfected tick colony (National Tick Research and Education Resource,
682 Oklahoma State University) were housed at 22 °C and 95% relative humidity. Egg
683 masses were allowed to mature to larval ticks 4 weeks prior to artificial infection. The
684 culture density of *B. burgdorferi* used for artificial infection was quantified using a
685 Petroff-Hauser Counter under darkfield microscopy, and cultures were diluted to a
686 density of 5 x 10^7 spirochetes · ml^{-1}. Cohorts of *I. scapularis* larval ticks were artificially

15

687 infected by submersion in 2.0 mL screw cap tubes containing 1.5 mL of *B. burgdorferi*
688 culture in BSK II medium for 2 hours, as previously described (81). Following
689 submersion, ticks were washed twice in phosphate buffered saline pH 8.0 and dried
690 with filter paper.
691   Artificially infected larval cohorts were allowed to attach to 6 – 8-week-old mice
692 for a bloodmeal. Larval ticks were allowed to molt to obtain *B. burgdorferi* infected *I.*
693 *scapularis* nymphs. Mice were anesthetized before *I. scapularis* were transferred to the
694 mice with a paint brush. Mice were placed in a wire bottom cage to allow for unattached
695 *I. scapularis* to fall into a live trap. Mice and *I. scapularis* were monitored continuously
696 for 7 days to observe the completion of the bloodmeals by all ticks. Fed larvae were
697 monitored for *B. burgdorferi* colonization and successful molt to nymphs. To quantify *B.*
698 *burgdorferi* within *I. scapularis* larvae and nymphs, cohorts of ticks were washed in 1.5
699 mL tubes with solutions of 3% H_2O_2, 70% ethanol, and PBS pH 8.0. Washed *I.*
700 *scapularis* were dissected with forceps on a microscope slide and placed in a tube
701 containing 600 µL BSK II medium. The dissected *I. scapularis* were further disrupted via
702 crushing with a pestle. Volumes of 5 µL, 50 µL, and 500 µL of BSK II medium containing
703 the crushed *I. scapularis* were plated as previously described (25) with semi-solid BSK
704 medium containing 2.5 µg / mL Amphotericin, 20 µg / mL Phosphomycin, and 50 µg /
705 mL Rifampicin.
706   The transmission of *B. burgdorferi* strains from nymphal *I. scapularis* were tested
707 following the molt of artificially infected larval *I. scapularis* to nymphs. Nymphs were
708 transferred to mice, and the mice were monitored as described above. *B. burgdorferi*
709 infection was determined by the same method described for the needle-inoculation
710 method of infection.
711
712 **Transcriptional profiling from cultures**
713   *B. burgdorferi* cultures were pelleted at 4°C, 3,200 x g for 20 minutes. Cell pellets
714 were washed once in HN buffer (10 mM HEPES, 10 mM NaCl, pH 8.0). RNA isolation
715 was performed using RNAzol (Sigma-Aldrich, St. Louis, MO, United States) according
716 to kit instructions. The RNA was quantified by spectrophotometry using a TAKE3 plate
717 in a Cytation 5 multi-mode plate reader (Biotek, Winooski, VT, USA), and RNA quality
718 was assessed by analysis of ribosomal RNA bands visualized following gel
719 electrophoresis by SYBR-safe dye incorporation. cDNA was generated from 500 ng of
720 total RNA from each sample with the High Capacity cDNA Reverse Transcriptase kit
721 (Applied Biosystems, Foster City, CA, United States) following kit instructions. RT-qPCR
722 was performed on the cDNA in the CFX Connect Real-Time PCR Detection System
723 (Bio-Rad, Hercules, CA, United States) using Bullseye EvaGreen Master Mix (MIDSCI,
724 St. Louis, MO, United States) reagents and oligonucleotide primers targeting the gene
725 of interest (Supplemental Table 1). The RT-qPCR data were analyzed using the ΔCq
726 method to indicate copies per 16S rRNA transcript levels.
727
728 **Western blotting**
729   Cultures were pelleted at 4 °C, 3,200 x g for 20 minutes. *B. burgdorferi* were
730 washed twice with HN buffer and subsequently lysed in lysis buffer (4% SDS, 0.1M Tris-
731 HCl). SDS-PAGE was performed on the Mini-Tetra System (Bio-Rad, Hercules, CA,
732 United States) using AnykD or 12% polyacrylamide gels and transferred to PVDF

733 membranes using the Transblot Turbo apparatus (Bio-Rad, Hercules, CA, United
734 States). PVDF membranes were blocked for 1-hour in TBST with 5% milk.
735 Commercially available antibodies for OspC (Rockland Immunochemicals, Pottstown,
736 PA), or rabbit polyclonal anti-DksA (11), anti-BBD18 (56), anti-RpoS antibody were
737 incubated with the PVDF membranes overnight at a dilution of 1:2000, 1:2000, 1:500, or
738 1:500, respectively, in TBST. Antibodies bound to the membrane were detected with the
739 incubation of anti-rabbit HRP-conjugated secondary antibodies for 1 hour followed by
740 five washes in TBST. Images were produced by the ChemiDoc apparatus (Bio-Rad,
741 Hercules, CA, United States) using ECL reagent (LI-COR, Lincoln, NE, United States).
742
743 **Quantification of GTP and (p)ppGpp**
744   *Borrelia* cell-free lysates were analyzed for GTP, ppGpp, and pppGpp
745 concentrations by HPLC. Wild-type *B. burgdorferi* 297 and its derivative 297 Δ*dksA*
746 strain were grown to mid logarithmic or stationary phase ($1 - 2 \times 10^8$ spirochetes $\cdot$ ml^{-1}).
747 One hundred ml cultures were centrifuged at 5,000 RPM and washed twice with HN
748 buffer. Cells were lysed by resuspension in distilled water followed by boiling at 99°C for
749 15 min. Aliquots were removed from each sample for total protein quantification by BCA
750 assay (Thermo Fisher Scientific, Grand Island, NY, United States). After boiling, the
751 suspensions were centrifuged, and the supernatants were extracted twice: first with 2 ml
752 95% ethanol, second with 1 ml 70% ethanol. The resulting extracts were combined and
753 evaporated under a nitrogen stream at 34°C. Upon evaporation, the samples were
754 resuspended in mobile phase A and filtered through a 0.45 µm nylon membrane syringe
755 filter (GE Healthcare, Chicago, IL, United States). Mobile phase A consisted of 0.15 M
756 triethylammonium acetate (TEAA) pH 5.0, and mobile phase B consisted 0.15M TEAA
757 in acetonitrile. Prior to use, mobile phases were filtered using a 0.2 µm filter with a
758 vacuum followed by ultrasonic degassing. Separations were conducted using a
759 SUPELCOSIL^{TM} LC-18T column, 25 cm x 4.6mm i.d., 5µm particle size (Supelco,
760 Bellefonte, PA, United States). Gradient elution was set to 0 – 4 min, 99% A to 99% A
761 (1 ml/min); 4 – 10 min, 99% A to 85% A (1 ml/min); 10 – 18 min, 85% A to 75% A (0.4
762 ml/min); 18 – 20 min, 75% A to 25% A (1 ml/min); 20 min – 24 min, 25% A to 97.5% A
763 (1 ml/min); 24 min – 24 min, 97.5% A to 99.0% A (1 ml/min). 20 µl sample injections
764 were used and guanosine triphosphate (Sigma-Aldrich, St. Louis, MO, United States)
765 and guanosine tetraphosphate (Jena Biosciences, Jena, Germany) were used as
766 standards for peak identification and quantification.
767
768 **Ethics statement.** *In vivo* studies were approved by the Institutional Animal Care and
769 Use Committee of the Rocky Mountain Laboratories (RML). Animal work was conducted
770 adhering to the institution's guidelines for animal use, and followed the guidelines and
771 basic principles in the United States Public Health Service Policy on Humane Care and
772 Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals
773 by certified staff in an Association for Assessment and Accreditation of Laboratory
774 Animal Care (AAALAC) International accredited facility.
775
776
777

17

778

779 1.   Orloski KA, Hayes EB, Campbell GL, Dennis DT. Surveillance for Lyme disease--
780 United States, 1992-1998. MMWR CDC Surveill Summ. 2000;49(3):1-11.
781 2.   Centers for Disease Control. 2018. Lyme Disease. Available:
782 https://www.cdc.gov/lyme/stats/tables.html. 2018.
783 3.   Bontemps-Gallo S, Lawrence K, Gherardini FC. Two Different Virulence-Related
784 Regulatory Pathways in Borrelia burgdorferi Are Directly Affected by Osmotic Fluxes in
785 the Blood Meal of Feeding Ixodes Ticks. PLoS Pathog. 2016;12(8):e1005791.
786 4.   Bourret TJ, Lawrence KA, Shaw JA, Lin T, Norris SJ, Gherardini FC. The
787 Nucleotide Excision Repair Pathway Protects *Borrelia burgdorferi* from Nitrosative
788 Stress in *Ixodes scapularis* Ticks. Front Microbiol. 2016;7:1397.
789 5.   Dulebohn DP, Richards CL, Su H, Lawrence KA, Gherardini FC. Weak Organic
790 Acids Decrease *Borrelia burgdorferi* Cytoplasmic pH, Eliciting an Acid Stress Response
791 and Impacting RpoN- and RpoS-Dependent Gene Expression. Front Microbiol.
792 2017;8:1734.
793 6.   Sonenshine DE. Biology of ticks. New York: Oxford University Press; 1991.
794 7.   Zhi H, Xie J, Skare JT. The Classical Complement Pathway Is Required to
795 Control *Borrelia burgdorferi* Levels During Experimental Infection. Front Immunol.
796 2018;9:959.
797 8.   Stewart PE, Wang X, Bueschel DM, Clifton DR, Grimm D, Tilly K, et al.
798 Delineating the requirement for the *Borrelia burgdorferi* virulence factor OspC in the
799 mammalian host. Infect Immun. 2006;74(6):3547-53.
800 9.   Bugrysheva JV, Pappas CJ, Terekhova DA, Iyer R, Godfrey HP, Schwartz I, et
801 al. Characterization of the Rel$_{Bbu}$ Regulon in *Borrelia burgdorferi* Reveals Modulation of
802 Glycerol Metabolism by (p)ppGpp. PLoS One. 2015;10(2):e0118063.
803 10.   Drecktrah D, Hall LS, Rescheneder P, Lybecker M, Samuels DS. The Stringent
804 Response-Regulated sRNA Transcriptome of *Borrelia burgdorferi*. Front Cell Infect
805 Microbiol. 2018;8:231.
806 11.   Boyle WK, Groshong AM, Drecktrah D, Boylan JA, Gherardini FC, Blevins JS, et
807 al. DksA Controls the Response of the Lyme Disease Spirochete *Borrelia burgdorferi* to
808 Starvation. J Bacteriol. 2019;201(4).
809 12.   Pappas CJ, Iyer R, Petzke MM, Caimano MJ, Radolf JD, Schwartz I. *Borrelia*
810 *burgdorferi* requires glycerol for maximum fitness during the tick phase of the enzootic
811 cycle. PLoS Pathog. 2011;7(7):e1002102.
812 13.   Tilly K, Elias AF, Errett J, Fischer E, Iyer R, Schwartz I, et al. Genetics and
813 regulation of chitobiose utilization in *Borrelia burgdorferi*. J Bacteriol.
814 2001;183(19):5544-53.
815 14.   Groshong AM, Dey A, Bezsonova I, Caimano MJ, Radolf JD. Peptide Uptake Is
816 Essential for Borrelia burgdorferi Viability and Involves Structural and Regulatory
817 Complexity of its Oligopeptide Transporter. mBio. 2017;8(6).
818 15.   Zhu M, Pan Y, Dai X. (p)ppGpp: the magic governor of bacterial growth
819 economy. Curr Genet. 2019;65(5):1121-5.
820 16.   Magnusson LU, Farewell A, Nystrom T. ppGpp: a global regulator in *Escherichia*
821 *coli*. Trends Microbiol. 2005;13(5):236-42.

822   17.   Traxler MF, Summers SM, Nguyen HT, Zacharia VM, Hightower GA, Smith JT, et
823   al. The global, ppGpp-mediated stringent response to amino acid starvation in
824   *Escherichia coli*. Mol Microbiol. 2008;68(5):1128-48.
825   18.   Sanchez-Vazquez P, Dewey CN, Kitten N, Ross W, Gourse RL. Genome-wide
826   effects on Escherichia coli transcription from ppGpp binding to its two sites on RNA
827   polymerase. Proc Natl Acad Sci U S A. 2019;116(17):8310-9.
828   19.   Holley CL, Zhang X, Fortney KR, Ellinger S, Johnson P, Baker B, et al. DksA and
829   (p)ppGpp have unique and overlapping contributions to *Haemophilus ducreyi*
830   pathogenesis in humans. Infect Immun. 2015;83(8):3281-92.
831   20.   Gourse RL, Chen AY, Gopalkrishnan S, Sanchez-Vazquez P, Myers A, Ross W.
832   Transcriptional Responses to ppGpp and DksA. Annu Rev Microbiol. 2018;72:163-84.
833   21.   Ross W, Sanchez-Vazquez P, Chen AY, Lee JH, Burgos HL, Gourse RL. ppGpp
834   Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on
835   Transcription Initiation during the Stringent Response. Mol Cell. 2016;62(6):811-23.
836   22.   Molodtsov V, Sineva E, Zhang L, Huang X, Cashel M, Ades SE, et al. Allosteric
837   Effector ppGpp Potentiates the Inhibition of Transcript Initiation by DksA. Mol Cell.
838   2018;69(5):828-39 e5.
839   23.   Lyzen R, Maitra A, Milewska K, Kochanowska-Lyzen M, Hernandez VJ,
840   Szalewska-Palasz A. The dual role of DksA protein in the regulation of *Escherichia coli*
841   pArgX promoter. Nucleic Acids Res. 2016;44(21):10316-25.
842   24.   Paul BJ, Berkmen MB, Gourse RL. DksA potentiates direct activation of amino
843   acid promoters by ppGpp. Proc Natl Acad Sci U S A. 2005;102(22):7823-8.
844   25.   Drecktrah D, Lybecker M, Popitsch N, Rescheneder P, Hall LS, Samuels DS.
845   The *Borrelia burgdorferi* RelA/SpoT Homolog and Stringent Response Regulate
846   Survival in the Tick Vector and Global Gene Expression during Starvation. PLoS
847   Pathog. 2015;11(9):e1005160.
848   26.   Bugrysheva J, Dobrikova EY, Sartakova ML, Caimano MJ, Daniels TJ, Radolf
849   JD, et al. Characterization of the stringent response and rel(Bbu) expression in Borrelia
850   burgdorferi. J Bacteriol. 2003;185(3):957-65.
851   27.   Bugrysheva J, Dobrikova EY, Godfrey HP, Sartakova ML, Cabello FC.
852   Modulation of Borrelia burgdorferi stringent response and gene expression during
853   extracellular growth with tick cells. Infect Immun. 2002;70(6):3061-7.
854   28.   Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS. ppGpp conjures
855   bacterial virulence. Microbiol Mol Biol Rev. 2010;74(2):171-99.
856   29.   Furman R, Danhart EM, NandyMazumdar M, Yuan C, Foster MP, Artsimovitch I.
857   pH dependence of the stress regulator DksA. PLoS One. 2015;10(3):e0120746.
858   30.   Fitzsimmons LF, Liu L, Kim JS, Jones-Carson J, Vazquez-Torres A. *Salmonella*
859   Reprograms Nucleotide Metabolism in Its Adaptation to Nitrosative Stress. MBio.
860   2018;9(1).
861   31.   Kvint K, Farewell A, Nystrom T. RpoS-dependent promoters require guanosine
862   tetraphosphate for induction even in the presence of high levels of sigma(s). J Biol
863   Chem. 2000;275(20):14795-8.
864   32.   Merrikh H, Ferrazzoli AE, Lovett ST. Growth phase and (p)ppGpp control of IraD,
865   a regulator of RpoS stability, in Escherichia coli. J Bacteriol. 2009;191(24):7436-46.

866 33.   Lin YH, Chen Y, Smith TC, 2nd, Karna SLR, Seshu J. Short-Chain Fatty Acids
867 Alter Metabolic and Virulence Attributes of *Borrelia burgdorferi*. Infect Immun.
868 2018;86(9).
869 34.   Carroll JA, Garon CF, Schwan TG. Effects of environmental pH on membrane
870 proteins in *Borrelia burgdorferi*. Infect Immun. 1999;67(7):3181-7.
871 35.   Yang X, Goldberg MS, Popova TG, Schoeler GB, Wikel SK, Hagman KE, et al.
872 Interdependence of environmental factors influencing reciprocal patterns of gene
873 expression in virulent *Borrelia burgdorferi*. Mol Microbiol. 2000;37(6):1470-9.
874 36.   Dunham-Ems SM, Caimano MJ, Eggers CH, Radolf JD. *Borrelia burgdorferi*
875 requires the alternative sigma factor RpoS for dissemination within the vector during
876 tick-to-mammal transmission. PLoS Pathog. 2012;8(2):e1002532.
877 37.   Yang XF, Lybecker MC, Pal U, Alani SM, Blevins J, Revel AT, et al. Analysis of
878 the *ospC* regulatory element controlled by the RpoN-RpoS regulatory pathway in
879 Borrelia burgdorferi. J Bacteriol. 2005;187(14):4822-9.
880 38.   Eggers CH, Caimano MJ, Radolf JD. Analysis of promoter elements involved in
881 the transcriptional initiation of RpoS-dependent *Borrelia burgdorferi* genes. J Bacteriol.
882 2004;186(21):7390-402.
883 39.   Iyer R, Caimano MJ, Luthra A, Axline D, Jr., Corona A, Iacobas DA, et al. Stage-
884 specific global alterations in the transcriptomes of Lyme disease spirochetes during tick
885 feeding and following mammalian host adaptation. Mol Microbiol. 2015;95(3):509-38.
886 40.   Tilly K, Krum JG, Bestor A, Jewett MW, Grimm D, Bueschel D, et al. *Borrelia*
887 *burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian
888 infection. Infect Immun. 2006;74(6):3554-64.
889 41.   Dunham-Ems SM, Caimano MJ, Pal U, Wolgemuth CW, Eggers CH, Balic A, et
890 al. Live imaging reveals a biphasic mode of dissemination of *Borrelia burgdorferi* within
891 ticks. J Clin Invest. 2009;119(12):3652-65.
892 42.   Samuels DS, Drecktrah D, Hall LS. Genetic Transformation and
893 Complementation. Methods Mol Biol. 2018;1690:183-200.
894 43.   Stevenson B, Seshu J. Regulation of Gene and Protein Expression in the Lyme
895 Disease Spirochete. Curr Top Microbiol Immunol. 2018;415:83-112.
896 44.   Burtnick MN, Downey JS, Brett PJ, Boylan JA, Frye JG, Hoover TR, et al.
897 Insights into the complex regulation of *rpoS* in *Borrelia burgdorferi*. Mol Microbiol.
898 2007;65(2):277-93.
899 45.   Smith AH, Blevins JS, Bachlani GN, Yang XF, Norgard MV. Evidence that RpoS
900 (sigmaS) in Borrelia burgdorferi is controlled directly by RpoN (sigma54/sigmaN). J
901 Bacteriol. 2007;189(5):2139-44.
902 46.   Miller CL, Karna SL, Seshu J. *Borrelia* host adaptation Regulator (BadR)
903 regulates rpoS to modulate host adaptation and virulence factors in *Borrelia burgdorferi*.
904 Mol Microbiol. 2013;88(1):105-24.
905 47.   Ouyang Z, Zhou J. BadR (BB0693) controls growth phase-dependent induction
906 of *rpoS* and *bosR* in *Borrelia burgdorferi* via recognizing TAAAATAT motifs. Mol
907 Microbiol. 2015;98(6):1147-67.
908 48.   Boylan JA, Posey JE, Gherardini FC. *Borrelia* oxidative stress response
909 regulator, BosR: a distinctive Zn-dependent transcriptional activator. Proc Natl Acad Sci
910 U S A. 2003;100(20):11684-9.

911   49.   Samuels DS. Gene regulation in Borrelia burgdorferi. Annu Rev Microbiol.
912   2011;65:479-99.
913   50.   Ouyang Z, Blevins JS, Norgard MV. Transcriptional interplay among the
914   regulators Rrp2, RpoN and RpoS in *Borrelia burgdorferi*. Microbiology. 2008;154(Pt
915   9):2641-58.
916   51.   Boardman BK, He M, Ouyang Z, Xu H, Pang X, Yang XF. Essential role of the
917   response regulator Rrp2 in the infectious cycle of *Borrelia burgdorferi*. Infect Immun.
918   2008;76(9):3844-53.
919   52.   Groshong AM, Gibbons NE, Yang XF, Blevins JS. Rrp2, a prokaryotic enhancer-
920   like binding protein, is essential for viability of *Borrelia burgdorferi*. J Bacteriol.
921   2012;194(13):3336-42.
922   53.   Grove AP, Liveris D, Iyer R, Petzke M, Rudman J, Caimano MJ, et al. Two
923   Distinct Mechanisms Govern RpoS-Mediated Repression of Tick-Phase Genes during
924   Mammalian Host Adaptation by *Borrelia burgdorferi*, the Lyme Disease Spirochete.
925   mBio. 2017;8(4).
926   54.   Sarkar A, Hayes BM, Dulebohn DP, Rosa PA. Regulation of the virulence
927   determinant OspC by *bbd18* on linear plasmid lp17 of *Borrelia burgdorferi*. J Bacteriol.
928   2011;193(19):5365-73.
929   55.   Hayes BM, Dulebohn DP, Sarkar A, Tilly K, Bestor A, Ambroggio X, et al.
930   Regulatory protein BBD18 of the lyme disease spirochete: essential role during tick
931   acquisition? mBio. 2014;5(2):e01017-14.
932   56.   Dulebohn DP, Hayes BM, Rosa PA. Global repression of host-associated genes
933   of the Lyme disease spirochete through post-transcriptional modulation of the
934   alternative sigma factor RpoS. PLoS One. 2014;9(3):e93141.
935   57.   Lybecker MC, Samuels DS. Temperature-induced regulation of RpoS by a small
936   RNA in *Borrelia burgdorferi*. Mol Microbiol. 2007;64(4):1075-89.
937   58.   Boyle WK, Hall LS, Armstrong AA, Dulebohn DP, Samuels DS, Gherardini FC, et
938   al. Establishment of an in vitro RNA polymerase transcription system: a new tool to
939   study transcriptional activation in Borrelia burgdorferi. Sci Rep. 2020;10(1):8246.
940   59.   Henard CA, Tapscott T, Crawford MA, Husain M, Doulias PT, Porwollik S, et al.
941   The 4-cysteine zinc-finger motif of the RNA polymerase regulator DksA serves as a thiol
942   switch for sensing oxidative and nitrosative stress. Mol Microbiol. 2014;91(4):790-804.
943   60.   Crawford MA, Tapscott T, Fitzsimmons LF, Liu L, Reyes AM, Libby SJ, et al.
944   Redox-Active Sensing by Bacterial DksA Transcription Factors Is Determined by
945   Cysteine and Zinc Content. MBio. 2016;7(2):e02161-15.
946   61.   Tapscott T, Kim JS, Crawford MA, Fitzsimmons L, Liu L, Jones-Carson J, et al.
947   Guanosine tetraphosphate relieves the negative regulation of *Salmonella* pathogenicity
948   island-2 gene transcription exerted by the AT-rich *ssrA* discriminator region. Sci Rep.
949   2018;8(1):9465.
950   62.   Pupov D, Petushkov I, Esyunina D, Murakami KS, Kulbachinskiy A. Region 3.2 of
951   the sigma factor controls the stability of rRNA promoter complexes and potentiates their
952   repression by DksA. Nucleic Acids Res. 2018;46(21):11477-87.
953   63.   Mason C, Thompson C, Ouyang Z. DksA plays an essential role in regulating the
954   virulence of *Borrelia burgdorferi*. Mol Microbiol. 2020.

64.   Richards CL, Lawrence KA, Su H, Yang Y, Yang XF, Dulebohn DP, et al. Acetyl-Phosphate Is Not a Global Regulatory Bridge between Virulence and Central Metabolism in Borrelia burgdorferi. PLoS One. 2015;10(12):e0144472.

65.   Hubner A, Yang X, Nolen DM, Popova TG, Cabello FC, Norgard MV. Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. Proc Natl Acad Sci U S A. 2001;98(22):12724-9.

66.   Jutras BL, Chenail AM, Stevenson B. Changes in bacterial growth rate govern expression of the *Borrelia burgdorferi* OspC and Erp infection-associated surface proteins. J Bacteriol. 2013;195(4):757-64.

67.   Zyskind JW, Smith DW. DNA replication, the bacterial cell cycle, and cell growth. Cell. 1992;69(1):5-8.

68.   Kasumba IN, Bestor A, Tilly K, Rosa PA. Virulence of the Lyme disease spirochete before and after the tick bloodmeal: a quantitative assessment. Parasit Vectors. 2016;9:129.

69.   Petushkov I, Esyunina D, Mekler V, Severinov K, Pupov D, Kulbachinskiy A. Interplay between sigma region 3.2 and secondary channel factors during promoter escape by bacterial RNA polymerase. Biochem J. 2017;474(24):4053-64.

70.   Igarashi K, Fujita N, Ishihama A. Promoter selectivity of *Escherichia coli* RNA polymerase: omega factor is responsible for the ppGpp sensitivity. Nucleic Acids Res. 1989;17(21):8755-65.

71.   Vrentas CE, Gaal T, Ross W, Ebright RH, Gourse RL. Response of RNA polymerase to ppGpp: requirement for the omega subunit and relief of this requirement by DksA. Genes Dev. 2005;19(19):2378-87.

72.   Chandrangsu P, Lemke JJ, Gourse RL. The *dksA* promoter is negatively feedback regulated by DksA and ppGpp. Mol Microbiol. 2011;80(5):1337-48.

73.   Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, et al. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature. 1997;390(6660):580-6.

74.   Zhou B, Yang Y, Chen T, Lou Y, Yang XF. The oligopeptide ABC transporter OppA4 negatively regulates the virulence factor OspC production of the Lyme disease pathogen. Ticks Tick Borne Dis. 2018;9(5):1343-9.

75.   Raju BV, Esteve-Gassent MD, Karna SL, Miller CL, Van Laar TA, Seshu J. Oligopeptide permease A5 modulates vertebrate host-specific adaptation of Borrelia burgdorferi. Infect Immun. 2011;79(8):3407-20.

76.   Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. Recent functional insights into the role of (p)ppGpp in bacterial physiology. Nat Rev Microbiol. 2015;13(5):298-309.

77.   Fernandez-Coll L, Potrykus K, Cashel M. Puzzling conformational changes affecting proteins binding to the RNA polymerase. Proc Natl Acad Sci U S A. 2018;115(50):12550-2.

78.   Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009;6(5):343-5.

79.   Frank KL, Bundle SF, Kresge ME, Eggers CH, Samuels DS. *aadA* confers streptomycin resistance in *Borrelia burgdorferi*. J Bacteriol. 2003;185(22):6723-7.

1000   80.   Blevins JS, Hagman KE, Norgard MV. Assessment of decorin-binding protein A
1001   to the infectivity of *Borrelia burgdorferi* in the murine models of needle and tick infection.
1002   BMC Microbiol. 2008;8:82.
1003   81.   Policastro PF, Schwan TG. Experimental infection of *Ixodes scapularis* larvae
1004   (Acari: Ixodidae) by immersion in low passage cultures of *Borrelia burgdorferi*. J Med
1005   Entomol. 2003;40(3):364-70.
1006
1007

1008 **Table 1**. Evaluation of the potential for DksA-deficient *B. burgdorferi* to cause infection.

1009

| Stain	Ear	Joint	Bladder	Control[a]	Serology
Wild-type	4/4[b]	5/5	5/5	1/1	5/5
∆*dksA*	0/5	0/5	0/5	1/1	0/5
∆*dksA* cDksA	5/5	5/5	5/5	1/1	5/5

Numbers indicate ratio of *B. burgdorferi* positive cultures from various tissue sites, ear, joint, or heart and positive serology over the number of individual mice tested. (a) control tubes tested the ability of each *B. burgdorferi* strain to grow in media containing antibiotics mixture. (b) one culture was lost due to contamination.

1010

1011

1012 **Table 2.** Evaluation of DksA role in *B. burgdorferi* transmission *I. scapularis* nymphs.

1013

1014

Stain	Ear	Joint	Bladder	Serology
Wild-type	2/2	2/2	2/2	2/2
Δ*dksA*	0/3	0/3	0/3	0/3
Δ*dksA* cDksA	1/3	1/3	1/3	1/3

1015
1016
1017
1018

1019

1020 Numbers indicate ratio of *B. burgdorferi* positive cultures from various tissue
1021 sites, ear, joint, or heart and positive serology over the number of individual
1022 mice tested.

1023

1024

1025

1026

Figure 1. Measurement of *B. burgdorferi* DksA α-helicity and Zn²⁺-binding. (A) Schematic of DksA predicted structure. (B) DksA purified to apparent homogeneity. DksA purity was assessed by Coomassie stained SDS-PAGE gel. (C) Circular dichroism spectra of recombinant *B. burgdorferi* DksA measured in 15 mM sodium phosphate buffer pH 7.4 and 100 mM NaCl. (D) Zn^{2+} release from 100 uM of DksA mixed with various concentrations of H_2O_2 was measured by a zinc sensitive dye 4-(2-pyridylazo)resorcinol (PAR). Increase in Zn^{2+} release with higher levels of H_2O_2 suggest 4-Cys zinc finger is capable of coordinating Zn^{2+}.

1035

1036

1037

1038

1039

1040
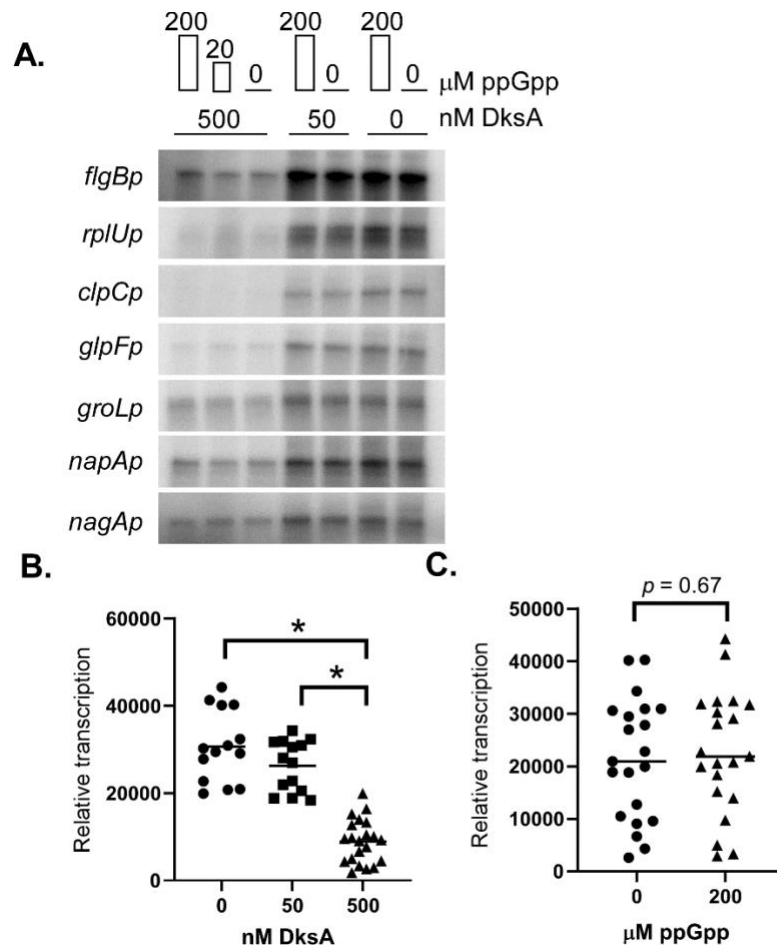
Figure 2. *In vitro* transcription reactions to assay ppGpp and DksA effects on transcription initiation from seven *B. burgdorferi* promoters. *In vitro* transcription reactions initiated on seven RpoD-dependent promoters were prepared containing 500, 50, or 0 nM DksA and 200, 20, or 0 µM of ppGpp. (A) RNA was separated by gel electrophoresis on a 10% urea gel are shown. First, fourth, and sixth lanes contained 200 µM of ppGpp. Second and fifth lane contained 20 µM ppGpp. Phosphor screen signals from all *in vitro* transcription were pooled to evaluate (B) DksA-dependent effects on transcription using a non-parametric ranked Kruskal–Wallis multiple comparisons test and (C) ppGpp-dependent effects using non-parametric ranked Mann-Whitney comparison test. Asterisk indicates p-value < 0.001 in multiple comparison testing.

1052
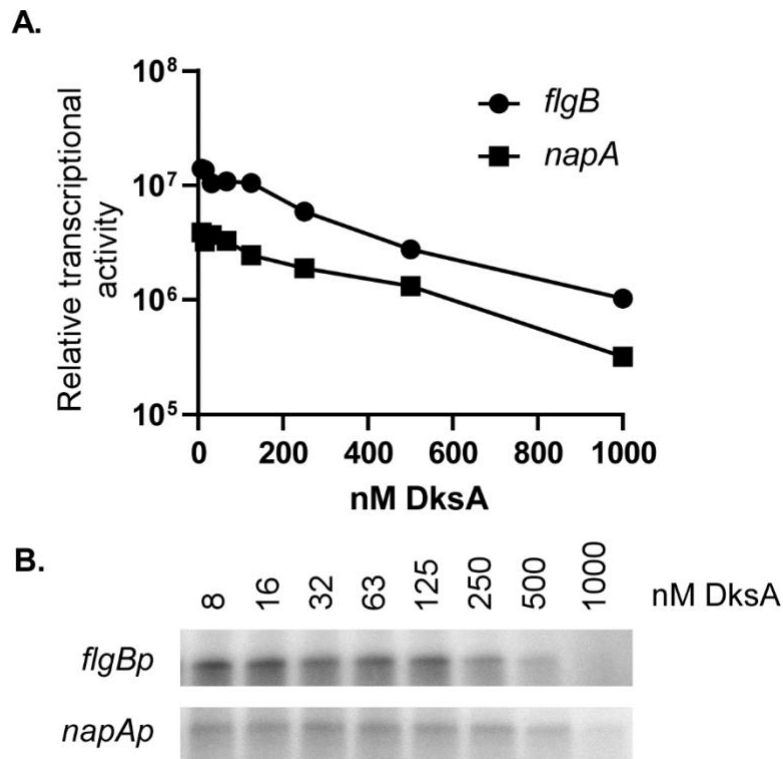
Figure 3. Test of DksA concentration-dependent regulation of *flgB* and *napA*.
Relative RNA product amounts after *in vitro* transcription reactions containing various
concentrations (8 – 1000 nM) of DksA. Two promoters *flgBp* and *napAp* which
appeared to have different levels of response to DksA in Figure 1 were tested. Signal
intensity as measured by isotopically labeled RNA and determined by densitometry **(A)**
of phosphor screen images **(B)** are shown.

1059
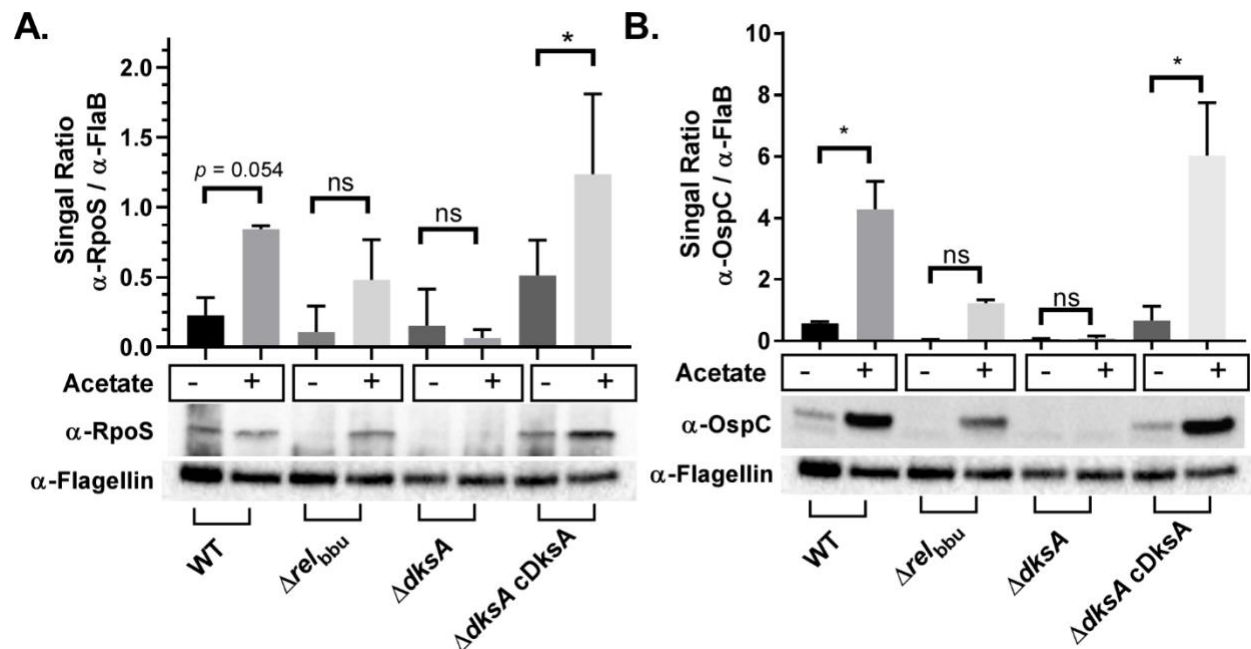
Figure 4. Relative expression of RpoS and OspC in wild-type (WT), Δrel_bbu, ΔdksA, and ΔdksA cDksA (cDksA) B. burgdorferi strains at mid-logarithmic growth.

Whole cell lysates were collected from *B. burgdorferi* 297 strain cultured to 5 x 10^7 spirochetes/ mL in either BSKII-pH 7.6 or BSKII-pH 6.7 with 20 mM acetate. Expression of RpoS and OspC protein levels were determined by normalizing densitometry of chemiluminescence signals obtained from western blot to signals obtained from flagellin. Data are representative of 3 replicate experiments. Asterisk indicate adjusted *p*-values < 0.05 in a Sidak multiple comparisons test.

1073

1074
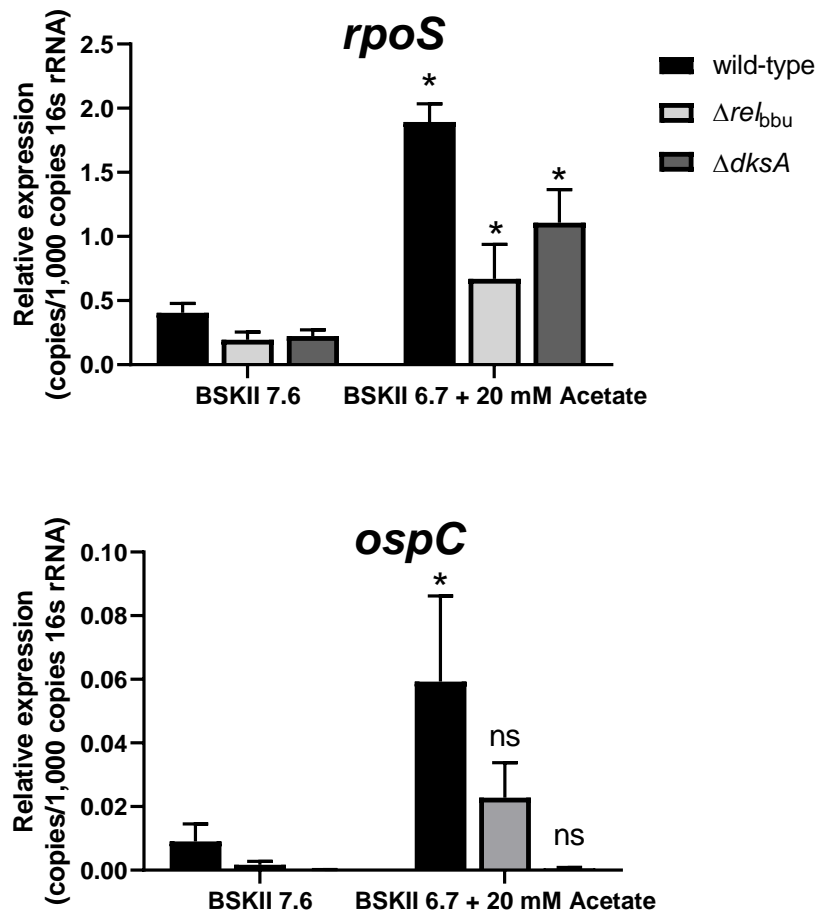
Figure 5. RT-qPCR analysis of *rpoS* and *ospC* transcripts in wild-type, Δ*rel*bbu and Δ*dksA* strains. RT-qPCR was performed on RNA extracted from wild-type (WT), Δ*rel*bbu, and Δ*dksA* mid-logarithmic phase cultures in BSKII 7.6 or BSKII 6.7 + 20 mM acetate. Relative expression of *ospC* and *rpoS* were normalized to 16s rRNA. Error bars represent standard deviation calculated from four biological replicates. Asterisk indicates *p*-value < 0.05 for relative expression between BSKII 7.6 and BSKII 6.7 + 20 mM acetate conditions in a Holm-Sidak multiple comparisons test.
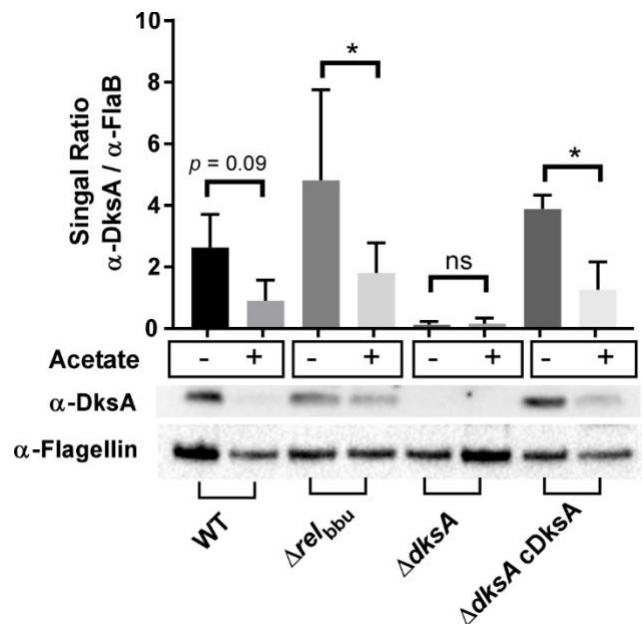
1082

1083

1084
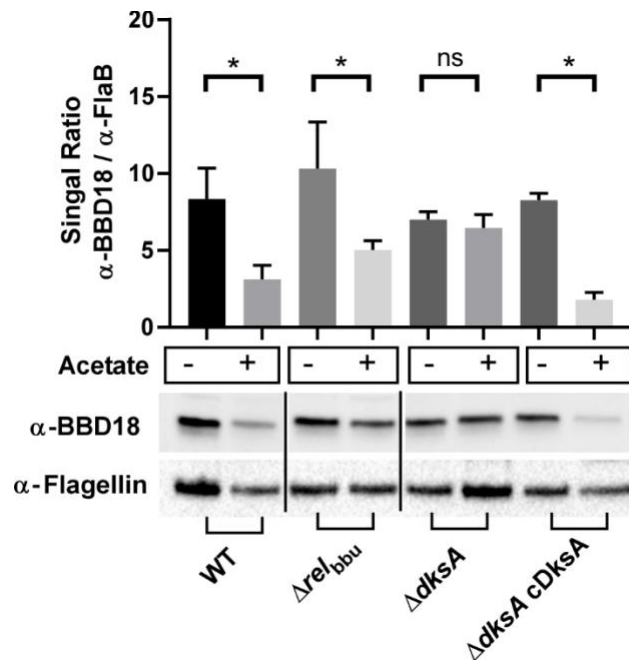
Figure 6. Relative expression of BBD18 by *B. burgdorferi* in response to growth in BSKII pH 7.6 and BSKII pH 6.7 + 20mM acetate. Whole cell lysates were collected from 297 wild-type (WT), Δ*rel*bbu, Δ*dksA*, and chromosomally complemented Δ*dksA* (Δ*dksA* cDksA) *B. burgdorferi* strains cultured to 5 x 10⁷ spirochetes / mL density in either BSKII-pH 7.6 or BSKII-pH 6.7 with 20 mM acetate. Expression of BBD18 protein levels were determined by densitometry of chemiluminescence signals and signals were normalized to expression of FlaB. Data are representative of 3 replicate experiments. Asterisk indicate adjusted *p*-values < 0.05 in a Sidak multiple comparisons test.

1093

1094

1095

1096

1097
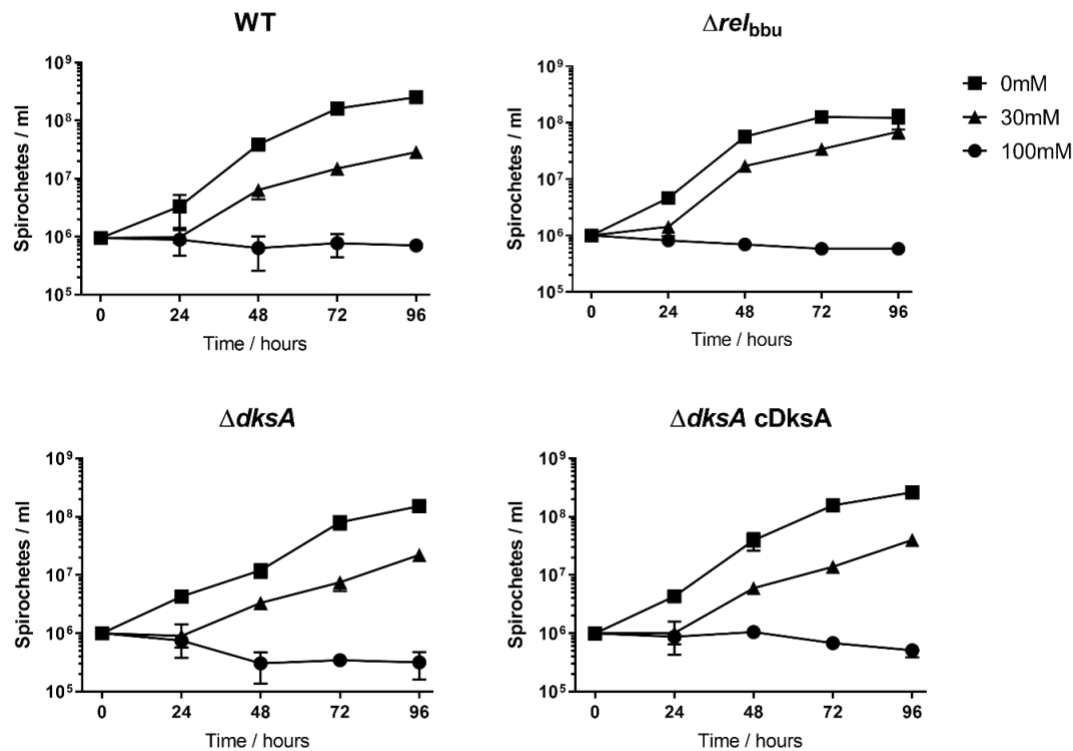
1098 **Figure 7. Growth rates of *B. burgdorferi* in acidic environment.** *B. burgdorferi* 297
1099 wild-type (WT), Δ*rel*Bbu, Δ*dksA*, and chromosomally complemented Δ*dksA* strain (Δ*dksA*
1100 cDksA) were cultured in BSK II containing 0 (square), 30 (triangle), and 100 mM (circle)
1101 of acetate. Media pH was not compensated for the addition of acetic acid and 0, 30, and
1102 100 mM medium also represent growth in pH 7.6, 6.1, and 4.6 respectively. Growth
1103 curves are representative of 3 replicate cultures and error bars represent standard
1104 deviation.

1105

1106

1107

1108

1109

1110

1111

1112

1113

1114

Figure 8. Detection of shifts in DksA protein levels with change in growth media pH and organic acid level at mid-logarithmic growth. Whole cell lysates were collected from 297 wild-type (WT), Δrel_{bbu}, $\Delta dksA$, and chromosomally complemented $\Delta dksA$ ($\Delta dksA$ cDksA) *B. burgdorferi* strains cultured to 5×10^7 spirochetes / mL density in either BSKII-pH 7.6 or BSKII-pH 6.7 with 20 mM acetate. Expression of DksA protein levels **(A)** and OspC protein levels **(B)** were determined by densitometry of chemiluminescence signals and signals were normalized to expression of FlaB. Data are representative of 3 replicate experiments. Asterisk indicate adjusted *p*-values < 0.05 a Sidak multiple comparisons test.

1124
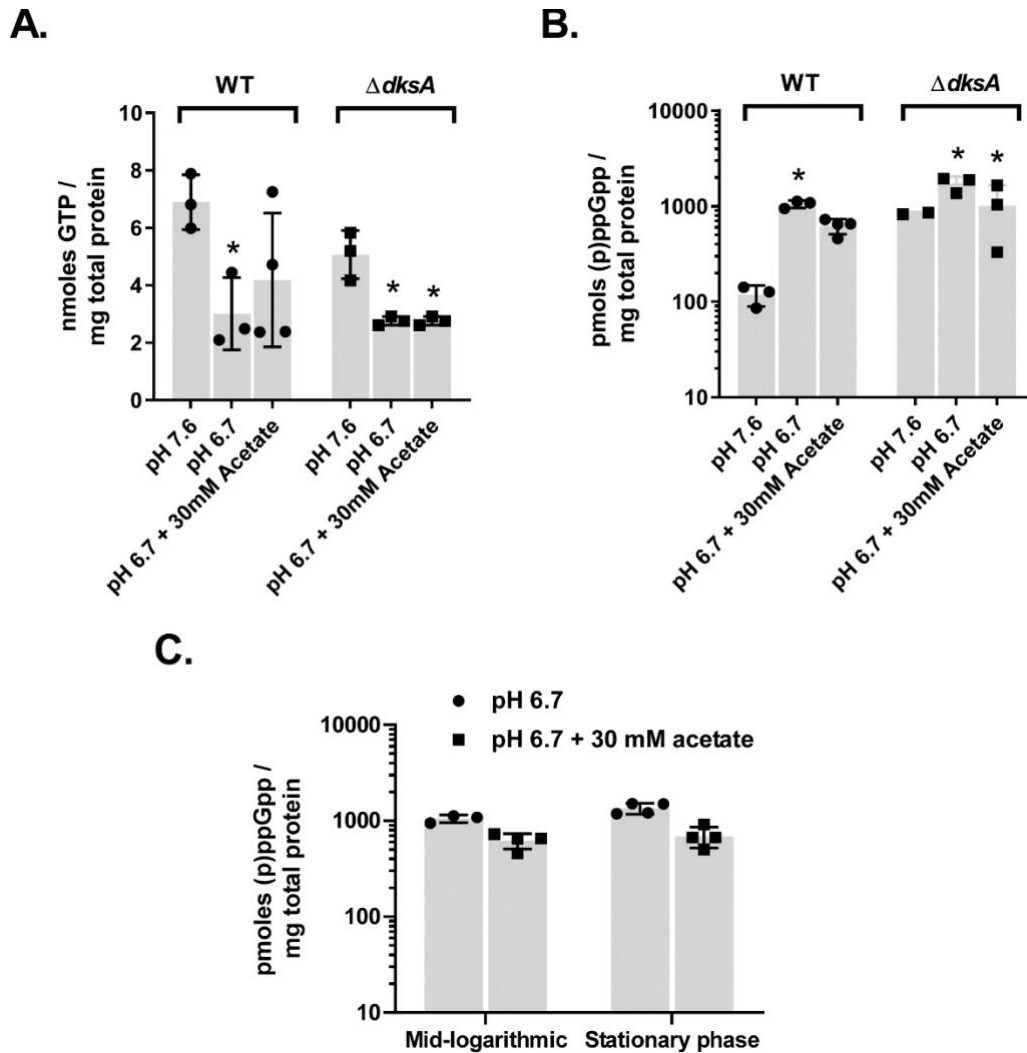
Figure 9. GTP and (p)ppGpp content of *B. burgdorferi* cells in response to culture media pH and organic acid content. Wild-type (WT) and Δ*dksA* strains cultured in BSK II pH 7.6 or in BSK II pH 6.7 with or without 30 mM acetate to $2 - 4 \times 10^7$ spirochetes · ml^{-1} were collected to measure (A) GTP and (B) (p)ppGpp content by HPLC. Three replicate experiments were performed. Asterisks indicate $p < 0.05$ for comparison between WT in BSKII 7.6 measurements and all other measurements in a one-way ANOVA with multiple comparisons test on levels of GTP and ppGpp. (C) Wild-type *B. burgdorferi* cultured in BSK II pH 6.7 with or without 30 mM acetate to either mid-logarithmic (2×10^7 spirochetes · ml^{-1}) or to stationary (2×10^8 spirochetes · ml^{-1}) phase were collected to measure cell density dependent (p)ppGpp content by HPLC. Relative measurement and identity of (p)ppGpp were verified by LC-MS.

34