Investigation of ospC Expression Variation among Borrelia burgdorferi Strains

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Outer surface protein C (OspC) is the most studied major virulence factor of Borrelia burgdorferi, the causative agent of Lyme disease. The level of OspC varies dramatically among B. burgdorferi strains when cultured in vitro, but little is known about what causes such variation. It has been proposed that the difference in endogenous plasmid contents among strains contribute to variation in OspC phenotype, as B. burgdorferi contains more than 21 endogenous linear (lp) and circular plasmids (cp), and some of which are prone to be lost. In this study, we analyzed several clones isolated from B. burgdorferi strain 297, one of the most commonly used strains for studying ospC expression. By taking advantage of recently published plasmid sequence of strain 297, we developed a multiplex PCR method specifically for rapid plasmid profiling of B. burgdorferi strain 297. We found that some commonly used 297 clones that were thought having a complete plasmid profile, actually lacked some endogenous plasmids. Importantly, the result showed that the difference in plasmid profiles did not contribute to the ospC expression variation among the clones. Furthermore, we found that B. burgdorferi clones expressed different levels of BosR, which in turn led to different levels of RpoS and subsequently, resulted in OspC level variation among B. burgdorferi strains.

Keywords: Borrelia burgdorferi, OspC, endogenous plasmid, multiplex PCR, BosR

INTRODUCTION

Lyme disease is the most common arthropod-born disease in the United States, Europe as well as Asia (Samuels, 2011; Radolf et al., 2012). The causative agent, Borrelia burgdorferi, is a spirochete that has a dual-membrane cell envelope. Unlike other Gram-negative bacteria that have lipopolysaccharide (LPS) on the outer membrane, B. burgdorferi outer membrane lacks LPS and contains numerous lipoproteins. These outer surface lipoproteins are differentially expressed and play key roles in host adaptation during its enzootic cycle between ticks and mammals (Radolf et al., 2012; Caimano et al., 2016). For example, the major outer surface lipoprotein C (OspC), is induced in B. burgdorferi during nymphal tick feeding and during early phase of mammalian infection, and it plays an essential role for spirochetes to establish early infection and may also be important for spirochetal transmission from tick to mammal (Grimm et al., 2004b; Pal et al., 2004; Carrasco et al., 2015a).

The genome of B. burgdorferi comprises of more than 21 linear and circular endogenous plasmids with 5–56 kb in size (Fraser et al., 1997; Cäsjens et al., 2000, 2012), making its genome
have the largest plasmid content among any reported bacteria (Chaconas and Norris, 2013). Many endogenous plasmids encode outer surface lipoproteins, and propagation of spirochetes in vitro can lead to plasmid loss, which often results in attenuation of infection in mice or a defect in ticks (Purser and Norris, 2000). As such, a crucial step for genetic manipulation of B. burgdorferi is to perform plasmid profile for each clone to be sure the phenotype is not due to a plasmid loss. In addition, studies on the correlation between the loss of a particular plasmid and an in vitro or in vivo phenotype have led to identification of several virulence-associated genes (Purser et al., 2003; Revel et al., 2005). However, the contribution of each plasmid-encoded genes to the life cycle of B. burgdorferi remains largely unknown.

B. burgdorferi sensu lato (s.l.) complex includes B. burgdorferi sensu stricto (s.s.), B. afzelii, and B. garinii. B. afzelii, and B. garinii are found outside of the United States. B. burgdorferi s.s. are highly heterogeneous with significant variation in virulence and endogenous plasmid content. B. burgdorferi s.s. strain B31, which was isolated from a tick collected on Shelter Island (Burgdorfer et al., 1982), is the first sequenced strain and has been widely used for Lyme disease research. Another widely used B. burgdorferi strain is strain 297, which was isolated from the cerebral spinal fluid (CSF) sample of a Lyme borreliosis patient (Steere et al., 1983; Hughes et al., 1992). The endogenous plasmid sequence of B. burgdorferi strain 297 was published in 2011 (Schutzer et al., 2011).

The advantage of using B. burgdorferi strain 297 to study gene regulation of B. burgdorferi is that ospC is highly expressed under the in vitro cultivation condition. Expression of ospC is induced by elevated temperature (37°C relative to ambient temperature, 25°C), low pH (pH 6.8–7.0), and high cell density (Yang et al., 2000). In fact, while high level of ospC expression can be achieved in B. burgdorferi strain B31 by temperature shift (first at 25°C then shift to 35°C), ospC expression in strain 297 can be easily achieved by directly culturing the spirochete at 35 or 37°C without temperature shift. The level of OspC in 297 strain can be readily visualized as one of the most dominant bands in the protein profile when separated on Coomassie stained gel. This advantage has contributed in part, to the discovery of the first regulatory pathway in B. burgdorferi, the Rrp2-RpoN-RpoS pathway. In this pathway, the two-component system Hk2-Rrp2 activates the σN-σS (RpoN-RpoS) sigma factor cascade, leading to the production of σS (RpoS), which in turn, controls expression of many virulence genes including ospC and many other genes (for review, see, Samuels, 2011; Radolf et al., 2012; Ouyang et al., 2014; Ye et al., 2016b). In addition, many other factors including BosR (Hyde et al., 2009; Ouyang et al., 2009, 2011), BadR (Miller et al., 2013; Ouyang and Zhou, 2015), DsrA (Lybecke and Samuels, 2007), Rrp1 (Rogers et al., 2009; Sze et al., 2013; He et al., 2014), BtmA (Troxell et al., 2013), SodA (Esteve-Gassent et al., 2015), BBD18 (Dulebohn et al., 2014), also influence rpoS and ospC expressions.

Performing plasmid profile for strain 297 has been challenging, as the endogenous plasmid sequences for this strain were not available until recently. In the past, primers used for tracking most of the plasmids of strain 297 clones were designed based on the sequence of strain B31, which is not appropriate. As such, it is difficult to determine whether the parental low passage 297 strain is a single or mixed clones. Similarly, it is also difficult to determine whether the commonly used 297 clones derived from the original 297 strain have a complete plasmid content. In this study, based on published plasmid sequences of B. burgdorferi strain 297 (Schutzer et al., 2011; Casjens et al., 2012), we developed a multiplex PCR method specifically for rapid plasmid profiling of strain 297.

We analyzed plasmid profiles of the original 297 strain and the derived clones with different levels of ospC expression. Since it has been reported that genes carried on certain plasmids in strain B31, e.g., bbd18 on lp17, influence ospC expression (Sarkar et al., 2011; Dulebohn et al., 2014), we tested whether the difference in plasmid contents contributes to the variation in OspC levels among the clones. We found that different clones expressed different levels of BosR, and such difference, not the difference in their plasmid profiles, contributes to the ospC expression variation among B. burgdorferi strains.

**MATERIALS AND METHODS**

**B. burgdorferi Strains and Culture Conditions**

The parental B. burgdorferi strain 297 is a non-clonal strain, originally obtained from culturing the CSF sample of a Lyme borreliosis patient (Steere et al., 1983; Hughes et al., 1992). No more than three passages from the original strain was used in this study. AH130 and PL133 were derived clones from original strain 297. Spirochetes were cultivated in complete Barbour-Stoenner-Kelly-II (BSK-II) medium (Barbour, 1984) at 37°C with 5% CO2. Semi-solid agar plating of B. burgdorferi was carried out as previously described [complete Barbour-Stoenner-Kelly-II (BSK-II) medium with 2.5% agar] (Samuels, 1995). Types of colony morphology were observed 8–12 days after plating.

**Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting**

The method for SDS-PAGE was described previously (Carrasco et al., 2015b). Briefly, spirochetes were cultured from 104 cells/ml and harvested at day 7 (stationary phase 108 cells/ml) by centrifugation at 8,000 g for 10 min and washed two times with PBS (pH 7.4) at 4°C. Pellets were suspended in SDS buffer containing 50 mM Tris–HCl (pH 8.0), 2% sodium dodecyl sulfate (SDS), and 10 mM dithiothreitol (DTT). Cell lysates (5 × 107 cells per lane) were separated by 12% SDS-PAGE and stained with Coomassie blue or transferred to nitrocellulose membranes (GE-Healthcare, Milwaukee, WI). Membranes were blotted with monoclonal antibodies against FlaB, RpoS, and BosR (He et al., 2008; Xu et al., 2010; Troxell et al., 2013) with 1:1,000, 1:50, and 1:500 dilutions, respectively, and then with goat anti-mouse IgG-HRP secondary antibody (1:1,000, Santa Cruz Biotechnology). Detection of horseradish peroxidase activity was determined using enhanced chemiluminescence method (Thermo Pierce ECL Western Blotting Substrate), and subsequently by exposure to X-ray film.
Primer Design
Multi-alignment among all 19 endogenous plasmid sequences of *B. burgdorferi* strain 297 (Table 1) were performed using ALIGNMENT tool in Software Vector NTI (Thermo Fisher Scientific, CA). The identified unique sequences of each primer pair for PCR of each plasmid were then subjected to BLAST analysis (National Center for Biotechnology Information) against all published *Borrelia* chromosomal sequences in order to confirm no potential homology with chromosomal sequence (note that *B. burgdorferi* strain 297 chromosomal sequence is not available). General guidelines for primer design were as follows: (a) Primers should be at least 20 nucleotides but not longer than 28 nucleotides, to minimize the possibility of primer-dimer formation and unspecific binding; (b) CG nucleotides should be distributed equally on both ends; (c) All PCR products should differ in size by at least 20 bp in order to allow efficient separation by gel electrophoresis. See primer sequences in Tables 2, 3.

Multiplex PCR and Gel Electrophoresis
PCR was performed by using TaKaRa Ex Taq kit (Clontech, USA). Twenty five microliters of optimized PCR reaction includes 100 ng of boiled whole cell lysate of *B. burgdorferi* (as DNA template), 2.5 µl of 10 × Ex Taq Buffer, 2.5 µl of 10 × dNTP Mixture, 1 µl of Ex Taq DNA polymerase, and 2 µl of 12.5 × primer mix. Given that each primer pair has different amplification efficiency, the concentrations for each primer pair used in the primer mix were adjusted based on the PCR result. For the primer mix to amplify linear plasmids (mixLp), 222 µl of 12.5 × mixLp primer stock includes12 µl of 50 µM primer stock for lp25, lp54, lp28-4, 15 µl for lp38, and 10 µl for lp28-1, lp28-3, lp28-5, lp28-6, lp17, lp36. For the primer mix to amplify circular plasmids (mixCp), 210 µl of 12.5 × mixCp primer stock includes 15 µl of 50 µM primer stock for cp32-3, 10 µl for cp26, 10 µl for cp32-1, cp32-4, cp32-5, cp32-6, cp32-9, cp32-11, cp32-12. The PCR reaction conditions were as the following: (1) 1 cycle of initial denaturation at 94°C for 5 mins; (2) 40 cycles of amplification including denaturation at 94°C for 30 S, annealing at 54°C for 1 min, and extension at 68°C for 1 min; (3) 1 cycle of final extension at 68°C for 5 min. PCR products were separated using 3% metaphor agarose gel (Lonza, Cohasset MN, USA) in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 2 mM EDTA, pH 8.2–8.4) by running 100 V for 1 h and then 80 V for 2 h.

RESULTS
Establish a Multiplex PCR Method for Rapid Plasmid Profiling of *B. burgdorferi* Strain 297
Genome sequence revealed that *B. burgdorferi* strain 297 contains at least 10 linear and 10 circular plasmids ranging from 17 to 54 Kb. The published plasmid sequences for strain 297 include 19 plasmids (Schutzer et al., 2011; Casjens et al., 2012). The strain 297 used for sequencing lost linear plasmid, lp25, so lp25 sequence of strain 297 is not available. In *B. burgdorferi* strain B31, lp25 contains at least two genes *pncA* and *bptA*, which are important for the enzootic cycle of *B. burgdorferi* (Purser et al., 2003; Grimm et al., 2004a; Revel et al., 2005). It was reported previously that strain 297 has lp25 (Grimm et al., 2004a; Revel et al., 2005). Of note, two previously reported circular plasmids, cp18-1 and cp18-2 in strain 297 (Caijano et al., 2000), were the result of ~9 Kbp truncation from cp32, and were subsequently renamed as cp32-7 and cp32-9 (Casjens et al., 2012).

All PCR primer pairs specific for each endogenous plasmid were designed based on multi-alignment of all published plasmid sequences of *B. burgdorferi* strain 297 (Schutzer et al., 2011), followed by BLAST analyses against published *B. burgdorferi* B31 chromosomal sequences (see Materials and Methods). Since the sequence of lp25 of strain 297 is not available, the primer pair for lp25 of strain 297 was designed based on the *bptA* gene sequence from lp25 of strain B31 (Revel et al., 2005). The specificity and efficiency of each primer pair for corresponding plasmid were confirmed by regular PCR (Figures 1A,B). Then, primer pairs for all linear plasmids or circular plasmids were mixed together (mixLp or mixCp), and two multiplex PCR reactions were performed accordingly. The sizes of PCR products in each of the two groups were designed to differ at least 20 bps, so that they could be readily separated by gel electrophoresis (Figure 1).

Comparison of Plasmid Contents among *B. burgdorferi* Clones with Different OspC Levels
Having established a rapid plasmid profiling method specific for strain 297, we sought to examine the plasmid contents of some commonly used clones of *B. burgdorferi* strain 297. Clone AH130 was isolated on semi-solid agar plate from the
TABLE 2 | PCR primer sequences for each linear plasmid.

| Linear plasmid | Sequence 5'->3' | Length (bp) | Coordination 5'->3' | Tm (°C) | Concentration in 12.5 x primer mix (µM) | Amplicon size (bp) |
|----------------|----------------|-------------|---------------------|---------|----------------------------------------|-------------------|
| lp25F*         | CGTTATCTACCGTTTATAGTTGGA | 25 | 266-290 | 52.5 | 2.7 | 100 |
| lp25R*         | TTAGAACCTCTACCGTTTATAGTTGGA | 27 | 367-341 | 55.3 | 2.7 | 125 |
| lp54F          | TCTTAATCTAACCCTAGAATATTC | 25 | 965-989 | 48.0 | 2.7 | 175 |
| lp54R          | TAACTTACCGTTTATAGTTGGA | 25 | 1,089-1,065 | 53.7 | 2.7 | 200 |
| lp28-4F        | CTGGGCTTATCAGACGAGGTTGGA | 25 | 53,000-5,324 | 53.0 | 2.25 | 251 |
| lp28-4R        | TTAACAGAGCTGAGGAGGTTGGA | 25 | 5,499-5,475 | 51.5 | 2.25 | 251 |
| lp28-1F        | TGAACCTCTACCGTTTATAGTTGGA | 25 | 5,400-5,324 | 53.0 | 2.25 | 251 |
| lp28-1R        | CGACTTCTTATATAGCTGAGGTTGGA | 25 | 5,425-5,400 | 53.0 | 2.25 | 251 |
| lp28-3F        | ATGGTCTACCGTTTATAGTTGGA | 25 | 5,830-5,755 | 54.9 | 2.7 | 251 |
| lp28-3R        | TTGGTCTACTCCCTTATAGTTGGA | 25 | 5,485-5,400 | 53.0 | 2.25 | 251 |
| *The primer sequences for lp25 were based on bptA gene sequence.

TABLE 3 | PCR primer sequences for each circular plasmid.

| Circular plasmid | Sequence 5'->3' | Length(bp) | Coordination 5'->3' | Tm (°C) | Concentration in 12.5 x primer mix (µM) | Amplicon size (bp) |
|-----------------|----------------|-------------|---------------------|---------|----------------------------------------|-------------------|
| cp32-4F         | TGACAAGCAACAGTAGATGATGCTT | 25 | 27,800-27,824 | 58.6 | 2.38 | 121 |
| cp32-4R         | CGGCGGATAATCGTCTCTTTATAGTTGGA | 23 | 27,920-27,898 | 56.8 | 2.38 | 153 |
| cp32-5F         | AAGGCGGTACAGCTATTACAGCTTTATAAACAA | 27 | 20,972-20,998 | 58.0 | 2.38 | 176 |
| cp32-5R         | AACCAAGGATATCAAGCTTTATAAACAA | 27 | 21,124-21,099 | 51.5 | 2.38 | 212 |
| cp32-1F         | AAACATTAGTAGAAAGTGAATTTGATTTAC | 30 | 21,109-21,138 | 51.5 | 2.38 | 225 |
| cp32-1R         | CCTATTGAAAGGATATCAAGCTTTATAAACAA | 25 | 21,280-21,196 | 51.5 | 2.38 | 225 |
| cp32-6F         | TGAGCAGCACAAGTAGATGATGCTT | 25 | 27,800-27,824 | 58.6 | 2.38 | 121 |
| cp32-6R         | CCGGGGATAATGCTAGTCAACAA | 23 | 27,920-27,898 | 56.8 | 2.38 | 153 |
| cp32-7F         | TTCAATGAATCCGGATGATGTTGA | 24 | 28,158-28,180 | 56.5 | 2.38 | 197 |
| cp32-7R         | TTAGTAGTTTATATAGTTGGA | 24 | 28,352-28,328 | 55.0 | 2.38 | 225 |
| cp32-3F         | CCATTATCTTAAAGGAGGAGGTTGGA | 25 | 18,535-18,558 | 54.7 | 2.38 | 225 |
| cp32-3R         | TGAGGATTTCTGTTGGAATTTGAGGTTGGA | 25 | 18,759-18,734 | 48.5 | 2.38 | 225 |
| cp32-12F        | TTTGTATCCTTATCTGTTATACCAT | 25 | 16,474-16,498 | 47.5 | 3.57 | 251 |
| cp32-12R        | TTAGGACTATTCGTTGGAATTTGAGGTTGGA | 25 | 16,724-16,700 | 56.8 | 2.38 | 251 |
| cp32-9F         | GGTGTTGCCATTATTTAAGGAGGAGGTTGGA | 25 | 1,341-1,364 | 49.1 | 2.38 | 295 |
| cp32-9R         | TTAGCACTCTTCGTTGGAATTTGAGGTTGGA | 25 | 1,681-1,657 | 55.3 | 2.38 | 341 |
| cp32-11F        | GTTTGTGCGCTTATTTAAGGAGGAGGTTGGA | 25 | 27,718-27,742 | 51.5 | 2.38 | 375 |
| cp32-11R        | GGATTTGTTTATCTGGTCTGTTGGA | 25 | 28,092-28,068 | 53.8 | 2.38 | 375 |
| cp26F           | GGAAAACTTACAAGGGATTTGAGGAGGTTGGA | 25 | 7,094-7,119 | 56.8 | 1.58 | 398 |
| cp26R           | TTAGGCTTCTCAGGAGGAGGCTTCCAT | 25 | 7,491-7,467 | 61.4 | 1.58 | 398 |

parental low passage B. burgdorferi strain 297 (Yang et al., 2004). Clone PL133 was isolated from mice after repeated rounds of needle inoculation by culturing ear-punch biopsies (Revel et al., 2005; Blevins et al., 2007). Although both AH130 and PL133 are infectious in mice, they produced different levels of OspC under the in vitro cultivation conditions: AH130 produces very
high level of OspC at 35 or 37°C at late logarithmic phase of growth (Yang et al., 2004; Gilbert et al., 2007; Ye et al., 2016a), even without performing the temperature shift from 23 to 35°C, a condition often required for OspC induction in strain B31 (Schwan and Piesman, 2000). On the other hand, PL133 had relative low level of OspC (Figure 2A).

Given that certain gene(s) on endogenous plasmids can affect OspC levels of *B. burgdorferi* (Sarkar et al., 2011), it is possible that AH130 and PL133 may have different plasmid content when isolated from parental 297 strain, resulting in a phenotype of different OspC expression. Although AH130 and PL133 were thought to have a complete plasmid content, the previous work was done using B31 plasmid sequences since the 297 plasmid sequences was not available till recently. Upon examining the plasmid content, we found that AH130 and PL133 actually do not have a complete plasmid content: AH130 lost the linear plasmid lp28-5, whereas PL133 lost a circular plasmid cp32-3 (Figure 2B). These results suggest that (1) the parental *B. burgdorferi* strain 297 is a mixed clone; (2) the difference in plasmid contents may contribute to the difference in OspC production between AH130 and PL133.

**Absence of lp28-5 is Not Associated with High OspC Level**

AH130 expressed very high level of OspC. One possibility is that lp28-5 may encodes a gene that is inhibitory to *ospC* expression, and absence of lp28-5 leads to high OspC level. Given that the parental clone 297 contains mixed clones with OspC high or low phenotypes, one cannot draw conclusion from parental 297 that the presence of lp28-5 can also have high OspC level. Thus, we investigated whether absence of lp28-5 leads to high OspC level by screening 297 clones isolated from semi-solid agar plate that lack lp28-5, and identified three such clones (A-1, B-2, D-1) (Figure 3A). While two lp28-5-lacking clones (B-2, D-1) exhibited high OspC level, one clone (A-1) had no detectable level of OspC on the Coomassie stained SDS gel (Figure 3B). This data suggests that absence of lp28-5 does not cause high level of *ospC* expression. Regarding the potential association between loss of cp32-3 and the low OspC level observed in PL133, we screened more than 50 clones to identify additional 297 clone lacking cp32-3 but were not successful.
Different Levels of RpoS and BosR among B. burgdorferi 297 Clones

To further investigate what may contribute to the ospC expression variation among strain 297 clones, we examined the upstream regulators that govern ospC expression. OspC has a RpoS-type promoter which is controlled by RpoS (Hübner et al., 2001; Yang et al., 2005). As shown in Figures 4A,B, the low OspC clones, PL133 and A-1, had very low or undetectable levels of RpoS in comparison to high OspC clones (AH130, B-2, D-1). This result indicates that variation in OspC levels is due to the difference in RpoS levels in B. burgdorferi 297 clones.

Many factors can influence rpoS and ospC expression such as Rrp2, RpoN, BosR, BadR, Rrp1, DsrA, BtmA, SodA, and BBD18 (Lybecker and Samuels, 2007; Hyde et al., 2009; Ouyang et al., 2009, 2011; Rogers et al., 2009; Miller et al., 2013; Sze et al., 2013; Troxell et al., 2013; Dulebohn et al., 2014; He et al., 2014; Esteve-Gassent et al., 2015; Ouyang and Zhou, 2015). In particular, expression of rpoS is directly activated by the alternative factor RpoN as well as the transcriptional factor, BosR. Activation of the RpoN pathway is governed by phosphorylation of the enhancer binding protein Rrp2 (Yang et al., 2003; Boardman et al., 2008). We recently showed that Rrp2 and RpoN is likely constitutively active under in vitro cultivation conditions, since phosphorylation of Rrp2 is vital for B. burgdorferi replication and Rrp2 is constitutively phosphorylated during in vitro replication (Groshong et al., 2012; Yin et al., 2016). Thus, Rrp2 is unlikely the factor that contributes to ospC expression variation among B. burgdorferi strains. Thus, we focused on the second factor, BosR. As shown in Figure 4B, PL133 and A-1 had very low or undetectable levels of BosR in comparison to high OspC clones (AH130, B-2, D-1). These results suggest that there is a difference in BosR levels among B. burgdorferi strains, which results in different levels of RpoS, and subsequently, ospC expression variation.

DISCUSSION

B. burgdorferi strain 297 is one of the widely used strain in borrelial research, especially for studying differential gene expression. Lack of a plasmid profiling method have made it difficult to track the plasmid contents of strain 297 during in vitro growth and genetic manipulation. Herein, we developed a multiplex PCR method that allows rapid plasmid profiling of strain 297. With such method, we found that the parental 297 strain is likely a mixed strain. Two of the commonly used infectious clones of strain 297, AH130 and PL133, which
were previously thought having a complete endogenous plasmid content based on previous plasmid analysis using B31 plasmid sequences-derived primers, actually lost lp28-5 and cp32-3, respectively. We further showed that the difference in plasmid profiles among B. burgdorferi clones could not explain ospC expression variation among the clones. On the other hand, we observed that there is a difference in BosR levels among the clones, which contributes to ospC expression variation.

Tracking each endogenous plasmid during every step of genetic manipulation of B. burgdorferi by PCR is time-consuming. Previously, Bunikis et al., developed a multiplex PCR as a tool for validating plasmid profiling of B. burgdorferi strain B31, which has significantly improved the efficiency of the procedure of plasmid profiling (Bunikis et al., 2011). Unfortunately, the optimized primer pairs for plasmid profiling of strain B31 cannot be applied for other B. burgdorferi strains such as strain 297, due to sequence variation of endogenous plasmids among B. burgdorferi strains. The rapid plasmid profiling specifically for strain 297 reported in this study should be widely useful for borrelial research whenever B. burgdorferi 297 is used.

The data presented here shows that lp28-5 does not contribute to ospC expression variation among B. burgdorferi strains. Although we were unable to identify other clones missing cp32-3, the redundancy of cp32 plasmids in B. burgdorferi makes it unlikely that loss of cp32-3 is responsible for the variation in OspC expression. Thus, this study suggests that other yet-to-be identified genetic differences between the two strains contributes to OspC expression variation. On the other hand, this result does not conclude that endogenous plasmids do not play a role in regulation of ospC expression. In fact, bbd18 on lp17 of B. burgdorferi strain B31 plays a negative role in regulation of rpoS and ospC expression (Sarkar et al., 2011; Dulebohn et al., 2014). The 297 strain also contains a similar lp17 plasmid. lp17 is one of the endogenous plasmids that are well-retained by B. burgdorferi under in vitro cultivation (Purser and Norris, 2000), so it is not surprising that we did not find a 297 clone lacking this plasmid.

The pattern of protein expression in the low OspC expressing clone A1 varied in different preparations and was sometimes significantly different from that seen in the high OspC expressing clones. For example, we noticed an increased intensity of a band below OspC in clone A1 in Figure 4A, but not in Figure 3. It has previously been proposed that downregulation of OspC is accompanied by upregulation of other lipoproteins (He et al., 2008; Xu et al., 2008). It is noteworthy that different clones derived from isolates of B. burgdorferi strain B31 have been shown to have considerable heterogeneity not only in protein profile and plasmid content, but also in colony phenotype on solid agar (Elias et al., 2002). We screened and compared strain 297 clones with different colony morphologies, and found that colony morphology is not associated with plasmid content (data not shown).

In addition, B. burgdorferi strain B31 presents different types of colony morphology on the semi-solid agar plate. Thus, we also screened and compared groups with different colony morphologies, and found that colony morphology is not associated with plasmid content (data not shown).

BosR is a FurR/PerR homolog (for review, see, Samuels and Radolf, 2009). It is required for activation of rpoS expression in B. burgdorferi, but the mechanism remains unknown. Recently, it was reported that BosR undergoes autoregulation (Ouyang et al., 2016). However, regulation of bosR expression in response to environmental signals by large remains unknown. The study herein demonstrates that the BosR levels are highly variable among B. burgdorferi strains. Continuing efforts on the investigation of clonal differences will likely help uncover the mechanism of regulation of BosR by environmental factors.

**ETHICS STATEMENT**

The work conducted in the manuscript was approved by Indiana University School of Medicine Biosafety Committee (protocol number 643).

**AUTHOR CONTRIBUTIONS**

XX, YY, XY, YL conceived and designed the experiments; XX, JD, and TL performed the experiments; XX and TC analyzed the data; XX, YL, and XY wrote the paper. XX and YY contributed equally to this work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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