Characterization of *Borrelia burgdorferi* Aggregates

Siddharth Y. Srivastava and Aravinda M. de Silva

Abstract

Lyme disease is caused by the tick-borne spirochete, *Borrelia burgdorferi*. It has been documented that *B. burgdorferi* form aggregates within ticks and during *in vitro* culture. However, *Borrelia* aggregates remain poorly characterized, and their functional significance is unknown. Here we have characterized *Borrelia* aggregates using microscopy and flow cytometry. *Borrelia* aggregation was temperature, pH, and growth phase dependent. Environmental conditions (high temperature, low pH, and high cell density) favorable for aggregation were similar to the conditions that increased the expression of *B. burgdorferi* genes, such as outer surface protein C (*ospC*), that are regulated by the RpoN/RpoS sigma factors. Experiments were conducted to determine if there is a relationship between aggregation and gene regulation through the RpoN/RpoS pathway. *ospC* Transcript levels were similar between aggregates and free cells. Moreover, no differences were observed in aggregate formation when null mutants of *rpoS*, *rpoN*, or *ospC* were compared to wild-type spirochetes. These results indicated that, despite the similar external signals that promoted aggregation and the RpoN/RpoS pathway, the two processes were not linked at the molecular level. The methods developed here to study *B. burgdorferi* aggregates will be useful for further studies on spirochete aggregates.

Key Words: *Borrelia*—Aggregate—Lyme disease.

Introduction

BORRELIA BURGDORFERI is a tick-borne spirochete that causes Lyme disease (Burgdorfer et al. 1982). Various investigators have reported that *Borrelia* make large aggregates and micro colonies within ticks and in culture (Burgdorfer et al. 1982, Barbour and Hayes 1986, Burgdorfer et al. 1989, Ewing et al. 1994, de Silva and Fikrig 1995, Sadzienie et al. 1995, Shi et al. 1998, Gilmore and Piesman 2000, Shang et al. 2000, Tilly et al. 2000, Bugrysheva et al. 2002, Blevins et al. 2007). In ticks, aggregates were observed during and after the blood meal when the spirochetes grow to high densities in the tick's gut. In the gut *Borrelia* formed aggregates that were localized in the intercellular spaces between epithelial cells and within microvillar brush border cells of the gut (Burgdorfer et al. 1989).

Bacterial aggregation is involved in transmission, host colonization, exchange of genetic information, and protection from adverse environmental conditions (Kjaergaard et al. 2000, Wimpenny and Colasanti 2004, Prentice and Rahalison 2007). In *Yersinia pestis*, clumping of bacteria plays an important role in transmission from fleas to animals (Prentice and Rahalison 2007). *Borrelia* form aggregates during tick blood feeding, when they also get transmitted from tick vector to mammalian host. During this process, many *B. burgdorferi*

genes required for transmission and host infection are expressed by a pathway regulated by the alternative sigma factors RpoN and RpoS. For example, the outer surface protein C (*ospC*) gene, which is required for transmission and host infection, is expressed by the RpoN/RpoS pathway. The timing of *ospC* expression within feeding ticks coincides with the formation of aggregates, indicating a possible relationship between aggregation and the RpoN/RpoS pathway. Here we report on studies that were done to characterize *B. burgdorferi* aggregates and to determine if aggregation was linked to the RpoN/RpoS pathway. Although the external signals for aggregation and activation of the RpoN/RpoS pathway are similar, our results demonstrate that the two processes are not linked at the molecular level.

Materials and Methods

Bacterial strains, growth analysis, and microscopy

A low passage culture of *B. burgdorferi* strain B31 (originally isolated from a tick in Shelter Island, NY) (Burgdorfer et al. 1982) was provided to us by the Centers for Disease Control and Prevention (Fort Collins, CO). The stock was cloned on solid BSK-H medium and named B31-C1. The clone was expanded in liquid BSK-H medium and frozen in aliquots that

were used for this study. Strain 297 was obtained from Dr. Kayla Hagman at the University of Texas Southwestern Medical Center (Dallas, TX). Strain B31A3 and mutants *ospC7*, *ospC7/ospC + 4*, and *A3rpoS* were obtained from Dr. Patricia A. Rosa, NIAID, Rocky Mountain Laboratories (Hamilton, MT) (Tilly et al. 2006), and strains *A3-Gm*, *A3ntrA-Gm*, *ntrA-comp*, and *ntrA-VC* were obtained from Dr. Frank Gherardini, NIAID, Rocky Mountain Laboratories (Fisher et al. 2005). Strains B31-CGFP and B31-FGFP were obtained from Dr. James Carroll, University of Pittsburgh (Pittsburgh, PA) (Carroll et al. 2003). B31-CGFP is B31 clone A3 harboring plasmid pBSV Φ (*ospCp-gfp*), and B31-FGFP is B31 clone A3 harboring plasmid pBSV Φ (*flaBp-gfp*). Plasmid pBSV Φ (*ospCp-gfp*) has *gfp* gene under control of *ospC* promoter, whereas in pBSV Φ (*flaBp-gfp*), *gfp* is under control of *flaB* promoter. Cultures were grown in BSK-H complete medium (Sigma, St. Louis, MO). The culture was grown at 35°C until the cells reached a density of 1×10^5 cells/mL. Cells were then transferred into fresh BSK-H complete medium for experiments. All cultures were vortexed before harvesting cells for counting or other analysis. Growth curves were generated for bacteria grown at 35°C by observing the bacteria by dark field microscopy at 20 $\times$ and counting the organisms with a Petroff Hausser chamber (Hausser Scientific, Horsham, PA). Pictures of free cells and aggregates were taken using bright field microscopy at 20 $\times$. *B. burgdorferi* was cultured under different temperature and pH conditions. For these experiments, *Borrelia* culture was grown at 35°C until the cells reached a density of 1×10^5 cells/mL and subsequently transferred into fresh BSK-H complete medium at 35°C and pH 7.0 or 23°C and pH 8.0, buffered with 25 mM HEPES. At equivalent cell densities of $5\text{--}7 \times 10^7$ cells/mL, *Borrelia* were analyzed from both cultures.

Scanning electron microscopy of *Borrelia* aggregates

Late log-phase (5×10^7 bacteria/mL) *Borrelia* culture was washed three times with PBS and fixed with 4% glutaraldehyde. Cell suspension was spread onto a poly-L-lysine-coated coverslip and incubated for 30 min at room temperature. The coverslip was submerged in one change of PBS for 10 min. Cells were then dehydrated by transferring through increasing concentrations of ethanol (30%, 50%, 75%, and anhydrous ethanol). Samples were transferred to the critical point dryer in 100% anhydrous ethanol. After the drying procedure, coverslips were mounted on 13 mm aluminum planchettes using silver, and coverslips were coated with a 15–20 nm thickness of gold palladium alloy (60:40) using a sputter coater or vacuum evaporator. Samples were observed using a Cambridge S200 scanning electron microscope.

Flow cytometry of *Borrelia*

To analyze the size and DNA content of *Borrelia*-free cells and aggregate populations, late log-phase (5×10^7 bacteria/mL) *B. burgdorferi* cultures grown at 35°C were incubated with Hoechst 33342 DNA stain (5 μ M) for 30 min at room temperature. They were subsequently flow sorted to analyze forward scatter (FSC), side scatter (SSC), and Hoechst 33342 staining intensity. Flow cytometer data were acquired using Summit V.3.1 (Cytomation, Fort Collins, CO). A MoFlo Modular flow Cytometer (Cytomation) was used to sort the *Borrelia*-free cells and aggregates. Aggregates and free cells were collected

based on their FSC and SSC values, where the mean values of (SSC, FSC) for free cells and aggregates were (39.11, 3.96) and (443.08, 102.65), respectively.

To analyze *ospC* promoter activity in single *Borrelia* cells, strain B31-CGFP was stained with Hoechst 33342 DNA stain (5 μ M) for 30 min at room temperature. Cells were then analyzed on MoFlo Modular flow cytometer. Control strain B31-FGFP was subjected to identical treatment.

Quantitative RT-PCR of *ospC* and *flaB* transcripts in flow-sorted *Borrelia*

Flow-sorted cells were collected in lysis buffer containing PBS, 100 mM DTT, RNase inhibitor (RNasin; Promega, Madison, WI), and DEPC water (Ambion, Austin, TX). Cells were then subjected to four freeze–thaw cycles. Genomic DNA in the cell lysates was removed using Turbo DNA-free kit (Ambion). Reverse transcription (RT) was performed using random primer (Invitrogen, Carlsbad, CA). Aliquots from RT reaction mix were used for quantitative PCR using SYBR green master mix (Applied Biosystems, Foster City, CA). The *flaB* cDNA was amplified using primers TTTCAGGGTCT CAAGCGTCT (forward) and TGTGAGCTCCTTCCTGTTG (reverse), whereas *ospC* cDNA was amplified using primers GAAAGAGGTTGAAGCTTGC (forward) and ATTGCAT AAGCTCCCGCTAA (reverse). Thermal cycles followed for amplification were 1 cycle at 95°C for 15 min and 50 cycles at 95°C for 15 s and 60°C for 1 min using ABI Prism 7000 Sequence Detection System (Applied Biosystems). Melting curve was generated by following 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. Relative quantitation of *ospC* transcripts was performed using comparative Ct method ($\Delta\Delta$ Ct method) to calculate fold differences in *ospC* transcript between free cells and aggregates (Livak and Schmittgen 2001). Expression levels of *ospC* transcript (target) and *flaB* transcript (endogenous control) are evaluated. The levels of these amplicons in aggregates are compared with free cells (calibrator).

Results

Aggregation of *B. burgdorferi* in culture medium

Initial experiments were done to characterize and quantify aggregate formation at different stages of growth in culture. *B. burgdorferi* strain B31-C1 was grown at 35°C in BSK-H complete medium and observed by light and dark field microscopy and scanning electron microscopy. At early log phase (4.3×10^6 cells/mL), almost all the spirochetes were present as individual cells (Fig. 1). As the cell density of the culture increased, the number of aggregates and the average size of each aggregate increased (Figs. 1 and 2A–D). A similar aggregation profile was also observed for *B. burgdorferi* strain RJ297 (data not shown). The earliest aggregates observed consisted of 2–6 spirochetes attached to each (Fig. 2 A, C). Most of the spirochetes in the aggregates appeared motile. When the culture reached a density 5×10^7 bacteria/mL, the average aggregate had greater than 20 bacteria (Fig. 2B, D). The center of each aggregate often appeared to have material without a clearly defined shape. Scanning electron microscopy demonstrated that this material was present toward the center of the aggregate and possibly holding the aggregate together (Fig. 2C, enlarged area). Once the culture reached stationary phase, the aggregates persisted, but the number of

FIG. 1. Growth curve and aggregation profile of a *B. burgdorferi* B31-C1 culture grown at 35°C. An aggregate was defined as a clump consisting of ≥ 20 bacteria. Values are average of four observations, and the figure is representative of two independent experiments.

aggregates dropped and the average size of each aggregate also decreased (Figs. 1 and 2E).

Experiments were conducted to determine if FACS could be used to separate aggregates and free cells. We predicted that owing to the large size of aggregates, they will have higher FSC and SSC than the free cells. When a late log-phase

culture was analyzed for the FSC and SSC properties of the bacteria, the majority of cells had low FSC and intermediate SSC (Fig. 3A, sector R2). A smaller population showed increasingly higher FSC and SSC values. We confirmed that the bacterial populations with lower scatter values were free cells (Fig. 3A, sector R2), while the ones with higher scatter values were aggregates by observing the bacteria by dark field microscopy (data not shown) and fluorescence microscopy (Fig. 3A, sector R24, R25). Further, flow analysis of *Borrelia* culture stained with Hoechst 33342 (which binds to DNA) showed that the FSC values of aggregates corresponded to their sizes because there was a strong positive correlation between FSC and the DNA content in each event (Fig. 3B).

At equivalent cell densities of $5\text{--}7 \times 10^7$ cells/mL, *Borrelia* grown at 35°C and pH 7.0 showed significantly higher aggregation than cultures grown at 23°C and pH 8.0 (Fig. 4). Larger aggregates were conspicuously missing in the cultures grown at low temperatures. Both sets of culture tubes were buffered with 25 mM HEPES, and at the end of the experiment no appreciable change in pH was observed from the starting pH (data not shown).

ospC Gene expression by free and aggregated spirochetes

The environmental conditions (high temperature, low pH, and high cell density) favorable for aggregation were similar to the conditions that increased the expression of *B. burgdorferi* genes, such as *ospC*, that are regulated by the RpoN/RpoS sigma factors (Schwan et al. 1995, Carroll et al. 1999, Hubner et al. 2001, Yang et al. 2005). Experiments were done to compare *ospC* expression per cell in aggregated versus free spirochetes using quantitative RT-PCR. Spirochetes were

FIG. 2. Typical aggregates of *Borrelia* viewed by bright field microscopy and scanning electron microscopy at different phases of growth at 35°C. A culture was grown to mid log (4.3×10^6 bacteria/mL; panels A, C), late log (7×10^7 bacteria/mL; panels B, D), and stationary (1.24×10^8 bacteria/mL; panel E) phase and observed by bright field microscopy (A, B, E) and scanning electron microscopy (C, D). At the center of each aggregate a poorly defined matrix was observed at the points of cell-to-cell contact (magnified in panel C).

FIG. 3. FACS analysis of a late log-phase *Borrelia* culture. Spirochetes were analyzed based on their FSC and SSC properties. **(A)** Three different sectors were selected having low (R2), intermediate (R24), and high (R25) FSC and SSC values. Events falling within the sectors were sorted, collected, and fixed with acetone and stained with α *Borrelia* polyclonal antibody. Most of the bacteria in sector R2 were free cells, whereas the bacteria in sectors R24 and R25 were in aggregates of increasing size. **(B)** To confirm that spirochetes with increasing FSC and SSC values correspond to bacterial aggregates of increasing size, a late log culture was stained with a fluorescent dye (Hoechst 33342) that binds to DNA. The culture was sorted, and populations of increasing FSC and SSC values (R1, R2, R13, and R14) were selected for further analysis. When the FSC values of the bacteria in each sector were plotted against the signal from the DNA stain, a positive correlation was observed. Thus, the FSC is a correlate for the number of bacteria in each aggregate.

sorted into free cells and aggregates. RNA was purified from each population; *ospC* and *flaB* transcript levels were estimated by quantitative RT-PCR (Table 1). The ratio of *ospC* to *flaB* transcript levels was compared for the two populations, and *ospC* mRNA levels were found to be similar in free cells and aggregates. The fact that transcription from the *ospC* promoter was similar in free cells and aggregated cells was confirmed using mutant B31-CGFP, transfected with green fluorescent protein fused to the *OspC* promoter. A culture of *ospCp-GFP* mutant was grown under conditions (35°C/cell density at 1×10^7 cells/mL) that favor *ospC* expression, and stained with Hoechst 33342 to stain DNA of *Borrelia* cells. During flow cytometry, GFP intensity and Hoechst 33342 staining intensity of free cells and aggregates were measured. Ratio of GFP/Hoechst staining intensities showed *ospC* promoter activity per cell in free cells and aggregates (Table 2). Aggregates showed only 1.8-fold more GFP expression per cell compared to free cells. Aggregates of *flaBp-GFP* mutant showed 1.4-fold more GFP expression compared to free cells

(data not shown). Thus, cells within aggregates showed insignificant increase in *ospC* promoter activity compared to free cells.

Aggregation studies with *B. burgdorferi* mutants

To further explore if aggregation and the RpoN/RpoS pathways were linked, experiments were done with null mutants of *ntrA* (coding for RpoN), *rpoS*, and *ospC*. The wild-type and *ospC* knockout strains aggregated to similar levels (WT 1.24% and Δ *ospC* 1.91% of cells in aggregates), and the aggregates that formed in wild-type and *ospC*-disrupted strains were structurally similar (data not shown). RpoS and RpoN together and independently regulate *OspC* and a large number of other genes and cellular processes (Hubner et al. 2001, Yang et al. 2005). Experiments were conducted with *rpoS* and *ntrA* (coding for RpoN) knockout mutants to determine if these genes influenced aggregate formation. Both *rpoS* and *ntrA* mutants aggregated to similar levels as wild type

FIG. 4. Effect of temperature, pH, and cell density on *B. burgdorferi* aggregation. The graphs show FSC and SSC profile of late log-phase cultures grown at 35°C/pH 7.0 (feeding tick conditions) and 23°C/pH 8.0 (unfed tick conditions) to a similar density. A greater number of aggregates were observed under conditions that mimicked the feeding tick compared to the unfed tick.

(WT 1.24%, $\Delta ntrA$ 2.03%, and $\Delta rpoS$ 2.04% of cells in aggregates), indicating that *Borrelia* aggregation was not dependent on genes expressed by RpoN or RpoS (data not shown).

Discussion

Investigators studying *Borrelia* have long known that these organisms often aggregate, but this phenomenon has not been systematically studied. Given the growing appreciation that bacterial aggregation is of biological significance, *Borrelia* aggregation deserves further study. *B. burgdorferi* persists after antibiotic treatment in mice (Hodzic et al. 2008), and there are conflicting reports about the effectiveness of antibiotic therapy in human Lyme disease (Moody et al. 1994, Nowakowski et al. 1995, Wang et al. 1998). In *Pseudomonas aeruginosa* aggregation has been linked to persistence and antibiotic resistance in cystic fibrosis patients (Drenkard and Ausubel 2002). Further studies are needed to explore the role of *Borrelia* aggregates in persistence both during normal infection and after antibiotic treatment. Here we demonstrate that high temperature, low pH, and high cell density enhance *Borrelia* aggre-

gate formation *in vitro*. Previous studies have demonstrated that high cell density, low pH, and high temperature cause expression of *ospC* and other *Borrelia* genes controlled by the global transcriptional regulators RpoS and RpoN (Schwan et al. 1995, Carroll et al. 1999, Elias et al. 2000, Yang et al. 2000, 2005, Hubner et al. 2001, Ramamoorthy and Scholl-Meeker 2001, Fisher et al. 2005, Burtnick et al. 2007, Smith et al. 2007). However, the results reported here demonstrate that spirochete aggregation is not linked to *ospC* or other genes regulated by the RpoN/RpoS pathway. These results corroborate an earlier report that showed that *Borrelia* mutant B313 strain that does not express OspA, B, C, or D formed microscopic and macroscopic aggregates (Sadziene et al. 1993, 1995). B313 strain of *Borrelia* has only 6 out of 21 plasmids. It lacks all plasmids except cp32-1, cp32-2, cp32-3, cp32-4, cp26, and lp17, indicating that gene responsible for *Borrelia* aggregation may be in either these plasmid(s) or in the genome.

It may be possible to correlate gene expression and protein production in an aggregate, although such an analysis would require estimation of live and dead cells, within an aggregate. For example, in live cells, *ospC* transcript and proportional

TABLE 1. COMPARISON OF *ospC* TRANSCRIPT LEVELS IN *B. BURGDORFERI* B31C1 USING QUANTITATIVE RT-PCR, IN FREE CELLS AND AGGREGATES

Fraction	<i>flaB</i> Average C_T	<i>ospC</i> Average C_T	ΔC_T <i>ospC-flaB</i> ^a	$\Delta \Delta C_T$ $\Delta C_T - \Delta C_T$ Free cells ^b	<i>ospC</i> Relative to free cells ^c
Free cells	29.97 $\pm$ 0.31	29.54 $\pm$ 0.65	-0.42 $\pm$ 0.72	0.00 $\pm$ 0.72	1.0 (0.27 - 1.72)
Aggregates	29.94 $\pm$ 0.08	30.36 $\pm$ 0.23	0.42 $\pm$ 0.24	0.85 $\pm$ 0.24	0.55 (0.61 - 0.79)

The fold change in the expression of *ospC* (target gene) relative to *flaB* (internal control gene) was studied in free cells and aggregates. Samples were analyzed using real-time PCR, and C_T data were imported into a Microsoft Excel sheet. The data are representative of two independent experiments, and each value in the table is mean of readings done in triplicate.

^aThe ΔC_T value was determined by subtracting the average *ospC* C_T value from the average *flaB* C_T value. The standard deviation of the difference was calculated from the standard deviations of the *ospC* and *flaB* values.

^b $\Delta \Delta C_T$ values were calculated by subtracting ΔC_T value of samples from the ΔC_T value of calibrator. Free cell fraction is designated as the calibrator.

^cThe range given for *ospC* relative to free cell fraction is determined by evaluating the expression: $2^{-\Delta \Delta C_T}$ with $\Delta \Delta C_T + s$ and $\Delta \Delta C_T - s$, where s = the standard deviation of the $\Delta \Delta C_T$ value.

TABLE 2. COMPARISON OF *ospC* TRANSCRIPTION IN FREE CELLS AND AGGREGATES OF *B. BURGDORFERI* B31-CGFP

	Mean GFP intensity (GFP)	Mean DNA staining intensity (DNA)	Ratio (GFP/DNA)
Free cells	6.23	86.80	0.27
Aggregates	22.42	176.14	0.49

Late log-phase B31-CGFP culture grown at 35°C was stained with Hoechst 33342. Free cells and aggregates were analyzed using MoFlo. Intensities of GFP and Hoechst 33342 were measured for both free cells and aggregates. Ratio of GFP/Hoechst 33342 shows *ospC* promoter activity per cell. Aggregated cells express 1.8-fold more GFP than free cells.

OspC protein can be detected. However, in cells within aggregates that are not alive, amount of OspC protein may not be proportional to *ospC* transcript, owing to shorter half-life of mRNA than protein. Using GFP reporter system may be more accurate, as cells showing active transcription of *ospC* would synthesize both GFP and OspC protein.

We and others have used flow cytometry to analyze protein and gene expression by spirochetes grown in culture (Caimano et al. 2005, Bakker et al. 2007, Motaleb et al. 2007, Srivastava and de Silva 2008). Here we also demonstrate the utility of flow cytometry to compare the properties of individual or aggregated spirochetes. With flow cytometry it was also possible to separate aggregates of different size. While the current study has demonstrated that the RpoN/RpoS pathway is not linked to aggregation, the tools developed here can be applied to further characterize aggregates and to evaluate their biological significance. This method could also be used to analyze difference in expression of gene(s) between *Borrelia* populations with different physical characteristics such as non-motile cystic forms and vegetative spirochetes, within the same culture (Murgia et al. 2002).

In summary, we have shown that high temperature, low pH, and high cell density enhance *Borrelia* aggregate formation *in vitro*. However, spirochete aggregation is not linked to *ospC* or other genes regulated by the RpoN/RpoS pathway.

Acknowledgments

This work was supported by Public Health Service Grant ROI AR47948 from the National Institute for Arthritis and Musculoskeletal and Skin Diseases. We thank Patricia Rosa, Frank Gherardini (NIAID), and Jay Carroll (University of Pittsburgh) for sending us *Borrelia* strains. Larry Arnold and Nancy Martin (UNC—Chapel Hill) assisted us with establishing the FACS assays for studying *Borrelia*. We also thank members of the de Silva laboratory for their advice.

Disclosure Statement

Siddharth Y. Srivastava: No competing financial interests exist. Aravinda M. de Silva: No competing financial interests exist.

References

Bakker, RG, Li, C, Miller, MR, Cunningham, C, et al. Identification of specific chemoattractants and genetic complemen-

tation of a *Borrelia burgdorferi* chemotaxis mutant: flow cytometry-based capillary tube chemotaxis assay. *Appl Environ Microbiol* 2007; 73(4):1180–1188.

Barbour, AG, Hayes, SF. Biology of *Borrelia* species. *Microbiol Rev* 1986; 50(4):381–400.

Blevins, JS, Revel, AT, Smith, AH, Bachlani, GN, et al. Adaptation of a luciferase gene reporter and lac expression system to *Borrelia burgdorferi*. *Appl Environ Microbiol* 2007; 73(5):1501–1513.

Bugrysheva, J, Dobrikova, EY, Godfrey, HP, Sartakova, ML, et al. Modulation of *Borrelia burgdorferi* stringent response and gene expression during extracellular growth with tick cells. *Infect Immun* 2002; 70(6):3061–3067.

Burgdorfer, W, Barbour, AG, Hayes, SF, Benach, JL, et al. Lyme disease—a tick-borne spirochetosis? *Science* 1982; 216(4552):1317–1319.

Burgdorfer, W, Hayes, SF, Corwin, D. Pathophysiology of the Lyme disease spirochete, *Borrelia burgdorferi*, in ixodid ticks. *Rev Infect Dis* 1989; 11 Suppl 6:S1442–S1450.

Burnnick, MN, Downey, JS, Brett, PJ, Boylan, JA, et al. Insights into the complex regulation of *rpoS* in *Borrelia burgdorferi*. *Mol Microbiol* 2007; 65(2):277–293.

Caimano, MJ, Eggers, CH, Gonzalez, CA, Radolf, JD. Alternate sigma factor RpoS is required for the *in vivo*-specific repression of *Borrelia burgdorferi* plasmid lp54-borne *ospA* and lp6.6 genes. *J Bacteriol* 2005; 187(22):7845–7852.

Carroll, JA, Garon, CF, Schwan, TG. Effects of environmental pH on membrane proteins in *Borrelia burgdorferi*. *Infect Immun* 1999; 67:3181–3187.

Carroll, JA, Stewart, PE, Rosa, P, Elias, AF, et al. An enhanced GFP reporter system to monitor gene expression in *Borrelia burgdorferi*. *Microbiology* 2003; 149(Pt 7):1819–1828.

de Silva, AM, Fikrig, E. Growth and migration of *Borrelia burgdorferi* in Ixodes ticks during blood feeding. *Am J Trop Med Hyg* 1995; 53(4):397–404.

Drenkard, E, Ausubel, FM. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 2002; 416(6946):740–743.

Elias, AF, Bono, JL, Carroll, JA, Stewart, P, et al. Altered stationary-phase response in a *Borrelia burgdorferi* *rpoS* mutant. *J Bacteriol* 2000; 182(10):2909–2918.

Ewing, C, Scorpio, A, Nelson, DR, Mather, TN. Isolation of *Borrelia burgdorferi* from saliva of the tick vector, Ixodes scapularis. *J Clin Microbiol* 1994; 32(3):755–758.

Fisher, MA, Grimm, D, Henion, AK, Elias, AF, et al. From the cover: *Borrelia burgdorferi* {sigma}54 is required for mammalian infection and vector transmission but not for tick colonization. *Proc Natl Acad Sci USA* 2005; 102(14):5162–5167.

Gilmore, RD, Jr., Piesman, J. Inhibition of *Borrelia burgdorferi* migration from the midgut to the salivary glands following feeding by ticks on OspC-immunized mice. *Infect Immun* 2000; 68(1):411–414.

Hodzic, E, Feng, S, Holden, K, Freet, KJ, Barthold, SW. Persistence of *Borrelia burgdorferi* following antibiotic treatment in mice. *Antimicrob Agents Chemother* 2008; 52(5):1728–1736.

Hubner, A, Yang, X, Nolen, DM, Popova, TG, et al. Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. *Proc Natl Acad Sci USA* 2001; 98(22):12724–12729.

Kjaergaard, K, Schembri, MA, Hasman, H, Klemm, P. Antigen 43 from *Escherichia coli* induces inter- and intraspecies cell aggregation and changes in colony morphology of *Pseudomonas fluorescens*. *J Bacteriol* 2000; 182(17):4789–4796.

- Livak, KJ, Schmittgen, TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25(4):402-408.
- Moody, KD, Adams, RL, Barthold, SW. Effectiveness of antimicrobial treatment against *Borrelia burgdorferi* infection in mice. *Antimicrob Agents Chemother* 1994; 38(7):1567-1572.
- Motaleb, MA, Miller, MR, Bakker, RG, Li, C, et al. Isolation and characterization of chemotaxis mutants of the Lyme disease Spirochete *Borrelia burgdorferi* using allelic exchange mutagenesis, flow cytometry, and cell tracking. *Methods Enzymol* 2007; 422:421-437.
- Murgia, R, Piazzetta, C, Cinco, M. Cystic forms of *Borrelia burgdorferi* sensu lato: induction, development, and the role of RpoS. *Wien Klin Wochenschr* 2002; 114(13-14):574-579.
- Nowakowski, J, Nadelman, RB, Forseter, G, McKenna, D, Wormser, GP. Doxycycline versus tetracycline therapy for Lyme disease associated with erythema migrans. *J Am Acad Dermatol* 1995; 32:223-227.
- Prentice, MB, Rahalison, L. Plague. *Lancet* 2007; 369(9568):1196-1207.
- Ramamoorthy, R, Scholl-Meeker, D. *Borrelia burgdorferi* proteins whose expression is similarly affected by culture temperature and pH. *Infect Immun* 2001; 69(4):2739-2742.
- Sadziene, A, Wilske, B, Ferdows, MS, Barbour, AG. The cryptic ospC gene of *Borrelia burgdorferi* B31 is located on a circular plasmid. *Infect Immun* 1993; 61(5):2192-2195.
- Sadziene, A, Thomas, DD, Barbour, AG. *Borrelia burgdorferi* mutant lacking Osp: biological and immunological characterization. *Infect Immun* 1995; 63(4):1573-1580.
- Schwan, TG, Piesman, J, Golde, WT, Dolan, MC, Rosa, PA. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc Natl Acad Sci USA* 1995; 92(7):2909-2913.
- Shang, ES, Champion, CI, Wu, XY, Skare, JT, et al. Comparison of protection in rabbits against host-adapted and cultivated *Borrelia burgdorferi* following infection-derived immunity or immunization with outer membrane vesicles or outer surface protein A. *Infect Immun* 2000; 68(7):4189-4199.
- Shi, W, Yang, Z, Geng, Y, Wolinsky, LE, et al. Chemotaxis in *Borrelia burgdorferi*. *J Bacteriol* 1998; 180(2):231-235.
- Smith, AH, Blevins, JS, Bachlani, GN, Yang, XF, et al. Evidence that RpoS (sigmaS) in *Borrelia burgdorferi* is controlled directly by RpoN (sigma54/sigmaN). *J Bacteriol* 2007; 189(5):2139-2144.
- Srivastava, SY, de Silva, AM. Reciprocal expression of ospA and ospC in single cells of *Borrelia burgdorferi*. *J Bacteriol* 2008; 190(10):3429-3433.
- Tilly, K, Elias, AF, Bono, JL, Stewart, P, Rosa, P. DNA exchange and insertional inactivation in spirochetes. *J Mol Microbiol Biotechnol* 2000; 2(4):433-442.
- Tilly, K, Krum, JG, Bestor, A, Jewett, MW, et al. *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection. *Infect Immun* 2006; 74(6):3554-3564.
- Wang, TJ, Sangha, O, Phillips, CB, Wright, EA, et al. Outcomes of children treated for Lyme disease. *J Rheumatol* 1998; 25(11):2249-2253.
- Wimpenny, J, Colasanti, R. A simple cellular automaton model for coaggregation. *Biofilms* 2004; 1:369-375.
- Yang, X, Goldberg, MS, Popova, TG, Schoeler, GB, et al. Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. *Mol Microbiol* 2000; 37(6):1470-1479.
- Yang, XF, Lybecker, MC, Pal, U, Alani, SM, et al. Analysis of the ospC regulatory element controlled by the RpoN-RpoS regulatory pathway in *Borrelia burgdorferi*. *J Bacteriol* 2005; 187(14):4822-4829.

Address correspondence to:

Siddharth Y. Srivastava

Carolina Cardiovascular Biology Center

CB #7126, 103 Mason Farm Road

2332 MBRB

Chapel Hill, NC 27599

E-mail: svastava@email.unc.edu