Spermine and Spermidine Alter Gene Expression and Antigenic Profile of *Borrelia burgdorferi*

Ying-Han Lin, a Jesus A. Romo, a Trever C. Smith II, a Ann N. Reyes, a S. L. Rajasekhar Karna, a Christine L. Miller, a Tricia A. Van Laar, b Raghunandan Yendapally, c James P. Chambers, a J. Seshu a

South Texas Center for Emerging Infectious Diseases, Center of Excellence in Infection Genomics and Department of Biology, The University of Texas at San Antonio, San Antonio, Texas, USA a; Department of Biology, California State University Fresno, Fresno, California, USA b; Feik School of Pharmacy, University of The Incarnate Word, San Antonio, Texas, USA c

**ABSTRACT** *Borrelia burgdorferi*, the agent of Lyme disease, responds to numerous host-derived signals to alter adaptive capabilities during its enzootic cycle in an arthropod vector and mammalian host. Molecular mechanisms that enable *B. burgdorferi* to detect, channel, and respond to these signals have become an intense area of study for developing strategies to limit transmission/infection. Bioinformatic analysis of the borrelial genome revealed the presence of polyamine transport components (PotA, PotB, PotC, and PotD), while homologs for polyamine biosynthesis were conspicuously absent. Although *potABCD* is cotranscribed, the level of PotA was elevated under in vitro growth conditions mimicking unfed ticks compared to the level in fed ticks, while the levels of PotD were similar under the aforementioned conditions in *B. burgdorferi*. Among several polyamines and polyamine precursors, supplementation of spermine or spermidine in the borrelial growth medium induced synthesis of major regulators of gene expression in *B. burgdorferi*, such as RpoS and BosR, with a concomitant increase in proteins that contribute to colonization and survival of *B. burgdorferi* in the mammalian host. Short transcripts of *rpoS* were elevated in response to spermidine, which was correlated with increased protein levels of RpoS. Transcriptional analysis of *rpoZ* and *B. burgdorferi rel* (*relBbu*, *bb0198*) in the presence of spermidine revealed the interplay of multiple regulatory factors in *B. burgdorferi* gene expression. The effect of spermidine on the levels of select borrelial proteins was also influenced by serum factors. These studies suggest that multiple host-derived signals/nutrients and their transport systems contribute to *B. burgdorferi* adaptation during the vector and vertebrate host phases of infection.

**KEYWORDS** *Borrelia burgdorferi*, Lyme disease, polyamines

Lyme disease is the most prevalent arthropod-borne infectious disease in the United States, with an estimated 300,000 cases in 2013 according to the Centers for Disease Control and Prevention (CDC). The causative agent of Lyme disease is the spirochetal pathogen *Borrelia burgdorferi*, which is transmitted to humans and other vertebrate hosts following the bite of infected *Ixodes scapularis* ticks (1, 2). The ability of this spirochete to recognize and respond to environmental signals and nutrients present in the vertebrate host or the tick vector plays a crucial role in the survival, transmission, and colonization capability, leading to Lyme disease (3–5). Numerous studies have established the significance of temperature (6–8), pH (9), dissolved gases (10, 11), host-specific stressors (12), and select nutrients (13–15) that play a role in a concerted fashion for expression and synthesis of key borrelial determinants that enable spirochete survival and colonization in different hosts. However, additional host signals and
signal transduction mechanisms are yet to be defined in understanding the dynamics of colonization of the vector and vertebrate hosts by Lyme spirochetes.

Since *B. burgdorferi* has a limited genome and is incapable of synthesis of amino acids, fatty acids, nucleotides, and other critical biomolecules, many of these substances are acquired from the host (16, 17). Therefore, analysis of the mechanisms of transport of these key host-derived molecules will not only extend our understanding of host-specific adaptation of spirochetes in general but also provide novel strategies to subvert and/or interdict these survival processes in hosts, thus potentially leading to a reduction in the incidence of Lyme disease. Moreover, host-derived components that are abundant in select sites of colonization in different hosts could potentially contribute to tissue-specific modulation of borrelial physiology, resulting in favorable or unfavorable conditions for adaptation.

Among the many host-derived components, polyamines are organic bases that are prevalent in millimolar ranges in many mammalian tissues (18). Polyamines, such as spermine (Spm), spermidine (Spd), cadaverine (Cad), and putrescine (Put), are cationic, exhibiting a net positive charge ranging from +2 to +4, while polyamine precursors such as ornithine (Ort) and arginine (Arg) carry a charge of +1. Both the structure and charge of polyamines facilitate their interaction with DNA, RNA, proteins, and phospholipids, thereby affecting a wide variety of biological functions such as increasing resistance to oxidative stress and regulating transcription/translation, among others (19–21). Moreover, polyamines are known to alter bacterial responses to antibiotics via structure/charge alterations as well as the functional efficacy of antimicrobial agents (22–24). There is a renewed interest in polyamine biogenesis and the role that polyamines play in regulating adaptive gene expression in many pathogenic bacteria and their ability to confer bacterial cell protection against oxidative and other host-derived physiological stressors. In *Escherichia coli*, polyamines increase the levels of RpoS (σ38 subunit of the RNA polymerase), leading to increased levels of the glutamate decarboxylase-dependent acid response system (25, 26).

Bioinformatic analysis of the *B. burgdorferi* genome revealed the presence of homologs that could be involved in polyamine transport, but no apparent homologs were found for biogenesis of polyamines, suggesting that *B. burgdorferi* acquires polyamines from the host (16). Moreover, a few enzymes involved in the conversion of precursors of polyamines such as arginine and ornithine are also apparent in the borrelial genome. While many bacteria have more than one transport system for polyamine transport, the absence of multiple polyamine binding and transport homologs suggested the presence of one polyamine transport (PotABCD) system within the genome of *B. burgdorferi* (16). Although recent transcriptional analyses have revealed altered levels of members of the polyamine transport system, the contribution(s) of polyamines in the pathogenesis of *B. burgdorferi* is yet to be uncovered (13).

In this study, we determined that the members of the polyamine transport system are cotranscribed and that supplementation of Spm and Spd drastically altered the *B. burgdorferi* protein profile, especially with respect to the key proteins critical for colonization in the vertebrate host. Regulators of gene expression in *B. burgdorferi* such as RpoS and BosR were elevated in response to increased levels of Spm and Spd. Effects of these polyamines on transcription of rpoZ and the bifunctional synthetase/hydrolase homolog *bb0198* (*B. burgdorferi* rel [relBbu]) suggest that the polyamines impact key borrelial regulatory proteins. Characterizing the role of polyamines in regulating multiple components of *B. burgdorferi* will pave the way for identifying new and novel strategies to reduce borrelial burden in the vector/vertebrate hosts, leading to a lowered incidence of Lyme disease.

**RESULTS**

Polyamine transport system of *B. burgdorferi*. Bioinformatic analysis of the *B. burgdorferi* genome revealed the presence of a transport system for polyamines and no apparent homologs associated with biogenesis of polyamines (16). Since *B. burgdorferi* has limited metabolic capabilities and is intimately associated with its hosts, analysis of
the key components of the polyamine transport system should afford a greater understanding of the adaptive capabilities of this spirochetal pathogen. Based on the organization of the polyamine transport system in other bacteria (19), *B. burgdorferi* has homologs of polyamine binding protein (PotD, *bb0639*), two channel-forming transmembrane proteins (PotC, *bb0640*, and PotB, *bb0641*) and a membrane-associated ATPase (PotA, *bb0642*) (Fig. 1A). The *potABCD* open reading frames (ORFs) are located adjacent to each other in the borrelial genome (Fig. 1B). Analysis of cDNA generated from total RNA using primers specific to *potA* and *potB* (primer sets 1 and 2), *potB* and *potC* (primer sets 3 and 4), and *potC* and *potD* (primer sets 5 and 6) revealed the members of the *potABCD* system in *B. burgdorferi* to be transcriptionally linked (Fig. 1C). Amplicon sizes were similar using cDNA (Fig. 1C, lane 3) and total genomic DNA (Fig. 1C, lane 4) as the templates. No amplification was observed using total RNA (Fig. 1C, lane 2) or PCR mix without template (Fig. 1C, lane 1), indicating no DNA contamination in the RNA sample used for generating cDNA. These observations suggested that the members of the polyamine transport system are transcriptionally linked in *B. burgdorferi*.

### Levels of PotA and PotD in *B. burgdorferi*

Immunoblot analysis of total lysates from *B. burgdorferi* grown under *in vitro* conditions mimicking the tick midgut before a blood meal (Fig. 2A, lane 1) (pH 7.6 at 32°C) compared to that after a blood meal (Fig. 2A, lane 2) (pH 6.8 at 37°C) was carried out using antiserum specific to PotA or PotD. Increased levels of PotA were observed in *B. burgdorferi* propagated in the tick under unfed-midgut conditions compared to levels under fed-midgut conditions (Fig. 2B, α-PotA). However, levels of PotD were similar in *B. burgdorferi* under both *in vitro* growth conditions (Fig. 2B, α-PotD). These findings suggested that the increased levels of PotA under conditions mimicking unfed ticks might reflect the kinetics of polyamine transport during the vector phase of borrelial infection (Fig. 2).

### Localization of PotD

The location of PotD, the substrate binding protein in the polyamine transport system, is critical for its role in responding to extracellular levels of...
different polyamines (18). In order to determine if PotD is localized to the outer surface, we performed proteinase K (PK) sensitivity assays as described previously (27). Immunoblot analysis revealed that PotD levels were similar in *B. burgdorferi* spirochetes with or without treatment with proteinase K (Fig. 3B, +PK α-PotD). As expected, there was no difference between treated and untreated samples in the levels of PotA due to its predicted localization in the inner membrane. The levels of another periplasmic protein, FlaB, were also similar in treated and untreated spirochetes while P66, an outer membrane protein, exhibited a reduction in size only in proteinase K-treated samples (Fig. 3B, +PK α-FlaB and +PK α-P66). Coomassie blue staining of the lysates from *B. burgdorferi* (Fig. 3A) before and after treatment with proteinase K exhibited similar

**FIG 2** Levels of PotA and PotD in *B. burgdorferi*. (A) Protein profile for *B. burgdorferi* grown under conditions mimicking the tick vector before (lane 1; pH 7.6 and 23°C) and after (lane 2; pH 6.8 and 37°C) a blood meal. (B) Immunoblot analysis was performed using anti-DbpA, anti-PotA, anti-PotD, and anti-FlaB antibodies, and blots were developed using sheep anti-mouse HRP-conjugated antibodies and an enhanced chemiluminescence system. Lane M, molecular mass marker (indicated in kilodaltons on the left).

**FIG 3** PotD is a periplasmic protein of *B. burgdorferi*. Proteinase K-treated (+PK) intact *B. burgdorferi* spirochetes or untreated (−PK) spirochetes were subjected to immunoblot analysis. (A) Coomassie blue-stained 12.5% SDS-PAGE gel of *B. burgdorferi* depicting the protein profile before and after PK treatment. (B) Immunoblot analysis using monoclonal antibody against FlaB or monospecific serum against PotD, PotA, and P66. Molecular weight (MW) markers (in thousands) are indicated to the left.
protein loading levels for each sample. This analysis revealed that PotD is not a surface-exposed protein in Lyme spirochetes.

**Effects of polyamines on the levels of key borrelial proteins.** We determined the effects of various polyamines on *B. burgdorferi* by supplementing the borrelial growth medium (Barbour-Stoenner-Kelly II [BSK-II]) with various concentrations of polyamines or polyamine precursors. We found that a 4 mM concentration of various polyamines, except Spm, did not significantly alter growth of *B. burgdorferi* at pH 7.6 and 32°C (data not shown). We then analyzed the levels of key borrelial proteins that reflect the physiological status of *B. burgdorferi* in the presence of a 4 mM concentration of the indicated polyamines (Fig. 4). Immunoblot analysis revealed that the levels of outer surface protein C (OspC) were elevated in *B. burgdorferi* propagated in the presence of Spd and Spm compared to growth in conventional BSK-II medium with no polyamine supplementation (control) or in the presence of Arg, Cad, Ort, and Put. Coincidentally, the levels of RpoS, a key regulator of the gene expression for the adaptation of *B. burgdorferi* to the mammalian host (3), were significantly elevated in Spm-supplemented samples and to a lesser extent in Spd-treated samples. The levels of RpoS were minimal in the control lysates and from lysates of *B. burgdorferi* grown in the presence of Arg, Cad, Ort, and Put. While the levels of PotA were elevated in *B. burgdorferi* grown in the presence of Cad, Ort, and Put, there was a marginal reduction in the levels of PotA in the Spd-, Spm-, and Arg-treated samples compared to levels in the control lysate. One possible explanation for this observation is that increased levels of PotA in the presence of Cad, Ort, and Put could be due to a competitive reduction in transport of other polyamines, such as Spd or Spm, that are known to reduce the levels of PotA although we have not measured the ATPase activity of PotA in the presence of various polyamines. The levels of Rho were similar in all borrelial lysates, while levels of RelBbu exhibited a modest reduction in *B. burgdorferi* supplemented with Arg and were relatively high in the presence of Spd compared to the level in the untreated sample. These observations indicate that...
increased levels of Spd and Spm altered the levels of a major regulator of gene expression, RpoS, and a key pathogenesis-associated protein, OspC, in *B. burgdorferi*. Therefore, we focused on determining the effects of these two polyamines to further assess their contributions toward modulating host adaptation of *B. burgdorferi* to the mammalian host.

**Effect of spermidine and spermine on expression of pathogenesis-related proteins of *B. burgdorferi***. Compared to lysates from *B. burgdorferi* grown in BSK-II medium at pH 7.6 and 32°C (Fig. 5, lane 3), there were increased levels of RpoS and OspC in lysates of *B. burgdorferi* grown in BSK-II medium supplemented with 4 mM Spd (Fig. 5B, lane 4) or Spm (Fig. 5B, lane 5). Moreover, the levels of other *rpoS*-regulated proteins, such as DbpA and BBK32 (28), were also elevated in the presence of Spd and Spm (Fig. 5C, α-DbpA and α-BBK32). The increase in RpoS and OspC in the presence of Spd or Spm was similar to that when *B. burgdorferi* was grown under conditions mimicking the midgut of fed ticks (Fig. 5, lane 1) (pH 6.8 and 37°C) compared to the level in unfed ticks (Fig. 5, lane 2) (pH 7.6 and 23°C). No significant differences were observed in the levels of FlaB, P66, and NapA in lysates of *B. burgdorferi* with or without Spd or Spm (Fig. 5C). These observations suggest that increasing levels of select polyamines, notably Spd and Spm, result in increased levels of proteins involved in the adaptation of *B. burgdorferi* to the mammalian host. Among all polyamines, Spm consistently induced higher levels of RpoS and other pathogenesis-related proteins, supporting its significance in contributing to the adaptive capabilities of *B. burgdorferi*. **Effect of polyamine transport inhibitors on *B. burgdorferi***. We determined the effect of a hydrophobic 1, 3-dicyclohexylcarbodi-imide (DCC) and a polar 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide (EDC) inhibitor of polyamine transport that has been shown to be effective with eukaryotic cells for their inhibitory effects on spirochetes (29). *B. burgdorferi* was grown in the presence of 4 mM Spm and 5 mM EDC or 5 mM DCC, and it was observed that the levels of PotA, RpoS, BosR, DbpA, and OspC were not altered in the presence of either polyamine transport inhibitor (Fig. 6B, lanes 3 and 4). However, consistent with previous observations in *B. burgdorferi* (Fig. 4 and 5), the levels of RpoS and other *rpoS*-regulated gene products, with the exception of PotA, were elevated following growth in Spm-supplemented medium (Fig. 6B, lane 2) compared to the level of the control (Fig. 6B, lane 1). Total protein staining of SDS-PAGE gels on protein samples from different lysates indicated (i) that loading was comparable and (ii) that the increased levels of OspC are apparent in spermine-treated samples (Fig. 6A).
Growth rates of *B. burgdorferi* in the presence of Spm and Spd. Growth rates of *B. burgdorferi* strain B31-A3 in the presence of 4 mM Spm and Spd were determined over 168 and 120 h, respectively, at pH 7.6 and 32°C until untreated control samples reached a density between $3 \times 10^{10}$ and $4 \times 10^{11}$ cells per ml. As shown in Fig. 7, a significant reduction in the *B. burgdorferi* growth was observed in the presence of Spm (Fig. 7A) at 168 h. There was no significant difference between growth rates in the

![Graph](A)

![Graph](B)

**FIG 6** Effects of polyamine transport inhibitors on *B. burgdorferi*. *B. burgdorferi* grown under laboratory growth conditions (lane 1, pH 7.6 and 32°C) and in the presence of 4 mM Spm (lane 2) was treated with 5 mM EDC (lane 3) or 5 mM DCC (lane 4). (A) Total cell lysates were separated on a 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue. (B) Immunoblot analysis with serum generated against PotA, RpoS, BosR, DbpA, and OspC. Molecular weight (MW) markers (in thousands) are indicated to the left.

**FIG 7** Growth rates of *B. burgdorferi* bacteria in the presence of spermidine or spermine. *B. burgdorferi* was grown in triplicate (1 x 10^7 spirochetes/ml as the starting density) in the absence or presence of 4 mM spermine or spermidine at 32°C and pH 7.6. Growth was monitored every 24 h for 168 (Spm) or 120 (Spd) h by examining the motility of spirochetes as a measure of viability using dark-field microscopy. Results of a representative experiment are shown. *, $P < 0.05$; ***, $P < 0.001$. 

Polyamines and *Borrelia burgdorferi* Infection and Immunity March 2017 Volume 85 Issue 3 e00684-16 iai.asm.org

Downloaded from http://iai.asm.org on October 24, 2017 by guest
presence or absence of Spd up to 120 h, with both the treated and untreated spirochetes reaching a density of greater than $3 \times 10^8$ spirochetes per ml during this period (Fig. 7B). It should be noted that the motilities of the spirochetes were comparable, indicating viability in both supplemented and control media, although the rate of growth over a period of time in the presence of Spm was significantly lower than that in control medium. While no significant difference in the growth rates of \textit{B. burgdorferi} at pH 7.6 and 23°C (conditions mimicking unfed ticks) was observed in the presence or absence of 4 mM Spd (see Fig. S1 in the supplemental material), significant differences in growth rates were observed in the presence of Spd when cultures were grown at pH 6.8 and 37°C (conditions mimicking fed ticks) (Fig. S2). These studies indicate that the effects of polyamines overlap other environmental signals encountered during the vector or mammalian phase of borrelial infection.

\textbf{Effects of spermidine and spermine on transcriptional rates of select borrelial determinants.} Transcriptional analysis of cDNA generated from total \textit{B. burgdorferi} RNA grown in the presence of Spd or Spm showed that ospC levels were significantly higher than the level of the control sample (Fig. 8). This was consistent with the increased levels of OspC observed in samples under these conditions by immunoblot analysis (Fig. 4). We also saw higher levels of rpoS with Spm treatment while levels of flaB were lower than the control level (Fig. 8). The transcriptional levels of rpoS were lower in the Spd-treated samples than in the untreated controls and were determined using primers specific to the long \textit{rpoS} transcript (Table 1). Additional transcriptional analysis using primers specific to the short \textit{rpoS} transcript (as shown in Fig. 9) was consistent with the increased RpoS levels in the Spd-treated samples as noted in Fig. 4 and S. \textit{ospA} levels were decreased with both Spd and Spm treatments (Fig. 8). These observations suggest that select polyamines can mediate changes in \textit{B. burgdorferi} at the transcriptional level by potentially stabilizing select transcripts, with minimal effects on their induction.

\textbf{Effects of cell density and polyamines on transcriptional levels in \textit{B. burgdorferi}.} Since there is an impact of the density of the spirochetes on borrelial gene expression, we determined the transcriptional levels of select borrelial determinants at low and high densities in the presence or absence of Spd. We focused on effects of Spd supplementation to discern whether there are other factors that contribute to the level of \textit{rpoS}, which was relatively low in the Spd-treated sample, and also to determine if there are differences in the long or short transcripts of \textit{rpoS} induced in response to Spd (30). As shown in Fig. 9A, when the medium was supplemented with Spd, the levels of \textit{rpoS} were significantly decreased in spirochetes grown to low density ($1 \times 10^7$ to $2 \times 10^8$)
completely to levels from spirochetes grown to high density (1 × 10^8 to 2 × 10^8 cells/ml). The levels of ospC were also lower when Spd-treated cells were harvested at low density, while there was a significant increase in ospC expression when cells were grown to high density with Spd addition. These transcriptional analyses underscore the importance of the role of polyamines and other growth-dependent conditions, such as cell density, in gene expression in *B. burgdorferi*. Additionally, we determined the levels of short and long rpoS transcripts induced in response to different environmental cues (30). As shown in Fig. 9B, the short rpoS transcript was upregulated in Spd-treated samples, while the long transcript was significantly down-regulated. In combination with density-dependent signals, these data reflect the role of RpoS in Spd-treated spirochetes. Both rpoZ and relBbu (bb0198) have been implicated in the metabolic response observed when spirochetes are shifted from minimal growth medium to nutritionally replete conditions that reflect the conditions in the midgut of tick before and after a blood meal (14, 15). The transcript levels of relBbu were significantly elevated in *B. burgdorferi* grown to low density in the presence of Spd, while relBbu was downregulated in the Spd-treated samples grown to high density (Fig. 9C). Levels of rpoZ were similar in treated and untreated low-density samples, while the addition of Spd led to downregulation of rpoZ when cells were grown to high density (Fig. 9C). These observations indicate that the overall effects of polyamines on borrelial gene expression could also be influenced by other environmental cues, and they open new avenues for regulating the adaptive responses of *B. burgdorferi* in response to host-specific signals.

**Effect of polyamines in the presence of serum.** It has previously been shown that Gram-negative bacteria such as *Neisseria gonorrhoeae* withstand the effects of cationic antimicrobial peptides and complement-mediated killing in the presence of polyamines (31). As shown in Fig. 10B, *B. burgdorferi* grown in medium supplemented with heat-inactivated rabbit serum ([IIS] 6%, vol/vol) plus 4 mM Spd had increased levels of OspC (Fig. 10B, IIS + Spd) compared to levels in spirochetes grown in the presence of

<table>
<thead>
<tr>
<th>Primer (restriction enzyme)</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>recAFq ATGCTTGATCCTGTTTATGCAA</td>
<td></td>
</tr>
<tr>
<td>recARq GGATCAGGCTAGTAAAGGCAA</td>
<td></td>
</tr>
<tr>
<td>flaBFq CAGCTAATGGCAATCTTCTCTT</td>
<td></td>
</tr>
<tr>
<td>flaBRq TTCTGTGAAACACCCCTTCTA</td>
<td></td>
</tr>
<tr>
<td>rpoSFq AGATATGCGGGTAAAGGGTTAAAA</td>
<td></td>
</tr>
<tr>
<td>rpoSRq CAGCAGCTCTTATTAATCCCAAGTT</td>
<td></td>
</tr>
<tr>
<td>ospCFq AATCAGTAGAGGTCTTGTCAAAAGCA</td>
<td></td>
</tr>
<tr>
<td>ospCRq CCACAACAGGGCTTGTAAGCATAGAA</td>
<td></td>
</tr>
<tr>
<td>ospAFq CCAAAAGAAGATCAACAACAGAAGAA</td>
<td></td>
</tr>
<tr>
<td>ospARq GGTTCCGCTGCTTGTATTATTT</td>
<td></td>
</tr>
<tr>
<td>PotA-B_int_F TGTATACGCCAGAGATAG</td>
<td></td>
</tr>
<tr>
<td>PotA-B_int_R TTTCATCCAGGCAATAAGT</td>
<td></td>
</tr>
<tr>
<td>PotB_C_int_F TGAAGCATCAACAAGATCTTG</td>
<td></td>
</tr>
<tr>
<td>PotB_C_int_R AGATTATGCTATTAAATATG</td>
<td></td>
</tr>
<tr>
<td>PotC_D_int_F ATCCGAAAATCGAGGAAG</td>
<td></td>
</tr>
<tr>
<td>PotC_D_int_R GCCCTCATTGAATATCTT</td>
<td></td>
</tr>
<tr>
<td>BB0639F (Ndel) ACGCATATGAAAATATTTATATATTAG</td>
<td></td>
</tr>
<tr>
<td>BB0639R (Xhol) ACGCCCTAGAGAAACAGAGAATTAATATTT</td>
<td></td>
</tr>
<tr>
<td>BB0642F (Nhel) TGCCCGCTAGGCCATAATTGATCTCAG</td>
<td></td>
</tr>
<tr>
<td>BB0642R (Xhol) TGGCGCCTGAGTCTCTTGTGACATAACATCATAT</td>
<td></td>
</tr>
<tr>
<td>BB0198 qRT F TGTATACCCCCAGAAGAGGAAG</td>
<td></td>
</tr>
<tr>
<td>BB0198 qRT Rv GCATAAAGAGCTTGATCCTCAATATATCT</td>
<td></td>
</tr>
<tr>
<td>RpoZ q RT Fw GCCGACTGAGAAAAATTATAGAG</td>
<td></td>
</tr>
<tr>
<td>RpoZ q RT Rv CGTGATCCTAAAAGCTGACCCTAAATAT</td>
<td></td>
</tr>
<tr>
<td>rpoS-short F AGATATGCGGGTAAAGGTTAAAA</td>
<td></td>
</tr>
<tr>
<td>rpoS-short R CACGACGCTCTATTAAACCAAGGT</td>
<td></td>
</tr>
<tr>
<td>rpoS-long F AAATACCCCTCCTAATAACTCCTCAAA</td>
<td></td>
</tr>
<tr>
<td>rpoS-long R TTCCATGCAAACACTGTGC</td>
<td></td>
</tr>
</tbody>
</table>

Restriction sites are underlined.
normal rabbit serum (NS 6%, vol/vol) (Fig. 10B, NS + Spd). Levels of OspC in the absence of Spd were comparable in B. burgdorferi bacteria grown in either IS (Fig. 10B, IS − Spd) or NS (Fig. 10B, NS − Spd). Immunoblot analysis also revealed increased levels of DbpA in lysates from B. burgdorferi grown with IS and Spd compared to levels in bacteria grown with NS and Spd. The levels of OspA and oligopeptide permease A1 (OppA1) were similar in borrelial cultures grown with IS or NS with or without Spd. These observations indicate that in vitro growth of B. burgdorferi in medium supplemented with IS and Spd leads to higher levels of OspC and DbpA than growth with NS and Spd, suggesting that the presence of heat-labile components in the serum potentially limits the effect of Spd. Although lipoproteins involved in the pathogenesis of B. burgdorferi, such as OspC and DbpA, are elevated in the presence of IS and Spd, the levels of another key borrelial lipoprotein, OspA, remained similar in the presence of either IS or NS plus Spd. The levels of OppA1 were similar in all samples examined, serving as a control reflecting the physiological status of the spirochetes grown in medium supplemented with IS or NS with Spd.

**DISCUSSION**

*B. burgdorferi* has limited metabolic capabilities, which imposes several limitations on its growth, metabolism, and virulence (3). The spirochetes, however, utilize a variety of transporters to acquire many host-derived components to overcome their metabolic insufficiency and achieve successful colonization in different compartments of the tick vector or vertebrate hosts (17). Thus, investigating the role played by key host-derived components in borrelial physiology will provide new avenues to alter the uptake of these biomolecules, limiting the survival and virulence potential. Among the multitude of host-derived molecules, polycations, polyamines, inorganic cations such as magnesium and calcium, and metals (among others) are known to contribute to functional conformations of nucleic acids, facilitating DNA replication, transcription, and translation (32). Notably, polyamines with a uniform distribution of positive charge serve as...
electrostatic bridges between the negatively charged phosphate backbone of nucleic acids and other biologically relevant, negatively charged polymers affecting multiple cellular processes (22). Several studies have shown that biogenesis and degradation of or acquisition of polyamines via active transport from the host modulate multiple biological processes such as nucleic acid and protein synthesis as well as cell growth, protection against oxidative stressors, and virulence (22, 33). However, the role of polyamines in regulating the adaptive response of the agent of Lyme disease, a host-adapted pathogen lacking multiple metabolic pathways, has remained unexplored.

Bioinformatic analysis of the borrelial genome revealed the presence of an ATP binding cassette (ABC) transport system comprised of a substrate binding protein (PotD), two channel-forming transmembrane proteins (PotB and PotC), and a membrane-associated ATPase (PotA) (Fig. 1A). The genes encoding these proteins are adjacent to each other in the borrelial chromosome and are cotranscribed, suggesting the possibility that the transport system is regulated in response to the availability of the binding substrate/cargo (Fig. 1B and C). The absence of apparent homologs of a polyamine biosynthetic pathway suggests that the levels of transport of polyamines in different microenvironments could potentially influence the adaptive capabilities of B. burgdorferi. Moreover, B. burgdorferi is exposed to variations in many signals and nutrients in different microenvironments, notably in the mammalian hosts and during transmission from a tick vector to the vertebrate host and vice versa.

Polyamine transport and utilization could therefore serve to enhance the survival of the spirochetes by altering a variety of physiological responses pertinent to the host-specific conditions. For example, we noted that B. burgdorferi bacteria propagated under in vitro growth conditions mimicking the tick midgut before a blood meal display higher levels of PotA, reflecting differences in the levels of polyamine transport under temperature/pH parameters mimicking the different phases of the infectious cycle of B. burgdorferi. It is possible that in B. burgdorferi increased levels of PotA, which exists as...
a membrane-permeant weak base when the pH of the medium is basic (pH 7.6 and 23°C), coincide with increased polyamine transport. Furthermore, the borrelial PotA homolog has several conserved residues that have been shown to play a role in ATPase nucleotide binding and Spd uptake in *E. coli*. These include Walker motif A (G<sup>40</sup>PSGC<sup>44</sup>GKT) and Walker motif B (L<sup>158</sup>LLLLD<sup>162</sup>E), with residues C<sup>44</sup> and D<sup>162</sup> potentially involved in ATPase activity (34). As shown in Fig. 4, the levels of PotA were lower when *B. burgdorferi* was propagated with increased Spd than in untreated spirochetes, suggesting that the Spd levels modulate levels and presumably the ATPase activity of PotA. The changes were similar to those observed for *E. coli*, in which the ATPase activity of PotA was shown to be lower with increasing concentrations of Spd, although we have not measured the ATPase activity of the borrelial PotA homolog (34). Even though the levels of polyamines are higher in the blood meal, the levels of PotA (ATPase energizing the transport system) are elevated in response to a lower concentration/availability of polyamines, suggesting that PotA levels reflect the need for increased transport. Recently, it was observed that the transcriptional levels of members of the polyamine transport system are elevated in *B. burgdorferi* during the tick phase of infection compared to the levels in mammalian host-adapted spirochetes (13). Moreover, as shown in Fig. 4, increased levels of Spd (4 mM) resulted in reduced levels of PotA, suggesting that the availability/concentration of polyamines under different host-mimicking environments modulates the levels of PotA.

The changes in the levels of PotD were not as dramatic as those of PotA in *B. burgdorferi* propagated under unfed- or fed-tick conditions (Fig. 2B, α-PotD). We did observe lower levels of PotD with increased levels of Spd and Spm (Fig. 4B, α-PotD), with no detectable changes in levels of PotD with elevated levels of other polyamines. Although the borrelial PotD homolog possesses S and D residues at positions 226 and 29, respectively, similar to the homolog in *E. coli*, it has been shown that these residues are not critical for Put binding. Moreover, there is limited sequence conservation in the region of PotD known to facilitate binding of Put in *E. coli* (34). It is possible that the borrelial PotD homolog binds to Put with less affinity than the binding of Spd or Spm, leading to changes in the levels of several borrelial determinants (Fig. 4). Since pot<sub>ABCD</sub> is cotranscribed, it is possible that higher concentrations of Spd could regulate the levels of transcription of pot<sub>ABCD</sub>, as previously demonstrated in *E. coli*. Although we have not directly evaluated the binding constants of various polyamines with PotD, of the polyamines or polyamine precursors, the addition of exogenous Spd and Spm exhibited the most dramatic effect on the borrelial proteins (Fig. 4B). Put and Spd are the two polyamines abundant in prokaryotes, and although the presence of Spm within bacterial cells is not well established, the concentrations of these proteins in *E. coli* are reported to be in the order of Put > Spd > Spm (18, 19). Second, Put and Spd are transported primarily via the PotABCD transport system in prokaryotes. Spm, on the other hand, is abundantly present in eukaryotic cells and is least prevalent within *E. coli*. The effects of Put on the gene/protein expression profiles of *B. burgdorferi* were minimal, while Spd and Spm had the greatest effects (Fig. 4). We therefore focused on Spd as it is the more physiologically relevant polyamine within the context of the PotABCD transport system. In all experiments that we have performed thus far, Spd is able to induce increased levels of RpoS and the rpoS-regulated genes (Fig. 4). Although the effect of Spm is more pronounced in terms of the levels of key determinants of *B. burgdorferi*, we rationalized that Spd is the most physiologically relevant polyamine to interrogate the polyamine transport system in *B. burgdorferi*. The increase in the levels of OspC and RpoS with addition of Spd and Spm was similar to the effects of pH and temperature mimicking the fed-tick midgut (Fig. 5B, lane 1) (pH 6.8 and 37°C). Importantly, it should be pointed out that the levels of polyamines present in the conventional BSK-II growth medium containing 6% normal rabbit serum were not sufficient to alter the levels of key borrelial proteins at pH 7.6 and 32°C, while supplementation with Spd and Spm resulted in increased levels of the aforementioned proteins. In summary, polyamines alter the levels of borrelial proteins in different microenvironments, supporting the metabolic needs of the spirochetes during different stages of their life cycle.
The effect of polyamines in response to growth medium pH has been studied extensively in *E. coli* (20, 25, 26, 35). When pH is low, a reduction in the entry of exogenous amines and an increase in the production of membrane-permeant amines via arginine/ornithine/lysine decarboxylases result in the generation and efflux of Put and Cad to neutralize the acidity in the external environment. This mechanism has been attributed to survival of enteric pathogens during their transit through the stomach. However, at high pH, deprotonated polyamines transit the cell membrane as weak bases, resulting in enhanced translation of RpoS, a critical sigma factor contributing to survival during stationary phase in *E. coli* (36–38). Although there are significant pathogen-specific differences in the roles of RpoS in *E. coli* and *B. burgdorferi* in the regulation of gene expression, increased levels of polyamines resulted in elevated levels of RpoS and *rpoS*-regulated proteins in *B. burgdorferi*. One possible mechanism for this regulation is the acid stress response in *B. burgdorferi*, although the borrelial genome does not contain a variety of genes contributing to polyamine-dependent modulation of the acid stress response as observed in *E. coli* (26). The specific ability of Spd and Spm to induce increased levels of RpoS and *rpoS*-regulated genes is indicative of the contributions of polyamines in modulating gene expression in *B. burgdorferi* in response to host-derived signals.

Since supplementation of Spd or Spm induced levels of RpoS and *rpoS*-dependent/independent pathogenesis-related proteins of *B. burgdorferi*, we determined if addition of hydrophobic 1, 3-dicyclohexylcarbodi-imide (DCC) or polar 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC), both of which are known polyamine transport inhibitors, alters the effects of 4 mM Spm on the borrelial protein profile. Both of these carbodi-imides readily interact with carboxy groups of aspartate or glutamate residues that stabilize carrier-substrate interactions during polyamine transport (29). We did not observe any major alterations in the levels of the RpoS, OspC, DbpA, and BosR proteins following addition of either DCC or EDC to the growth medium with Spm (Fig. 6). Consistent with previous observations, addition of Spm induced increased levels of the above proteins compared to the level in the untreated control. The levels of PotA were higher in the control samples but lower in the presence of Spm, independent of addition of DCC or EDC. It is possible that the lack of an effect in using these compounds could be due to the inaccessibility of carboxy groups of residues involved in stabilizing the substrates of the polyamine transport system in *B. burgdorferi*. However, nonmetabolizable polyamine analogs offer a rational strategy to limit *B. burgdorferi* survival in infected reservoir hosts and thereby limit the transmission of the spirochetes in nature.

Lower levels of the long form of *rpoS* were observed in *B. burgdorferi* exposed to Spd than in untreated or Spm-treated spirochetes, although the mRNA levels for *ospC* were significantly higher following treatment with both polyamines (Fig. 8). The short *rpoS* transcript, however, was significantly higher in the Spd-treated spirochetes, indicating differences in the transcriptional levels of *rpoS* (Fig. 9B). Significant differences were also noted in the levels of *rpoS* transcripts in spirochetes grown to low or high densities in the presence of polyamines. Interestingly, an increase in transcript levels specific to *ospA* was observed at low density, while both *rpoS* and *ospC* levels were higher in cultures grown to higher density in the presence of Spd. We used primers specific to the short transcript of *rpoS* and determined that the levels of short *rpoS* transcripts were higher in the Spd-treated samples than in the untreated samples, as shown in Fig. 9B. Although we did not check the stability of the long *rpoS* transcript, the levels of the short transcripts of *rpoS* are reflected in increased translational levels of RpoS. These observations are consistent with previous reports in the field (30). The growth rates of spirochetes in the presence of 4 mM Spm and Spd also reflected the differences noted in the levels of RpoS and OspC, with Spm-treated spirochetes showing a significantly lower cell density after 96 h (Fig. 7). These observations indicate that the effects of polyamines on borrelial gene expression/growth rates are affected by additional culture conditions, reflecting the complex regulatory nature of gene expression in *B. burgdorferi*. 
Previous studies have shown that polyamines increase the viability of E. coli in the stationary phase by increasing the levels of ppGpp regulatory protein (SpoT) and the omega (ω) subunit of the RNA polymerase (RpoZ) (39). We found that the transcript levels of rpoZ were similar at low density with and without Spd, while the addition of Spd and growth to high density led to a significant decrease in transcription. On the other hand, relBbu (bb0198) was significantly upregulated in the lower-density culture with the addition of Spd (Fig. 9C). RelBbu is a bifunctional synthetase/hydrolase controlling the levels of guanosine tetra/pentaphosphate [(p)ppGpp]. (p)ppGpp has been shown to play a role in the regulation of the stringent response during the survival of B. burgdorferi in the tick vector or during starvation (14, 15). While temperature, growth phase, or pH of the medium did not affect the transcriptional level of relBbu, shifting the spirochetes from nutritionally starved medium to that supplemented with rabbit serum significantly induced its transcription (15). Although the culture conditions used in this study are different, we did observe that the addition of Spd induced the levels of RelBbu (Fig. 4 and 9B). These observations indicate that polyamines, specifically Spd and Spm, may contribute to B. burgdorferi adaptation as the bacteria transit from nutrient-limited to nutrient-enriched conditions within the midgut of the tick or during various stages of dissemination through different tissues.

A key in vitro growth component that allows the spirochetes to recover from starvation is rabbit serum, and this was reflected with increased transcription of relBbu (14, 15). Importantly, polyamines affect the surface composition, notably porins, in many bacterial species. Furthermore, polyamines increase the ability of Neisseria gonorrhoeae to withstand the effects of cationic antimicrobial peptides and complement-mediated killing (31). While the protein profiles of spirochetes grown in medium with heat-inactivated or normal rabbit serum were similar (Fig. 10), supplementation with 4 mM Spd with heat-inactivated serum resulted in a noticeable increase in the levels of OspC and DbpA compared to levels with normal serum, although the levels of OspA and OppA1 were similar under all conditions (Fig. 10B). It is interesting to speculate that the presence of heat-labile serum components interferes with the availability of Spd or, alternatively, that the heat-inactivated serum contains components that contribute to increased induction of levels of OspC and DbpA in the presence of 4 mM Spd.

Lyme spirochetes lack the full spectrum of homologs of four different pathways that have been characterized in other bacterial systems for catabolism of the polyamine precursor Arg, i.e., the (i) arginase, (ii) arginine deiminase (ADI), (iii) arginine succinyltransferase (AST), and (iv) arginine transaminase pathways; however, some members of the ADI pathway are present, such as ArcA (BB0841; arginine deiminase), ArgF (BB0842; ornithine carbamoyltransferase), and ArcD (BB0843; arginine/ornithine antiporter) (40). ArcA is the first enzyme that converts arginine to citrulline with the release of the NH₃ molecule. Citrulline is then converted to carbamoyl phosphate by ArcB or to ornithine by ArgF. Since B. burgdorferi has an ORF annotated as ArgF, it is possible that increased amounts of arginine lead to increased levels of ornithine, which would presumably be effluxed out via ArcD (arginine/ornithine antiporter). Since there is no apparent homolog of ArcC (carbamate kinase) in B. burgdorferi, it is unknown if carbamoylphosphate is indeed generated via the effects of ArcB and in turn converted to ammonia and carbon dioxide, with concomitant generation of ATP by an ArcC-like homolog. Taken together, these observations indicate that B. burgdorferi presumably possesses a truncated arginine deiminase pathway and cannot utilize arginine for the synthesis of polyamines, suggesting the importance of polyamine transport in the physiological responses of B. burgdorferi in different hosts. In summary, understanding the role of polyamines in connecting the physiology and virulence of B. burgdorferi will help identify novel tools to reduce the borrelial burden in infected reservoir hosts, altering transmission kinetics to reduce the incidence of Lyme disease.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and plasmids.** B. burgdorferi strain B31-A3 was cultured in BSK-II medium supplemented with 6% (vol/vol) normal rabbit serum (Pel-Freez, AR) at 32°C and pH 7.6.
in 1% CO₂ in a CO₂ incubator. Borrelial cultures grown in BSK-II medium at pH 7.6 and 23°C were shifted to pH 6.8 and 37°C to mimic conditions within the tick midgut before and after a blood meal, respectively, as described previously (41, 42). Growth of B. burgdorferi in the presence of 4 mM Spm or Spd at pH 7.6 and 32°C with a starting density of 1 × 10⁶ spirochetes/ml was monitored every 24 h for 168 or 120 h, respectively. Cultures grown to a density of less than 1 × 10⁷ to 2 × 10⁷ or 1 × 10⁸ to 2 × 10⁹ spirochetes/ml were considered low- and high-density cultures, respectively. A pCR2.1 cloning vector (Invitrogen) was routinely used for cloning procedures and transformed into E. coli TOP10 cells, followed by blue/white screening (Table 2). Recombinant PotA (BB0642) and PotD (BB0639) were obtained by generating the respective amplicons using primers (Table 1) with appropriately engineered restriction enzyme sites that facilitated cloning into pET23a (Novagen), followed by overexpression using the E. coli Rosetta strain, as reported previously (43, 44). Histidine-tagged (6 copies; C-terminus) PotA and PotD were purified using nickel affinity column chromatography and used to generate specific antisera (45).

**Generation of antisera against PotA and PotD.** Six- to 8-week-old mice (n = 5) were immunized subcutaneously with 50 μg of recombinant PotA or PotD in phosphate-buffered saline (PBS) with an equal volume of the adjuvant TiterMax (Sigma). Booster immunizations were given at days 14 and 21, and immune serum was collected on day 28 (45). Serum was checked for reactivity to PotA or PotD in B. burgdorferi by immunoblot analysis as described below. All animal procedures were performed in accordance with the animal use protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Texas at San Antonio.

**Preparation of B. burgdorferi lysates.** Spirochetes were grown in the presence or absence of various polyamines (4 mM) and monitored for growth. When the density of the culture reached 1 × 10⁶ bacteria/ml, spirochetes were washed three times in Hanks balanced salt solution (HBSS) and processed for SDS-PAGE analysis. B. burgdorferi was grown in the absence or presence of spermine (4 mM) in BSK-II growth medium supplemented with 6% (vol/vol) normal or heat-inactivated (56°C for 1 h) rabbit serum. In order to determine if PotD is surface exposed, the spirochetes were washed in PBS (pH 7.4) supplemented with 5 mM MgCl₂ and 50 mM sucrose as described previously (27). Samples were split in two and incubated either with 50 μl of sterile water (proteinase K negative control) or with proteinase K (final concentration, 200 μg/ml) at 20°C for 40 min. The protease activity was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) 1 mM final concentration, and the spirochetes were examined by dark-field microscopy for motility as a measure of viability. Samples were washed and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.

**SDS-PAGE and immunoblot analysis.** B. burgdorferi whole-cell lysates were prepared and separated by SDS–12.5% PAGE as described previously (44). The separated proteins were either visualized by Coomassie brilliant blue staining or transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P; GE Healthcare, Buckinghamshire, UK) and subjected to immunoblot analysis as described previously (46). Membranes were probed with monoclonal antibodies or monospecific serum against a variety of borrelial proteins. Blots were developed following incubation with appropriate dilutions of horse radish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-rat secondary antibody using ECL Western blotting reagents (GE Healthcare).

**RNA extraction and quantitative RT-PCR analysis.** Transcriptional analysis of key genes relevant to this study was carried out using quantitative real-time PCR (RT-PCR) analysis as described previously (47, 48). Total RNA was extracted as previously described from B. burgdorferi cultures grown in the presence or absence of 4 mM spermine to low density (2 × 10⁶ to 3 × 10⁷ spirochetes per ml) or high density (1 × 10⁸ spirochetes per ml) (46). Total RNA was treated twice at 37°C for 45 min with DNase I (Ambion, Austin, TX) to remove any contaminating DNA and quantified spectrophotometrically. RNA purity was determined using real-time PCR with recA primers (recAFq and recARq) to rule out the presence of contaminating DNA. RNA samples were reverse transcribed to cDNA using a Bio-Rad iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was carried out using iTaq Universal SYBR green Supermix with various oligonucleotide primers (Table 1) at a final concentration of 100 nM, and quantitative real-time PCR was done using an ABI Prism 7300 system (Applied Biosystems) as described previously (46). The cycle threshold (Cₜ) values of each of the genes were averaged following normalization. Levels of induction were determined using the ∆∆CT method where the quantity of each transcript was determined by the calculation of 2^(-∆∆C_T), as described previously (46, 47). Normalized Cₜ

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>PCR cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pET23a</td>
<td>Expression vector with a C-terminal 6× His tag</td>
<td>Novagen</td>
</tr>
<tr>
<td>pJR-100</td>
<td>bb0639 cloned into pCR2.1</td>
<td>This study</td>
</tr>
<tr>
<td>pJR-101</td>
<td>bb0639 cloned into pET23a</td>
<td>This study</td>
</tr>
<tr>
<td>pJR-102</td>
<td>bb0642 cloned into pCR2.1</td>
<td>This study</td>
</tr>
<tr>
<td>pJR-103</td>
<td>bb0642 cloned into pET23a</td>
<td>This study</td>
</tr>
<tr>
<td>B. burgdorferi strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B31-A3 (wild type)</td>
<td>B31, low-passage number, virulent isolate</td>
<td>49</td>
</tr>
</tbody>
</table>
values were subjected to an unpaired Student’s t test implemented in GraphPad Prism software. P values of less than 0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00684-16.

REFERENCES


25. Chatteropadhyay MK, Keembiyehetty CN, Chen W, Tabor H. 2015. Polyamines stimulate the level of the outer membrane subunit (RpoS) of Escherichia coli RNA polymerase, resulting in the induction of the glutamate


