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*Borrelia burgdorferi* genes, *bb0639-0642*, encode a putative putrescine/spermidine transport system PotABCD that is spermidine specific and essential for cell survival

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Running Head: PotABCD is essential for *Borrelia burgdorferi*

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1 **Summary**

2 Polyamines are an essential class of metabolites found throughout all kingdoms in life.  
3 *Borrelia burgdorferi* harbors no enzymes to synthesize or degrade polyamines yet does  
4 contain a polyamine uptake system, *potABCD*. In this report, we describe the initial  
5 characterization of this putative transport system. After several unsuccessful attempts to  
6 inactivate *potABCD*, we placed the operon under the control of an inducible LacI  
7 promoter expression system. Analyses of this construct confirmed that *potABCD* was  
8 required for *in vitro* survival. Additionally, we demonstrated that the *potABCD* operon  
9 were upregulated *in vitro* by low osmolarity. Previously, we had shown that low  
10 osmolarity triggers the activation of the Rrp2/RpoN/RpoS regulatory cascade which  
11 regulates genes essential for the transmission of spirochetes from ticks to mammalian  
12 hosts. Interestingly, induction of the *pot* operon was only affected in an *rpoS* mutant but  
13 not in a *rpoN* mutant, suggesting that the genes were RpoS-dependent, RpoN-  
14 independent. Furthermore, *potABCD* was upregulated during tick feeding concomitant  
15 with the initiation of spirochete replication. Finally, uptake experiments determined the  
16 specificity of *B. burgdorferi*'s PotABCD for spermidine. Our studies demonstrate that  
17 *potABCD* was essential for survival, regulated in an RpoS-dependent, RpoN-independent  
18 manner, and regulated concurrent with the drop in osmolarity observed in the midgut of  
19 feeding ticks.  
20

## 21 **Introduction**

22 *Borrelia burgdorferi*, one of the causative agents of Lyme disease (Pritt *et al.*, 2016), is  
23 maintained in a life cycle that involves a vertebrate host (*e.g.* rodent) and a hard tick:  
24 *Ixodes scapularis* (Stewart & Rosa, 2017). During its infectious cycle, *B. burgdorferi*  
25 adapts its physiology and metabolism in response to changing environmental conditions  
26 in the mammal or the arthropod vector (Caimano *et al.*, 2016, Gherardini *et al.*, 2010).  
27 The mammalian host offers this spirochete a nutrient-rich, homeostatic environment  
28 (temperature, pH, osmolarity, etc.) (Waymouth, 1970, The staff of the Jackson  
29 Laboratory, 1966). In addition, *B. burgdorferi* uses several mechanisms for evading the  
30 aggressive host immune system, allowing it to successfully colonize various mammalian  
31 species, including humans (Norris, 2014, Petzke & Schwartz, 2015, Radolf *et al.*, 2012).  
32 In contrast, environmental parameters inside the arthropod vector are quite dynamic over  
33 the course of its life cycle. Studies have shown that the *I. scapularis* midgut has shifting  
34 physio-chemical parameters (temperature: 28°C → 37°C; osmolarity: 600 → 300 → 600  
35 mOsM) (Bontemps-Gallo *et al.*, 2016, Schwan *et al.*, 1995, Stevenson *et al.*, 1995) and,  
36 nitrosative and oxidative bursts that peak during the feeding process (Bourret *et al.*, 2016,  
37 Sonenshine & Anderson, 2014). In response to fluctuating environmental conditions, *B.*  
38 *burgdorferi* modulates its gene expression program to allow it to successfully colonize  
39 both host and vector.

40 A small set of regulators allow *B. burgdorferi* to adapt to changing conditions  
41 throughout the infectious process. Upon colonization of *I. scapularis*, the spirochetes  
42 upregulate the expression of genes involved in carbohydrate utilization, cell envelope  
43 biosynthesis and long-term survival through the two-component system, Hk1-Rrp1  
44 (Caimano *et al.*, 2011, He *et al.*, 2011, Kostick *et al.*, 2011). This two-component system  
45 regulates c-di-GMP turnover through the diguanylate cyclase activity on Rrp1 (Caimano  
46 *et al.*, 2011, He *et al.*, 2011). *In vitro*, Hk1-Rrp1 is stimulated by high osmolarity (600  
47 mOsM) (Bontemps-Gallo *et al.*, 2016). Long-term survival of the spirochetes, after  
48 digestion of the blood meal and subsequent molt, is coordinated via Rel<sub>Bbu</sub> (RelA/SpoT  
49 homolog) (Drecktrah *et al.*, 2015), BosR and RpoS (Samuels, 2011, Skare *et al.*, 2010).  
50 After the molt, *I. scapularis* begins its next feeding which provides an influx of nutrients  
51 and affects multiple environmental parameters inside the midgut, including temperature

52 and osmolarity (Blevins *et al.*, 2009, Bontemps-Gallo *et al.*, 2016, Caimano *et al.*, 2004,  
53 Caimano *et al.*, 2007, Elias *et al.*, 2000, Fisher *et al.*, 2005, Hubner *et al.*, 2001). Growth  
54 and changes in environmental conditions activate the Rrp2/RpoN/RpoS regulatory  
55 cascade which is required for successful transmission from a tick to a mammal (Caimano  
56 *et al.*, 2016). Over the last two decades, several signals responsible for activating this  
57 pathway have been identified including a temperature shift (23 → 34°C) (Schwan *et al.*,  
58 1995, Stevenson *et al.*, 1995), cell density ( $3 \times 10^8$  cells ml<sup>-1</sup>) (Indest *et al.*, 1997,  
59 Ramamoorthy & Philipp, 1998, Yang *et al.*, 2000), low osmolarity (250 mOsM)  
60 (Bontemps-Gallo *et al.*, 2016) and acid stress (Carroll *et al.*, 1999, Yang *et al.*, 2000,  
61 Dulebohn *et al.*, 2017).

62 In other bacteria, polyamines (*e.g.* putrescine, spermidine, spermine) are involved in  
63 both the oxidative and osmotic stress responses (Altendorf *et al.*, 2009, Chattopadhyay *et*  
64 *al.*, 2003, Cohen, 1998, Munro *et al.*, 1972, Shah & Swiatlo, 2008), both of which are  
65 encountered by *B. burgdorferi* during tick feeding. In general, the polyamine content in  
66 bacterial cells is regulated by both biosynthesis and transport (Igarashi & Kashiwagi,  
67 1999). In *E. coli*, the most commonly used bacterial model for polyamine research, the  
68 intracellular concentration of putrescine (22.1 nmol/10<sup>9</sup> cells) is higher than spermidine  
69 (3.8 nmol/10<sup>9</sup> cells) (Morris & Jorstad, 1970). Uptake experiments in *E. coli* demonstrate  
70 that spermidine/putrescine transport (via PotABCD) is a spermidine-preferential uptake  
71 system (Kashiwagi *et al.*, 1993) that also transports putrescine but with lower affinity.  
72 Both molecules can bind nucleic acids and proteins to stabilize their interactions (Childs  
73 *et al.*, 2003), modulate the rate of transcription (Thomas *et al.*, 1995) and stimulate  
74 growth (Igarashi & Kashiwagi, 2010). Despite extensive research efforts, the role of  
75 putrescine and spermidine in bacterial cells remains unclear (Miller-Fleming *et al.*, 2015).

76 Since *B. burgdorferi*'s genome does not appear to contain the genes encoding enzymes  
77 with the capacity to synthesize, modify or degrade either putrescine, spermidine or  
78 spermine, the bacteria is thought to be a spermidine auxotroph (Fraser *et al.*, 1997, Wyss  
79 & Ermert, 1996). Only one putative spermidine/putrescine ABC-transporter, called  
80 PotABCD (encoded by *bb0639-642*) is annotated in *B. burgdorferi*'s genome (Fraser *et*  
81 *al.*, 1997, Lin *et al.*, 2017). *bb0642* (*potA*) encodes a potential ATP-binding protein while  
82 *bb0641* (*potB*) and *bb0640* (*potC*) encode proteins with 6 transmembrane domains

83 suggesting a role in membrane translocation. Finally, *bb0639* (*potD*) encodes a putative  
84 spermidine/putrescine periplasmic binding protein which could bind polyamines  
85 facilitating transport from the periplasm space into the cytosol. It has been shown that  
86 these four genes are expressed as an operon in a polycistronic mRNA (Lin *et al.*, 2017).

87 In this study, we further characterize PotABCD, the spermidine/putrescine transport  
88 system from *B. burgdorferi*, using an inducible expression system (*lacUV5* p/o) to  
89 regulate *potABCD* expression. Using this genetic construct, we were able to demonstrate  
90 that this system was essential for survival *in vitro*. Interestingly, analyses of specific  
91 RpoS, BosR and RpoN mutants indicated that *potABCD* was regulated in an RpoS-  
92 dependent, RpoN-independent manner, and that regulation was concurrent with the drop  
93 in osmolarity measured in the midgut of feeding ticks (600 – 250 mOsM). Finally, uptake  
94 experiments strongly suggested that PotABCD was specific for spermidine in *B.*  
95 *burgdorferi*.

96

## 97 **Results**

98

99 *The putative PotABCD transport system is essential for B. burgdorferi growth.*

100 Spermidine and spermine have been shown to trigger the expression and synthesis of  
101 proteins required for mammalian invasion through RpoS and BosR (Lin *et al.*, 2017). To  
102 better understand the significance of this transport system, we attempted to inactivate the  
103 *potABCD* operon. Unfortunately, multiple attempts were unsuccessful. In addition, the  
104 comprehensive STM mutant library did not detect any transposon insertions in any of the  
105 genes in the operon (Lin *et al.*, 2012) supporting the assumption that the *potABCD*  
106 system could be essential to *B. burgdorferi*. Therefore, we generated an inducible *pot*  
107 operon using the inducible *lac* promoter expression system developed by Gilbert *et al.*  
108 (Gilbert *et al.*, 2007). In this system, *lacI* under control of the *flgB* promoter leads to  
109 constitutive synthesis of the LacI repressor which binds the *flacp* promoter and represses  
110 the expression of the *potABCD* operon. When added, IPTG will bind LacI in a dose-  
111 dependent manner, allowing controlled expression of the *potABCD* operon. Using *B.*  
112 *burgdorferi* strain B31-68-LS (Chu *et al.* 2016), we obtained three clones harboring the

113 *flacp* promoter fused to *potABCD* and confirmed these putative mutants by sequencing.  
114 One clone, designated BBi-*potABCD*, was selected for further characterization.

115 To confirm that polyamine utilization and the *pot* operon were essential, the wild-type  
116 (B31-68-LS) parent and BBi-*potABCD* strains were grown in BSK-II, pH 7.5 with 0.5  
117 mM IPTG to  $5 \times 10^7$  cells/ml. Cells from the cultures were harvested, washed, sub-  
118 cultured (to  $\sim 5 \times 10^5$ ) and grown in BSK-II with or without IPTG. Growth of each  
119 culture was monitored every 24 h by dark-field microscopy and aliquots were removed  
120 and the cells were grown and quantified on BSK-II plates with IPTG (Fig. 1). In the  
121 presence of IPTG, the cells exhibited a normal growth rate and reached  $10^8$  cells/ml,  
122 while without IPTG, growth completely stopped. More importantly, the number of cells  
123 that could be recovered from cultures without IPTG decreased dramatically with time  
124 suggesting that the cells lost viability without the expression of *potABCD*. These data  
125 indicated that *potABCD* is an essential operon in *B. burgdorferi*.

126

127 *Spermidine is specifically transported by B. burgdorferi*

128

129 Previous studies of *potABCD* systems in other bacteria have demonstrated that these  
130 transporters are capable of a broad range of uptake selectivity for polyamines (Kashiwagi  
131 *et al.*, 1993, Yao & Lu, 2014). Some transport spermine, spermidine and/or putrescine  
132 with equal efficiency while others demonstrate a selective preference. To determine if  
133 *potABCD* in *B. burgdorferi* demonstrated preferential uptake of polyamines, we  
134 performed kinetic uptake assays with tritium ( $^3\text{H}$ ) labelled polyamines. *B. burgdorferi*  
135 cells were grown to a density of  $\sim 5 \times 10^7$  cells/ml, harvested, washed thoroughly and  
136 resuspended to a cell density of  $6 \times 10^8$  cells/ml. Cells were incubated with either  $^3\text{H}$ -  
137 spermidine or  $^3\text{H}$ -putrescine and cell-associated radioactivity was analyzed at 1, 2, 6 and  
138 10 min (Fig. 2). Uptake of  $^3\text{H}$ -spermidine increased linearly 2.4-fold between 1 and 10  
139 min, while no  $^3\text{H}$ -putrescine uptake was observed during the same time interval. Because  
140 the inducible strain was unable to survive without IPTG, we were unable to assay the  
141 uptake selected polyamines in BBi-*potABCD*. These data indicated that, unlike the  
142 characterized polyamine transport systems in other bacteria (Kashiwagi *et al.*, 1993, Yao  
143 & Lu, 2014), *potABCD* in *B. burgdorferi* seemed to be specific for the uptake of

144 spermidine. Previous studies have shown that spermidine is more abundant in human  
145 blood (9 uM) than putrescine (0.13 uM) (Wishart *et al.*, 2007, Wishart *et al.*, 2009,  
146 Wishart *et al.*, 2013) and is the only polyamine available in the midgut of nymphal ticks.  
147 The availability of spermidine in the various hosts and vectors harboring *B. burgdorferi*  
148 and its selective transport strongly suggests that spermidine is the essential polyamine  
149 required by *B. burgdorferi* (Narasimhan *et al.*, 2017).

150

151 *potABCD* is up-regulated during nymph tick feeding

152

153 To shed light on the role of *potABCD* during tick feeding, we analyzed the expression of  
154 the four genes in the operon by RT-qPCR during nymph feeding. Additionally, *rpoS* and  
155 *ospC* were assayed as markers for the activation of the Rrp2/RpoN/RpoS pathway (Fig.  
156 3). Ticks were removed daily from mice infected with wild-type *B. burgdorferi* (B31-A3)  
157 and the scutal index was measured to standardize feeding progress (Bontemps-Gallo *et al.*,  
158 2016, Falco *et al.*, 1996) before pooling for RNA extraction. For each time-point  
159 examined, RNA was extracted from 3 groups of 5 *I. scapularis* ticks. The expression  
160 level of *enoS* was using as a housekeeping gene

161 As expected, *rpoS* transcript increased (6.1-fold) at the first day post attachment (scutal  
162 index 2.09) and stayed at a similar level during the feeding process (Fig. 3). Activation of  
163 the Rrp2-RpoN-RpoS pathway increases expression of RpoS-dependant genes (e.g.  
164 *ospC*) (Caimano *et al.*, 2004). Expression of *ospC* increased 5.6-fold on day one post-  
165 attachment and 5.9-fold more on the second day post-attachment after which the  
166 expression slowly decreased. The four genes of the *potABCD* operon followed the same  
167 pattern of transcription (Fig. 3). On the second day of tick feeding (scutal index of 2.9),  
168 the expression of *potABCD* increased 1.9-fold, 2.3-fold, 3.1-fold and 1.8-fold,  
169 respectively. In contrast, the expression of the *potABCD* operon decreased about 7-fold in  
170 the replete ticks (scutal index of 5.1) compared to the value measured on the second day  
171 of feeding (scutal index of 2.9). These data showed that during tick feeding *B.*  
172 *burgdorferi* upregulated the expression of genes encoding the polyamine transport system  
173 (*potABCD*) and this expression corresponded with the increase of RpoS-dependent genes.

174



175 *potABCD* is regulated by low osmolarity

176

177 During the feeding process of the tick vector, *B. burgdorferi* encounters and must survive  
178 two significant stresses in the midgut: oxidative and osmotic (Bontemps-Gallo *et al.*,  
179 2016, Sonenshine & Anderson, 2014). Furthermore, putrescine and spermidine have been  
180 shown to be involved in protecting bacteria from both stresses (Chattopadhyay *et al.*,  
181 2003, Cohen, 1998, Munro *et al.*, 1972, Shah & Swiatlo, 2008). We hypothesized that  
182 one or both stresses was responsible for the activation of the *potABCD* operon during tick  
183 feeding. To test this hypothesis, we followed the expression of each gene by RT-qPCR at  
184 250, 450, and 650 mOsM and after exposure to *tert*-butyl hydroperoxide (Fig. 4).  
185 Expression of the *potA*, *potB* and *potC* genes encoding the putative ATP-binding protein  
186 and the two membrane transport proteins increased 10-fold at low osmolarity compared  
187 to medium or high osmolality (Fig. 4A). Surprisingly, expression of the *potD* gene,  
188 encoding the periplasmic binding protein, increased 19-fold (Fig. 4A). The higher  
189 expression of *potD* compared to the three other genes suggests an internal promoter for  
190 *potD*. Recently, Arnold *et al.* demonstrated by RNA-seq that the *potABCD* operon had  
191 only one promoter regardless of the phase of growth (Arnold *et al.*, 2016). However,  
192 Adams *et al.* showed by using an *in vivo* expression technology-based approach that the  
193 *potABCD* operon had one primary promoter and multiple secondary promoters (Adams *et*  
194 *al.*, 2017). These data together with our data from feeding ticks (Fig. 3), strongly  
195 suggested that the *potABCD* operon could be expressed as one polycistronic mRNA from  
196 a single promoter or as multiple transcripts from secondary promoters to control the ratio  
197 of the mRNA of each gene and the stoichiometry of the different proteins. These data  
198 may also suggest that increased expression of the putative periplasmic binding protein  
199 (PotD) was required to increase the uptake of polyamines.

200 No induction of the *potABCD* operon was observed under oxidative stress while  
201 *coADR* and *napA*, both known to be involved in the oxidative stress response (Boylan *et*  
202 *al.*, 2003, Hyde *et al.*, 2009), were induced (Fig. 4B). Additionally, we tested the  
203 potential of spermidine and putrescine to function as a protective molecule against  
204 osmotic or oxidative stress (Fig. S1). No effects on the growth or on the susceptibility to  
205 both osmotic and oxidative stresses were observed. We were unable to use our *BBi*-

206 *potABCD* mutant to assay if the PotABCD system was required for protection against  
207 oxidative or osmotic stresses, because the strain did not grow without the IPTG (Fig. 1).  
208 It seems very likely that the *in vitro* conditions tested do not accurately reflect the  
209 combination of stresses and changing conditions that challenge *B. burgdorferi* *in vivo*.

210

211 *potABCD* is regulated by *RpoS* independent of *RpoN*

212

213 Because *potABCD* expression followed a similar pattern to genes regulated by the  
214 Rrp2/RpoN/RpoS regulon in a feeding tick under low osmolarity, we hypothesized that  
215 this regulatory pathway controlled *potABCD* expression. To test this hypothesis, we  
216 assayed the expression levels of *potABCD* in mutant strains deficient in the key  
217 regulatory factors ( $\Delta rpoN$ ,  $\Delta rpoS$ , and  $\Delta bosR$ ) known to directly or indirectly modulate  
218 the Rrp2/RpoN/RpoS cascade (Caimano *et al.*, 2016, Samuels, 2011). Wild-type *B.*  
219 *burgdorferi*,  $\Delta rpoN$ ,  $\Delta rpoS$ , and  $\Delta bosR$  mutant strains were grown in BSK-II at 250 or  
220 450 mOsM to approximately  $\sim 5 \times 10^7$  cells/ml and RNA was harvested for RT-qPCR  
221 analyses. As observed previously, the *potABCD* operon was induced in wild-type cells at  
222 low osmolarity (Fig. 4 and 5). *potABCD* induction in the  $\Delta rpoN$  mutant was similar to  
223 levels observed in WT cells, while the  $\Delta rpoS$  mutant exhibited significantly reduced  
224 transcript levels under low osmolarity (Fig 5). These data were surprising since the  
225 current understanding is that that inactivation of either *rpoN* or *rpoS* results in the same  
226 phenotype (Caimano *et al.*, 2016, Samuels, 2011). Fisher *et al.* previously demonstrated  
227 using microarray analyses that a small set of genes in the Rrp2/RpoN/RpoS regulon were  
228 RpoS-dependent, RpoN-independent (Fisher *et al.*, 2005). In their study, they found that  
229 *potD* expression was decreased in an *rpoS* mutant but not in an *rpoN* mutant. Those  
230 previous observations and the data presented here demonstrate that *potABCD* is regulated  
231 in an RpoS-dependent, RpoN-independent manner.

232 It has been shown that *rpoS* expression can be modulated by BosR, a transcription  
233 factor that is activated by oxidative stress (Boylan *et al.*, 2003, Ouyang *et al.*, 2011). We  
234 found that inactivation of *bosR* had no effect on the induction of *potABCD* at low  
235 osmolarity. This result was expected since oxidative stress had no effect on *potABCD*  
236 gene expression (Fig. 4). Taken together, these data suggest that *potABCD* is RpoS-

237 dependent and BosR- and RpoN-independent. These results also demonstrate that various  
238 *in vivo* stresses can be regulated independently [e.g. oxidative stress via BosR (Boylan *et*  
239 *al.*, 2003, Hyde *et al.*, 2009) and acid stress via RpoS (Dulebohn *et al.*, 2017)] from the  
240 Rrp2/RpoN/RpoS cascade.

241

## 242 **Discussion**

243

244 Polyamines such as putrescine, spermidine, spermine and cadaverine are found in both  
245 prokaryotes and eukaryotes and are essential for normal cell growth, modulating the rate  
246 of transcription (Childs *et al.*, 2003), promoting efficient translation, altering and  
247 stabilizing the structure of RNA (Thomas *et al.*, 1995) and protecting the cell from  
248 deleterious effects of acid, oxidative and nitrosative stresses (Miller-Fleming *et al.*,  
249 2015). Typically, bacterial cells transport, synthesize and/or modify polyamines to  
250 maintain an intracellular concentration that is optimal to promote maximum function and  
251 these processes are coordinately regulated (Miller-Fleming *et al.*, 2015). Most bacteria  
252 maintain higher intercellular levels of spermidine than putrescine. *E. coli* is a notable  
253 exception in that higher levels of putrescine (22.1 nmol/10<sup>9</sup> cells) are detected compared  
254 to spermidine (3.8 nmol/10<sup>9</sup> cells) (Morris & Jorstad, 1970). The synthesis of putrescine  
255 and spermidine is accomplished by the decarboxylation of precursor amino acids such as  
256 ornithine (via ornithine decarboxylase, encoded by *speC*) or arginine (arginine  
257 decarboxylase, encoded by *speA*), while the synthesis of spermine or cadaverine are rare  
258 (Miller-Fleming *et al.*, 2015).

259 The genome of *B. burgdorferi* lacks the genes encoding proteins for the *de novo*  
260 synthesis of putrescine or spermidine and it has been proposed that *B. burgdorferi* is a  
261 spermidine auxotroph (Wyss & Ermert, 1996). However, it does harbor genes encoding a  
262 single putative putrescine/spermidine transport system, *potABCD*, suggesting that this  
263 putrescine/spermidine system may be responsible for polyamine utilization in *B.*  
264 *burgdorferi*. Recently, Lin *et al.* showed that high concentration of spermidine (4 mM) *in*  
265 *vitro* triggers the activation of the Rrp2/RpoN/RpoS cascade (Lin *et al.*, 2017),  
266 suggesting that this polyamine may affect the regulation of key virulence factors during  
267 the infectious cycle. During the infectious process, *B. burgdorferi* switches between an

268 arthropod vector and a mammalian host. To be successful during this transition, the  
269 bacteria needs to adapt to take advantage of the resources available, optimize growth,  
270 correctly alter gene expression and protect itself from deleterious environmental  
271 conditions. The purpose of this study is to determine the role of *potABCD* and  
272 polyamines in these progressions.

273 To determine the role of *potABCD* in *B. burgdorferi*, we initially attempted to generate  
274 a mutant by deleting the entire operon. However, multiple attempts to inactivate the  
275 putative polyamine transport system were unsuccessful. Therefore, an inducible  
276 *potABCD* (strain BBi-*potABCD*) was generated. Growth studies indicated that without  
277 inducer, cell division ceased, and the cells lost viability after 24 h, suggesting that  
278 *potABCD* was required for bacterial replication and survival (Fig. 1) *in vitro*. Compared  
279 to other bacteria which can synthesize and/or transport putrescine, spermidine or  
280 spermine, *B. burgdorferi* is quite metabolically limited. Uptake experiments indicate that  
281 the bacteria use the PotABCD transport system to specifically transport spermidine and  
282 suggests that this polyamine is an essential metabolite for *B. burgdorferi* (Fig. 2).

283 Since we could not introduce strain BBi-*potABCD* directly into ticks, we evaluated the  
284 expression patterns of *potABCD* in ticks colonized with strain B31-A3. In the tick,  
285 spirochetes up-regulated the *potABCD* operon during the early stage of feeding  
286 concomitant with the activation of RpoS-dependent genes (Fig. 3). Additionally, we  
287 demonstrated that the *potABCD* operon is a part of a small subset of genes that are RpoS-  
288 dependent, RpoN-independent (Fig. 5). This result agrees with previous observations by  
289 Fisher and colleagues (Fisher *et al.*, 2005). Our understanding of the regulation of this  
290 small set of genes by RpoS, independent of RpoN, is incomplete. More investigation will  
291 be required to evaluate the importance of this regulation beyond Rrp2/RpoN/RpoS  
292 regulation (Samuels, 2011).

293 Finally, we investigated the potential biological roles of polyamines in *B. burgdorferi*  
294 as well as the cues that trigger the activation of the *potABCD* in *B. burgdorferi*.  
295 Polyamines, such as putrescine or spermidine, are required for cell growth and, in some  
296 bacteria, for protecting cells from oxidative or osmotic stress (Altendorf *et al.*, 2009,  
297 Igarashi & Kashiwagi, 2010, Miller-Fleming *et al.*, 2015). First, we tested the ability of  
298 polyamines to protect *B. burgdorferi* in protecting the bacterium from the adverse effects

299 of osmolarity or oxidative stress (Fig. S1). Under the conditions tested, spermidine was  
300 not effective at expanding the survivability of cells from osmotic conditions outside the  
301 range of those which have been shown to exist in the tick midgut before, during or after  
302 feeding (250-600 mOsM) (Bontemps-Gallo *et al.*, 2016). Additionally, it did not increase  
303 the resistance of cells to ROS.

304 However, *potABCD* was up-regulated when the cells were exposed to low osmolarity  
305 but not when cells were exposed to ROS (Fig. 4). Also, *potABCD* was upregulated in the  
306 tick midgut during feeding, paralleling the drop in osmolarity that has been measured in  
307 ticks at the initial stages of feeding (Bontemps-Gallo *et al.*, 2016). While these data  
308 suggest a role for *potABCD* in the tick, we cannot assign a specific function for  
309 spermidine in *B. burgdorferi*. It is interesting to note that the increase in expression of  
310 *potABCD* occurs at a time during tick feeding which corresponds with maximum cell  
311 growth. Because of the complexity of the environment in the tick midgut, it seems likely  
312 that the conditions tested *in vitro* do not accurately reflect the multiple parameters  
313 (osmolarity, ROS, RNS) to which *B. burgdorferi* is exposed in the tick midgut. While  
314 spermidine is essential for *B. burgdorferi* survival and growth *in vitro*, its exact  
315 biochemical role remains to be determined.

316

## 317 **Experimental Procedures**

318

### 319 *Bacterial strains, media and growth conditions*

320 The bacterial strains used in this study are described in the Table 1. *B. burgdorferi* strains  
321 were grown in BSK-II medium (Barbour, 1984) at 34°C under microaerobic conditions  
322 (3-5% O<sub>2</sub>, 5% CO<sub>2</sub>, 90% N<sub>2</sub>). Cell densities were determined by dark-field microscopy.  
323 The osmolality of BSK-II medium is 450 mOsM. High and low-osmolality media, were  
324 generated as previously described (Bontemps-Gallo *et al.*, 2016). Every 24 h, an aliquot  
325 from each culture was examined by dark-field microscopy and plated on BSK-II. Plates  
326 were incubated at 34°C under a microaerobic atmosphere for 7-14 d to determine CFU.  
327 Spermidine and putrescine (Sigma-Aldrich, St. Louis, MO, USA) were added to BSK-II  
328 as indicated. Osmolarity of the growth media (mOsM) was measured with a vapor  
329 pressure osmometer (Model 3320, Advanced Instruments, Inc., Norwood, MA, USA).

330

331 *Construction of the BBi-potABCD (B. burgdorferi inducible – pot operon) strain*

332 Plasmids and primers designed for PCR are listed in Tables 1 and 2, respectively. A  
333 fragment containing *bb0643-flaBp-Kan-flacp-potA* was synthesized by Genscript USA  
334 and cloned into pUC57 and was named pBBi- *potABCD*. The plasmid was transformed  
335 into *B. burgdorferi* strain B31-68-LS (Chu *et al.*, 2016) as described previously (Samuels,  
336 1995) and kanamycin-resistant colonies were analyzed by PCR and sequenced to confirm  
337 the mutation (Table 2). The mutant strain was designated as BBi-*potABCD*.

338 Plasmid profile of the wild-type and the BBi-*potABCD* were performed by using the  
339 multiplex PCR system from Bergström laboratory (Bunikis *et al.*, 2011). Wild-type B31-  
340 68-LS was missing cp9 and lp5. BBi-*potABCD* was missing cp9, lp28-1 and lp5.

341

342 *Uptake of <sup>3</sup>H-Spermidine and <sup>3</sup>H-Putrescine*

343 Polyamine transport was determined using a previously described assay by Tilly *et al.*  
344 (Tilly *et al.*, 2004). Briefly, cells were grown in BSK-II to mid log phase (~5 x 10<sup>7</sup>  
345 cells/ml), harvested by centrifugation, washed three times with an equal volume of HN  
346 buffer (50 mM Hepes, 20 mM NaCl, pH 7.6) supplemented with 6 mM glucose and  
347 resuspended to a cell density of 6x10<sup>8</sup> cells/ml in HN buffer supplemented with 6 mM  
348 glucose. A 50 µl aliquot was reserved for protein quantification. Tritium labeled  
349 polyamine (5 µCi) was added to 1.0 ml cell suspensions and incubated at 34°C for the  
350 duration of the experiment. 100 µl aliquots were removed at each time point and applied  
351 to a 0.22 µm AcetatePlus filter (Osmonics, Inc., Minnetonka, MN) using a vacuum  
352 manifold and washed three times with 5 ml HN buffer before measuring scintillation  
353 counts. Spermidine [terminal methylene-<sup>3</sup>H] trihydrochloride (ART1749) and Putrescine  
354 [2,3-<sup>3</sup>H] dihydrochloride (ART0279) were purchased from American Radiolabeled  
355 Chemicals, Inc, (St. Louis, MO). Polyamine amounts (pmol) in samples were determined  
356 against a calibration performed with authentic labeled substrate. The protein  
357 concentrations were determined using the Pierce<sup>TM</sup> BCA Protein Assay with bovine  
358 serum albumin as a standard (ThermoFisher Scientific, Rockford, IL, USA).

359

360 *Tick rearing and feeding*

361 *I. scapularis* egg masses (Oklahoma State University) were allowed to hatch and mature  
362 in a controlled temperature, humidity and photoperiod environment. RML mice, an  
363 outbred strain of Swiss-Webster mice reared at the Rocky Mountain Laboratories  
364 breeding facility, were needle inoculated by intradermal injection with 100 µl of BSK-II  
365 containing  $1 \times 10^5$  *B. burgdorferi* B31-A3 and after three weeks, infection was confirmed  
366 by re-isolation of spirochetes from ear punch biopsies. Larval ticks were fed to repletion  
367 on infected mice, collected and allowed to molt into nymphs in a controlled environment.  
368 Nymphs were then fed on naïve RML mice, mechanically removed during the feeding  
369 and the scutal index was determined to standardize the feeding progression (Bontemps-  
370 Gallo *et al.*, 2016, Falco *et al.*, 1996).

371

#### 372 *RNA purification and RT-qPCR*

373 RNA samples were extracted from *B. burgdorferi* cultures using the RNeasy mini kit  
374 (Qiagen, Valencia, CA) according to the manufacturer's protocol. Three independent  
375 culture samples were used for each condition. RNA samples were also extracted from *B.*  
376 *burgdorferi* infected ticks during feeding. Ticks were frozen at  $-80^{\circ}\text{C}$ , crushed and  
377 resuspended in TRIzol (Life technologies, Carlsbad, CA) with chloroform. After  
378 centrifugation, the upper phase was mixed with 70% ethanol (1:1) and loaded onto  
379 RNeasy column (Qiagen) according to the manufacturer's protocol. The removal of  
380 contaminating DNA was accomplished using TURBO DNA-free DNase I (Life  
381 technologies, Carlsbad, CA). The cDNA was synthesized from the purified RNA using  
382 Superscript IV reverse transcriptase with random primers (Invitrogen, Carlsbad, CA). To  
383 compare gene expression, a relative quantification method was employed using *enoS* as a  
384 reference gene (Pfaffl, 2001, Bontemps-Gallo *et al.*, 2016). All samples were analyzed in  
385 triplicate on a Roche LightCycler 480 System (Indianapolis, IN) using *Power SYBR*<sup>®</sup>  
386 Green PCR Master Mix according to the manufacturer's instructions (Life technologies,  
387 Carlsbad, CA). The primers used for the qPCR are listed in Table 2.

388

#### 389 *ROS susceptibility and protection assays.*

390 Strains were grown in pyruvate-free BSK-II medium under microaerobic conditions at  
391  $34^{\circ}\text{C}$  to a cell density of  $2 \times 10^7$  cells/ml and cells were exposed to the different

392 concentrations of *tert*-Butyl hydroperoxide (Luperox<sup>®</sup> TBH70X, Sigma-Aldrich, St.  
393 Louis, MO, USA) for 3 h in pyruvate-free BSK-II. Samples were plated in BSK-II and  
394 incubated at 34°C under microaerobic conditions for 7-14 d to allow enumeration of  
395 CFU. When indicated, Spermidine (Sigma-Aldrich, St. Louis, MO, USA) was added to  
396 the BSK-II.

397

#### 398 **Ethics statement**

399 Mouse infection studies were carried out in accordance with the Animal Welfare Act  
400 (AWA1990) and followed the guidelines of the National Institutes of Health, Public  
401 Health Service Policy on Humane Care (PHS 2002) and Use of Laboratory Animals and  
402 the United States Institute of Laboratory Animal Resources, National Research Council,  
403 Guide for the Care and Use of Laboratory Animals. All animal work was done according  
404 to protocols approved by the Rocky Mountain Laboratories, NIAID, NIH Animal Care  
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408

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423

424 **Author Contributions**

425 S.B-G. designed and performed experiments, interpreted data and wrote manuscript.

426 K. L. and C. L. R. performed experiments and edited manuscript.

427 F. C. G. contributed to experimental design, data analysis, and wrote manuscript.

428

429 **Conflict of Interest**

430 The authors declare that they have no conflict of interest with the contents of this article.

431

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Table 1. Bacterial Strains and Plasmids used in this study

<b>Strains</b>	<b>Source</b>
<i>B. burgdorferi</i>	
B31-A3	(Elias <i>et al.</i> , 2002)
B31-A3 $\Delta$ <i>rpoN</i>	(Fisher <i>et al.</i> , 2005)
B31-A3 $\Delta$ <i>rpoS</i>	(Burtnick <i>et al.</i> , 2007)
B31-A3 $\Delta$ <i>bosR</i>	(Katona, 2015)
B31-68-LS	(Chu <i>et al.</i> , 2016)
B31-68-LS – BBi- <i>potABCD</i>	This study
<i>Escherichia coli</i>	
Top10F': <i>mcrA</i> , $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ), $\Phi$ 80 <i>lacZ</i> $\Delta$ M15, $\Delta$ <i>lacX74</i> , <i>recA1</i> , <i>araD139</i> , $\Delta$ ( <i>ara-leu</i> )7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> , <i>endA1</i> , <i>nupG</i>	Invitrogen
<b>Plasmids</b>	
pUC57	Genscript
pBBi- <i>potABCD</i>	This Study

740



741 Table 2. PCR primers used in this study.

PCR Primers	Sequence	Source
potAF	GCCATATGGATAATTGTATTATCCTAGAGATTAA	This study
potAR	CGGATCCTTATTCCTTATGCATAACATGAAT	This study
GTPF- <i>EcoRI</i>	ACTGCTGAATTCTCCACCTATTTTGGCTGCCA	This study
GTPR- <i>aatII</i>	ACTGCTGACGTCACATCCGGATGGGCCTAGTA	This study
QrpoSF	CTGGACAAAGAAATAGAGGGATCTG	This study
QrpoSR	CAAGGGTAATTTTCAGGGTTAAAAGAA	This study
QospCF	TGGTACTAAAATAAAGGTGCTGAAGAA	This study
QospCR	GCATCTCTTTAGCTGCTTTTGACA	This study
QpotAF	GCTGGTTGTAAGTTTGCTTGG	This study
QpotAR	ATCTTCTGGGCGTATTACAAGG	This study
QpotBF	AGATCTTGGAGCAAGAATGTG	This study
QpotBR	GTTTAGAGCCTCCTAGCAAATC	This study
QpotCF	CGGAAATCGCAGGAAGCATAG	This study
QpotCR	ATCCCTGTCCAGTGGTGAAA	This study
QpotDF	GCACAAAGCGCTATGCTAAA	This study
QpotDR	TGGAGCATCAATAGGAATTACA	This study
QnapAF	CCCTCAATGGAAAGCATTGTTTG	This study
QnapAR	GCATCCATAAATGTTTCTCAAGATCAC	This study
QcoADRF	AGCTGGGAATCATAACAGCATTTA	This study
QcoADRR	GTCCTGTTCTTGCAGCTTCT	This study
QenoSF	GTGCACACTCTGACAACTCT	This study
QenoSR	ACCTCTGCTGCCATTCTTATT	This study

742

743 *Figure Legends*

744 **Fig. 1.** The PotABCD uptake system is essential for *B. burgdorferi*.

745 Growth and survival curve of wild-type B31-68-LS and BBi-*potABCD* strains with or  
 746 without 500  $\mu$ M IPTG. Cells were enumerated by plating in BSK-II plating. Data  
 747 represent means  $\pm$  standard deviation of three independent experiments.

748

749 **Fig. 2.** Spermidine is the preferred polyamine transported by *B. burgdorferi*.

750 Curve of uptake of radioactive spermidine and putrescine by *B. burgdorferi* B31-A3  
 751 strain was measured over a 10-min time course. Cells were grown to mid-log phase,  
 752 washed and resuspended to a cell density of  $6 \times 10^8$  cells/ml in HN buffer supplemented  
 753 with 6 mM glucose. Tritium labelled polyamine (5  $\mu$ Ci) was added to the cell

754 suspensions and incubated at 34°C for the duration of the experiment. Polyamine  
755 amounts (pmol) in samples were determined against a calibration performed with  
756 authentic labeled substrate. The protein concentration was determined using a BCA  
757 Protein Assay with bovine serum albumin as a standard. Dotted lines represent the linear  
758 regression. Data represent means ± standard deviation of two independent experiments.

759

760 **Fig. 3.** Relative expression of *potABCD* operon genes during nymph feeding.

761 Graph of the expression of *rpoS* and *ospC* (as markers for the induction of the Rrp2-  
762 RpoN-RpoS pathway), and the four genes of the *potABCD* operon were analyzed by RT-  
763 qPCR. RNA was extracted from 3 pools of 5 ticks and cDNA was generated as described  
764 in the methods section. Scutal index was used to standardize feeding progress  
765 (Bontemps-Gallo *et al.*, 2016, Falco *et al.*, 1996). The scutal index is indicated under  
766 each bar. Relative gene expression was calculated using levels expression of *enoS* as  
767 reference. Data represent means ± standard deviation of three independent pools for each  
768 condition.

769

770 **Fig. 4.** Expression of *potABCD* operon under osmotic or oxidative stress.

771 (A) Graph of the expression analyses of the *potABCD* operon of *B. burgdorferi* grown in  
772 BSK-II at 250, 450 and 650 mOsM. (B) Graph of the expression analyses of the  
773 *potABCD* operon of *B. burgdorferi* grown in BSK-II after a treatment with 5 mM *tert*-  
774 butyl hydroperoxide compared to an untreated culture. The expression of *coADR*, *napA*  
775 (as marker of the induction of the oxidative stress response), and the four genes of  
776 *potABCD* operon were analyzed by RT-qPCR. Relative gene expression was calculated  
777 using levels expression of *enoS* as reference. Data represent means ± standard deviation  
778 of three independent experiments.

779

780 **Fig. 5.** Expression of *potABCD* operon in wild-type,  $\Delta rpoN$ ,  $\Delta rpoS$  and  $\Delta bosR$  mutants.

781 Graph of the expression analyses of the *potABCD* operon of *B. burgdorferi* grown in  
782 BSK-II at 250 and 450 mOsM. Relative gene expression was calculated using levels  
783 expression of *enoS* as reference. Data represent means ± standard deviation of three  
784 independent experiments.

785

786 *Supplemental Material*

787 **Fig. S1.** Spermidine is not an osmoprotective or oxidative protective molecule.

788 (A) Growth of wild-type B31-A3 strain at 150, 250, 450, 650, 850 mOsM with or without  
789 5 mM spermidine (SPD). An aliquot of each culture was examined daily by dark-field  
790 microscopy and plated on solid BSK-II. (B) Early stationary phase ( $5 \times 10^7$  cells/ml)  
791 cultures were incubated for 3 h with 0, 1, 5 or 10 mM of *tert*-butyl hydroperoxide (*t*-  
792 butyl) and plated on BSK-II. Plates were incubated at 34°C under microaerobic  
793 conditions for 7-14 d to allow enumeration of CFU. Untreated samples were standardized  
794 to 100%. Data represent means  $\pm$  standard deviation of three independent experiments.