

# The Oligopeptide ABC transporter OppA4 negatively regulates the virulence factor OspC production of the Lyme disease pathogen

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Running Title: OppA4 negatively regulates *ospC* expression

## ABSTRACT

*Borrelia burgdorferi* sensu lato, the agent of Lyme disease, exists in nature through a complex enzootic life cycle that involves both ticks and mammals. The *B. burgdorferi* genome encodes five Oligopeptide ABC transporters (Opp) that are predicted to be involve in transport of various nutrients. Previously, it was reported that OppA5 is important for the optimal production of OspC, a major virulence factor of *B. burgdorferi*. In this study, possible role of another Oligopeptide ABC transporter, OppA4 in *ospC* expression was investigated by construction of an *oppA4* deletion mutant and the complemented

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strain. Inactivation of *oppA4* resulted an increased production of OspC, suggesting that OppA4 has a negative impact on *ospC* expression. Expression of *ospC* is controlled by Rrp2-RpoN-RpoS, the central pathway essential for mammal infection. We showed that increased *ospC* expression in the *oppA4* mutant was due to an increased *rpoS* expression. We then further investigated how OppA4 negatively regulates this pathway. Two regulators, BosR and BadR, are known to positively and negatively, respectively, regulate the Rrp2-RpoN-RpoS pathway. We found that deletion of *oppA4* resulted in an increased level of BosR. Previous reports showed that *bosR* is mainly regulated at the post-transcriptional level by other factors. However, OppA4 appears to negatively regulate *bosR* expression at the transcriptional level. The finding of OppA4 involved in regulation of the Rrp2-RpoN-RpoS pathway further reinforces the importance of nutritional virulence to the enzootic cycle of *B. burgdorferi*.

Key Words: *Borrelia burgdorferi*, OppA4, OspC

## INTRODUCTION

*B. burgdorferi* sensu lato, the Lyme disease/borreliosis pathogen, is maintained in nature in an enzootic cycle between *Ixodes* tick vectors and certain vertebrates, so-called reservoir hosts (Lane et al., 1991; Radolf et al., 2012). This spirochetal pathogen dramatically regulates its gene expression during its transition between ticks and the reservoir hosts (for recent reviews, see Caimano et al., 2016; Samuels, 2011; Stevenson and Seshu, 2017; Ye et al., 2016). During the process of transmission, spirochetes in the midgut of nymphal ticks begin replication triggered by tick blood meal engorgement process, activate a

two-component response regulator and enhancer-binding protein Rrp2, which in turn, activates the alternative sigma factor RpoN ( $\sigma^N$ ), leading to the transcription of *rpoS* that encodes for another alternative sigma factor and global regulator RpoS ( $\sigma^S$ ). This Rrp2-RpoN-RpoS pathway controls production of a few of surface proteins that have been shown to be important for mammalian infection (e.g., OspC, DbpA/B, BBK32, BBA33, BBA64, BBA66, etc), concomitant with downregulating several factors important for spirochetal colonization in ticks (e.g., OspA, P22, lp6.6) (for review and initial discoveries of this pathway, see Caimano et al., 2016; Hübner et al., 2001; Radolf et al., 2012; Samuels, 2011; Yang et al., 2003). On the other hand, this pathway is repressed in the tick midgut during the process of tick acquisition of spirochetes (i.e., spirochetes migrate from infected vertebrate hosts to uninfected ticks), with downregulation of mammalian infection-associated proteins while upregulation of proteins important for tick colonization (Ouyang et al., 2008; Radolf et al., 2012).

Given the importance of the Rrp2-RpoN-RpoS pathway, many signals and factors have shown to regulate *rpoS* expression. *rpoS* has a  $\sigma^{54}$ -type promoter, whose expression is under the control of Rrp2 and RpoN ( $\sigma^{54}$ ) and. In addition, a Fur/PerR-like transcription factor, BosR, is also required for *rpoS* and *ospC* expression (Hyde et al., 2009; Ouyang et al., 2011; Ouyang et al., 2009; Wang et al., 2017). BosR is capable of binding to the promoter region of *rpoS in vitro*, the mechanism of how BosR is involved in activating *rpoS* transcription from a  $\sigma^{54}$  promoter remains unclear (Hyde et al., 2009; Ouyang et al., 2011; Ouyang et al., 2009; Wang et al., 2017). Another transcription factor, BadR, directly represses *rpoS* expression (Miller et al., 2013; Ouyang and Zhou, 2015). Additional factors, including DsrA (Lybecker and Samuels, 2007), Rrp1 and PlzA (He et al., 2014; Rogers et al., 2009; Sze et al., 2013), BtmA (Troxell et al., 2013), SodA (Esteve-Gassent et al., 2015), BBD18 (Dulebohn et al., 2014), are also shown to influence *rpoS* and *ospC* expression (for review, see Samuels, 2011; Stevenson and Seshu, 2017).

However, what signal(s) *B. burgdorferi* senses and how the signals feed into this pathway, are largely unknown.

*B. burgdorferi* has a reduced genome and lacks many biosynthetic pathways for amino acids and other essential nutrients, and thus relies on transporters to obtain nutrients from vertebrate and tick hosts (Casjens et al., 2000; Fraser et al., 1997; Groshong et al., 2017; Radolf et al., 2012). The oligopeptide permease (Opp) transporter system is an efficient system for bacteria to transport multiple amino acids (Garai et al., 2017; H., 2010). It consists of an oligopeptide-binding protein (OppA), a membrane permease (OppBC), and a nucleotide-binding domain (NBD) protein (OppDF) (Garai et al., 2017; Monnet, 2003). *B. burgdorferi* has five OppA proteins (OppA1 to OppA5), two OppBC permeases, and one OppDF (Bono et al., 1998; Raju et al., 2011; Wang et al., 2004). *oppA1-oppA3*, *oppB1*, *oppC1*, *oppD*, *oppF* are linked together and located in the chromosome, and *oppB2-oppC2* are located at different loci of the chromosome. Two additional oligopeptide-binding proteins *oppA4* and *oppA5*, encode on the endogenous plasmids cp26 and lp54, respectively (Bono et al., 1998; Fraser et al., 1997; Groshong et al., 2017). Previous studies using the *Escherichia coli* as a surrogate system showed that the OppA systems of *B. burgdorferi* can import small peptides in *E. coli*, and the specificity of peptide binding for OppA1, OppA2, and OppA3 were determined (Lin et al., 2001; Wang et al., 2004). All five OppA proteins have conserved residues for structure folding and peptide binding found in other OppA proteins (Lin et al., 2001; Wang et al., 2004). Sequence analysis indicates that OppA4 and OppA5 cluster together, OppA1 and OppA3 form another group, and OppA2 forms its own clade (Groshong et al., 2017). Recently, the structures of five OppA proteins of *B. burgdorferi* were analyzed based on the crystal structure of OppA4 (Fairman JW et al., 2012; Groshong et al., 2017), suggesting that OppA2 and OppA4 likely bind to negatively charged peptides, OppA2 and OppA5 prefer positively charged peptides, and OppA3 may bind a neutral peptides (Groshong et al., 2017).

The *oppA* genes of *B. burgdorferi* were differentially regulated by environmental conditions as well as during the enzootic cycle of *B. burgdorferi* (Troy et al., 2016; Drecktrah et al., 2015; Groshong et al., 2017; Iyer et al., 2015; Medrano et al., 2007; Wang et al., 2002). *oppA1* and *oppA3* are expressed in the feeding tick, and *oppA2* is expressed at relatively high levels throughout the enzootic cycle (Groshong et al., 2017). The two plasmid-coded *oppA* genes, *oppA4* and *oppA5*, are the most differentially expressed: *oppA5* is preferably expressed in mammalian host condition, whereas *oppA4* is expressed ticks during the larval and nymphal blood meals (Groshong et al., 2017; Wang et al., 2002). *oppA5* expression is positively regulated by the Rrp2-RpoN-RpoS pathway, but not other *oppA* genes (Medrano et al., 2007). Targeted mutagenesis showed that inactivation of *oppA5* impaired expression of *rpoS* and *ospC* (Raju et al., 2011), but did not affect mammalian infection. However, whether *OppA4* influences virulence gene expression and whether it plays a role in the enzootic cycle of *B. burgdorferi* have not been determined. In this study, we report targeted inactivation of *oppA4* and the complemented strain, and studied its influence on the Rrp2-RpoN-RpoS pathway and the mammalian infection.

## MATERIALS AND METHODS

### Bacterial strains

All strains and plasmids used in this study are described in **Table 1**. Low-passage, virulent *B. burgdorferi* strain 5A18NP1 was kindly provided by Drs. Norris and Kawabata (Kawabata et al., 2004). *B. burgdorferi* were cultivated in Barbour-Stoenner-Kelly (BSK-II) medium supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR) at 37°C with 5% CO<sub>2</sub>. Relevant antibiotics were added to the cultures at the following final concentrations: 300 µg/ml for kanamycin (Kan), 50 µg/ml for streptomycin (Strep), and 50 µg/ml for gentamicin (Gent). The constructed suicide vectors and shuttle vector were maintained in *Escherichia coli* strain DH5α.

### Construction of the *oppA4* deletion mutant

To inactivate *oppA4* gene in 5A18NP1, a suicide vector pZB020 was constructed for homologous recombination as following: the regions of DNA corresponding to 1.1 kb upstream and 1.5 kb downstream regions of *oppA4* were PCR amplified from 5A18NP1 genomic DNA with primer pair PRBB067/PRBB068 and PRBB049/PRBB050 (**Table 1**), respectively. The resulting PCR fragments were cloned into pMP025 and confirmed by restriction enzyme digestion and sequencing. Firstly, pMP025 and the fragment amplified by PRBB067/PRBB068 were digested by ApaI and SalI and ligated to generate pZB020A. Then, pZB020A and the fragment amplified by PRBB049/PRBB050 were digested by XmaI and BamHI and ligated. pZB020 was constructed and confirmed by both restriction enzyme digestion and sequencing, then it is used for transformation. Transformants were selected with Kan and Strep, and twelve

positive clones were obtained. PCR analysis was used to confirm correct marker insertion and inactivation of the chromosomal copy of *oppA4*. Endogenous plasmid profiles were performed as previously described (Bunikis et al., 2011; Xiang et al., 2017; Ye et al., 2014). The positive clones were named BBZB014.

### **Construction of the *oppA4* complemented strain.**

To complement *oppA4* in BBZB014, we constructed a shuttle vector, pZB028, that harbors a wild-type *oppA4* gene (with a human influenza virus hemagglutinin [HA] tag at the 5' end of the coding region) driven by *flaB* promoter. Wide-type *oppA4* with a HA-tag was amplified by PRBB034/PRBB056 and digested by NdeI and PstI, the resulting fragment was ligated into pYY025, which harbors a *flgB* promoter (*flgBp*)-*aadA* cascade (streptomycin-resistant gene), resulting in pZB028A. The positive clones were identified by restriction enzyme digestion and sequencing. Then pZB028A was digested by BglII and AatII to remove *flgBp-aadA* (streptomycin-resistant gene) and ligated with *flgBp-aacC1* (gentamicin-resistant gene) digested by BglII and AatII. To get rid of BglII cutting site in *aacC1*, we introduced a point mutation to change BglII cutting site from AGATCT to AAATCT without change of amino acid sequence by overlap PCR. To make this, we use pYY020 as template and amplify *flgBp*-partial *aacC1* and the rest partial *aacC1* by PRYY049/PRBB053, PRBB052/PRYY056, respectively. And the whole *flgBp-aacC1* without BglII was amplified by PRYY049 and PRYY056 using the two fragments amplified above. The new *flgBp-aacC1* without BglII was ligated into pZB028A, resulting in pZB028. Positive clones were confirmed by restriction enzyme digestion and sequencing. Then pZB028 was transformed to BBZB014 for complementation. Complemented strains were selected by Kanamycin, Streptomycin and Gentamicin. Positive clones were analyzed by PCR, SDS-PAGE and immunoblotting and named as BBZB018.

**SDS-PAGE and immunoblotting.**

SDS-PAGE and immunoblotting were performed as previously described (Troxell et al., 2013). The images were developed using chemiluminescent method (Pierce ECL Western Blotting Substrate, Thermo Scientific, IL). All proteins were separated on 12.5% SDS-PAGE. Spirochetes were collected at both early phase and late-log phase for immunoblotting. Antibodies directed against BosR, RpoS, BadR, OspC and FlaB have been described previously (Xu et al., 2010).

**RNA isolation and quantitative RT-PCR**

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To reduce trace amounts of DNA contamination, samples were further digested with RNase-free DNaseI (Qiagen), and purified using the RNeasy kit (Qiagen). DNA-free RNA was confirmed by PCR. cDNA was generated using the SuperScript III First-Strand Synthesis System according to the manufacturer's protocol (Invitrogen). Real-time quantitative PCR was performed using the RT2 SYBR Green ROX qPCR Mastermix (Qiagen) on an ABI 7000 sequence detection system. As previously described (Yang et al., 2009), changes in gene expression level were analyzed through relative quantification method ( $\Delta\Delta CT$ ) using *flaB* of *B. burgdorferi* for normalization. Statistical analysis of the data were performed using an unpaired, two-tailed Student's t-test, in which statistical significance was determined when  $P < 0.05$ .

**Mouse infection.** All mouse experiments were approved by the IACUC committee of Indiana University School of Medicine under the protocol number # 11339 MD/R/USDA/HZ/AR. Four-week-old C3H/HeN mice (Harlan, Indianapolis, IN) were subcutaneously inoculated with  $1 \times 10^5$  spirochetes. Ear punch was done 4 weeks after infection and cultivated in 2 ml of the BSK-II medium (Sigma-Aldrich, St. Louis, MO) containing an antibiotic mixture of phosphomycin (2 mg/ml), rifampin (5 mg/ml), and amphotericin B



(250 mg/ml) (Sigma-Aldrich). All cultures were maintained at 37°C and examined for the presence of spirochetes by dark-field microscopy beginning from 5 days after inoculation. Mouse infection was determined by positive growth of ear punch culture.

ACCEPTED MANUSCRIPT

## RESULTS

### Construction of the *oppA4* mutant and complemented strain

The *oppA4* mutant was generated by allele exchange via homologous recombination by transforming a suicide vector (pZB020) carrying *aadA* (Strep-resistant cassette) flanked by 1.1 kb upstream and 1.5 kb downstream regions of *oppA4* into an infectious *B. burgdorferi* strain B31-5A18NP1 (**Fig. 1A**). Twelve positive clones were obtained and PCR analysis was performed to confirm that *oppA4* was replaced by the *aadA* marker (**Fig. 1C**). The *oppA4* mutant was designated OppA4-Mut. For complementation, a shuttle vector containing a *flaB*-driven *oppA4* gene (pZB028, **Fig. 1B**) was transformed with the *oppA4* mutant. Positive clones were confirmed by PCR (**Fig. 1C**), and the complemented strain was designated OppA4-Com. qRT-PCR analysis showed that the *oppA4* mutant lost *oppA4* expression (**Fig. 1D**). The complemented strain showed much higher *oppA4* expression level, as the result of the *flaB* promoter (**Fig. 1D**). Endogenous plasmid profiles were examined, and one clone of the *oppA4* mutant and the complemented strain with identical plasmid contents with the parental strain was chosen for further study (**Fig. 2**).

### Inactivation of *oppA4* increased the OspC level

The *oppA4* mutant had similar growth rate *in vitro* as that of parental strain (data not shown). To examine the effect of *oppA4* deletion on protein profile of *B. burgdorferi*, spirochetes were grown in the BSK-II medium at 37°C, and harvested at the mid-logarithmic phase (M,  $1 \times 10^7$  cells/ml, M) and stationary phase (S,  $5 \times 10^8$  cells/ml) (**Fig. 3**). As expected, wild-type *B. burgdorferi* did not have visible OspC production on SDS-PAGE gel at the mid-log phase, but readily detected at the stationary phase (**Fig. 3**). In contrast, the *oppA4* mutant showed readily visible OspC production even at the mid-log phase, and the level of

OspC was further increased at the stationary phase (**Fig. 3**). Enhanced OspC levels in the *oppA4* mutant in both mid-log and stationary phase spirochetes were more apparent in the immunoblot assay using monoclonal antibody against OspC (**Fig. 4**). Noted that 50-fold less samples were loaded for immunoblotting than that used for SDS-PAGE gel because of high levels of OspC. Further qRT-PCR analysis demonstrated that the enhanced OspC production in the *oppA4* mutant occurred at the transcription level (**Fig. 5**). Complementation restored the OspC pattern close that in wild-type spirochetes (**Fig. 4 & 5**). These data suggest that OppA4 negatively regulates *ospC* expression.

### **Inactivation of *oppA4* increased the RpoS and BosR levels**

*ospC* has an RpoS-type promoter and its expression is under the direct control of RpoS (Hübner et al., 2001; Lybecker and Samuels, 2007; Yang et al., 2005). Accordingly, expression of *rpoS* at mRNA and protein levels were examined. The result showed that the *oppA4* mutant had increased levels of *rpoS* expression at both mRNA (~8 folds) and protein levels (~50 folds) (**Fig. 4 & 5**). As an initial attempt to understand how OppA4 negatively regulates *rpoS* and subsequently *ospC* expression, we investigated the known factors involved in the Rrp2-RpoN-RpoS pathway. Specifically, we focused on two major factors BosR and BadR. BosR positively regulates *rpoS* expression, while BadR negatively regulates *rpoS* expression. The result showed that the BosR level, not BadR, was upregulated in the *oppA4* mutant in spirochetes harvested at both mid-log and stationary phases (**Fig. 4**), suggesting that OppA4 down-regulates *rpoS* and *ospC* expression via BosR.

### **OppA4 negatively regulates *bosR* at the transcriptional level**

The BosR level is often regulated at the post-transcription level (Hyde et al., 2007; Troxell et al., 2013). Transcriptional regulation of *bosR* was also reported previously (He et al., 2014; Ouyang and Zhou,

2015). Thus, levels of *bosR* mRNA were determined in the *oppA4* mutant. As shown in **Fig. 5**, there was a significant increase in *bosR* mRNA levels in the *oppA4* mutant in comparison to wild-type and the complemented strains. This result indicates that OppA4 negatively regulates *bosR* at transcriptional level.

#### **OppA4 is not essential for mammalian infection.**

To examine the *oppA4* mutant's ability to infect mice, groups of C3H/HeN mice were needle inoculated with wild-type, the *oppA4* mutant, or the complemented strain with a dose of  $1 \times 10^5$  spirochetes per mouse. Ear punch biopsies were collected 4 weeks post-infection and then cultured for presence of spirochetes. As shown in **Table 2**, all mice inoculated with wild-type *B. burgdorferi* were culture-positive. Four out of five mice infected with the *oppA4* mutant showed culture positive. Since the complemented strain also had the same number of mice infected, this minor difference in infection was not related to *oppA4* inactivation. These data suggested that OppA4 is not essential for *B. burgdorferi* to establish infection in mice.

## DISCUSSION

In this study, we successfully constructed an *oppA4* deletion mutant and the complemented strain in *B. burgdorferi*. These results showed that the *oppA4* mutant showed increased *ospC* expression via influencing BosR and RpoS levels. These data are different from previous findings for OppA5, another substrate-binding protein, in which deletion of *oppA5* resulted in an impaired activation of the Rrp2-RpoN-RpoS pathway (Raju et al., 2011). These observations indicate that although they all involve in acquiring amino acids from either tick or vertebrate hosts, effects of OppA1 to OppA5 on the Rrp2-RpoN-RpoS pathway are different. This is consistent with the observations that expressions of each *oppA* genes are different in the enzootic cycle of *B. burgdorferi* (Groshong et al., 2017; Wang et al., 2002).

The findings of Opp transporter systems influencing virulence gene expression of *B. burgdorferi* from this and previous studies highlight the important role of nutrients in regulating bacterial virulence, which represents one aspect of the concept of “nutritional virulence” (Garai et al., 2017; Kwaik and Bumann, 2013). During tick feeding, a flux of blood meals provides rich nutrient conditions that allows the most robust replication of *B. burgdorferi* in the tick midgut (de Silva and Fikrig, 1995; Schwan and Piesman, 2000). Thus, it is the spirochete’s advantage to sense such change in nutritional conditions as a signal for triggering virulence gene expression. In this regard, we and other showed that metals are important signals for regulating *B. burgdorferi* virulence gene expression (Troxell and Yang, 2013; Troxell et al., 2013; Wang et al., 2017). Pyruvate, a key molecule in the central metabolic pathway could help *B. burgdorferi*, an organism that lacks catalase, defending against H<sub>2</sub>O<sub>2</sub> (Troxell et al., 2014). More importantly, we and others showed that acetyl phosphate, an intermediate molecule of AckA-Pta pathway that *B. burgdorferi* employs to synthesize the essential molecule, acetyl-coA, from acetate, contributes to the activation of the Rrp2-RpoN-RpoS pathway (Raju et al., 2011; Van Laar et al., 2012; Xu et al., 2010).

Recent data showed that during *in vitro* cultivation conditions, the AckA-Pta pathway is not essential for the activation of the Rrp2-RpoN-RpoS pathway (Richards et al., 2016). Such finding suggests that multiple mechanisms are present for Rrp2 phosphorylation, but this study does not dispute the data from earlier studies showing that acetate and acetyl phosphate play an important role in the activation of the Rrp2-RpoN-RpoS pathway during tick feeding (Xu et al., 2010). All these data support the notion that nutritional factor is one of the key player in regulating virulence gene expression of *B. burgdorferi*.

The Rrp2-RpoN-RpoS pathway is repressed when spirochetes migrate to the tick midgut from the vertebrate host (for review, see Radolf et al., 2012; Samuels, 2011). The signaling pathway that leads to this repression remains largely unknown. BadR is the repressor of *rpoS*, and it was proposed that phosphorylated sugar may be one of the signals (Miller et al., 2013). We previously showed that the metal ion manganese ( $Mn^{2+}$ ) represses *rpoS* expression while zinc ( $Zn^{2+}$ ) enhances *rpoS* expression. Recently, Wang et al., reported that another transition metal, copper ( $Cu^{2+}$ ) also plays a represses *rpoS* expression via inhibiting BosR binding to DNA. Given that *oppA4* is expressed ticks during the larval and nymphal blood meals (Groshong et al., 2017; Wang et al., 2002), and inactivation of *oppA4* leads to increased activation of the Rrp2-RpoN-RpoS pathway, we postulate that the ligand that OppA4 transports may be a cue to downregulates the Rrp2-RpoN-RpoS pathway during the acquisition phase of the enzootic cycle of *B. burgdorferi*.

Several regulators were known to regulate *rpoS* and subsequently, *ospC* expression in *B. burgdorferi* (Radolf et al., 2012; Samuels, 2011; Stevenson and Seshu, 2017). In this study, we only examined two major transcription factors, BosR and BadR, and uncovered that the OppA4 negatively regulates the *rpoS* and *ospC* levels via alteration of *bosR* expression. Note that the complemented strain had lower levels of *bosR* than that of wild-type strain (**Fig. 5**). This is likely due to the high level of constitutive expression of *oppA4* driven by the *flaB* promoter in the complemented strain. We did not

examine the level of *rrp2* and *rpoN* in the *oppA4* mutant, as activation of *rpoS* by Rrp2 and RpoN is controlled by phosphorylation of Rrp2. We and others recently showed that under the *in vitro* cultivation conditions, Rrp2 phosphorylation appears to be constitutive, as Rrp2 phosphorylation is vital for *B. burgdorferi* replication (Groshong et al., 2012; Ouyang and Zhou, 2016; Yin et al., 2016). How OppA4 negatively regulates *bosR* expression warrants further investigation.

In this study, we showed that the *oppA4* mutant is capable of infecting mice via needle infection, indicating that OppA4 is not essential for mammalian infection. It remains possible that the *oppA4* mutant has reduced infectivity. Performing ID<sub>50</sub> experiment or competition assay by co-infection wild-type *B. burgdorferi* are needed to determine whether OppA4 contributes partially to mammalian infection of *B. burgdorferi*. Furthermore, whether OppA4 is required for mammalian infection via tick bite remains to be determined. If OppA4 plays a role in the tick vector, the outcomes for infectivity via tick infection would be different from that of needle inoculation. One caveat of this study is that the complete enzootic cycle of the *oppA4* mutant was not performed. The parental strain of *B. burgdorferi* used in this study is 5A18NP1, which does not contain the endogenous plasmids lp56 and lp28-4: both plasmids were reported to contribute to colonize ticks (Jacobs et al., 2006; Strother et al., 2005). Given that expression of *oppA4* is preferably expressed in ticks, we postulate that OppA4 plays a more prominent role in the tick part of the cycle of *B. burgdorferi*. Generating an *oppA4* mutant in a *B. burgdorferi* strain with complete endogenous plasmids is warranted to examine the exact role of OppA4 in the enzootic cycle of *B. burgdorferi*.

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Table 1. Strains, plasmids, and primers used in this study.

Strains, plasmids or primers	Description or sequence	Purpose or source
<b>Strains</b>		
5A18NP1	Wild-type strain with <i>bbe02</i> gene disrupted by Kan <sup>r</sup>	(Kawabata et al., 2004)
OppA4-mut	<i>oppA4</i> deletion mutant; 5A18NP1 transformed with pZB20	In this study
OppA4-com	<i>oppA4</i> complementation; OppA4-mut transformed with pZB028	In this study
<b>Plasmids</b>		
pZB020	Suicide vector for constructing <i>oppA4</i> deletion; Str <sup>r</sup>	In this study
pZB028	Shuttle vector carrying <i>oppA4</i> gene; Gen <sup>r</sup>	In this study
<b>Primers</b>		
PRBB067 (2F)	GCAGGGCCCTTAACTTTTATAATCTTTATTTTCAATCGATT CAACCATTAC	Amplify <i>oppA4</i> upstream region
PRBB068	GCAGTCGACACAAGCATCCTTACAAGTTTTTATAAATTTA AATTTTGCC	Same as above
PRBB049	GCACCCGGGTTTAAAGTAAAAATGGTTTATAGCTAGATC	Amplify <i>oppA4</i> downstream region
PRBB050	GCAGGATCCATGCCAAATAAGATAACAAAAGAAGCTTTA A C	Same as above
PRBB034 (1R)	GCACTGCAGTCATTTAATTGGTTTTATTTTCAGATAAATTA AATC	Amplify <i>oppA4</i> with NdeI and PstI
PRBB056	GCACATATGATGTACCCATACGATGTTCCAGATTACGCTA AAATATTGATAAAAAAGTTAAAAG	Same as above
PRYY049	GATCAGATCTCAGCTTTTTTTTGA	Amplify <i>flgBp</i> -partial <i>aacC1</i> with BglII site

PRBB053	GCGAGATCATAGATATAAATCTCACTACGCGGCTGCTC	Same as above
PRBB052	GAGCAGCCGCGTAGTGAAAT CTATATCTATGATCTCGC	Amplify partial <i>aacCI</i> with AatII site
PRYY056	TCCGGCGACGTCATTAGGTGGCGGTA CTG	Same as above
PRBB033 (1F)	GCACATATGATGAAAATATTGATAAAAAAGTTAAAAGTT GTA	Amplify <i>oppA4</i> for identification
PRBB097 (2R)	GCTGCGTAACATATATAGAAACCTCCCTC	Amplify <i>BB_B14</i> for identification
PRBB003 (3F, 4F)	GAGGAATGAATGAGGGAAGCGGTGATCGCC	Amplify <i>aadA</i> for identification
PRBB135 (3R)	ACAACAAGAATCGTTGCGGG	Amplify <i>guaB</i> for identification
PRBB009 (4R)	GCAACGCGTTATTTGCCGACTACCTTGGTGATC	Same as above
qPCR-flaB-F	CAGCAATAGCTTCATCTTGGTTTG	qPCR for <i>flaB</i>
qPCR-flaB-R	ACCAGCATCTTCAGGGTCTCA	qPCR for <i>flaB</i>
qhx-rpoS-F	ATTTTCAACTTATGCATCATTTTG	qPCR for <i>rpoS</i>
qhx-rpoS-R	TTTTCTTTTCTGTATGGGACTTTT	qPCR for <i>rpoS</i>
qhx-ospC-F	TAGCGGGAGCTTATGCAATATCAACC	qPCR for <i>ospC</i>
qhx-ospC-R	CATCAATTTTTTCCTTTAATCCTTCA	qPCR for <i>ospC</i>
PRBB132	GGATACAGACCGGGACTTGC	qPCR for <i>oppA4</i>
PRBB133	CCTTCGGCAGTAATGGAAACTC	qPCR for <i>oppA4</i>

**Table 2. Mouse infection of the OppA4-mut and OppA4-com**

	WT <sup>†</sup>	OppA4-mut <sup>†</sup>	OppA4-com
No. of infected mice /Total No. of mice	3/3	4/5	4/5

\* C3H/HeN mice were needle infected with  $10^5$  spirochetes/mouse.

<sup>†</sup> Fisher Exact Test statistic value between WT and OppA4-mut is 1.

## FIGURE LEGEND

**Fig. 1. Construction of the *oppA4* mutant and the complemented strain.** (A) Strategy for constructing the *oppA4* mutant. WT: wild type. (B) Diagram of the shuttle vector used for complementation. (C) PCR analysis of the *oppA4* mutant (Mut) and the complemented strain (Com). Primers used are labeled on top and their locations are indicated in (A). (D) qRT-PCR analysis of *oppA4* mRNA levels in wild-type strain (WT), the *oppA4* mutant (OppA4-mut), and the complemented strain (OppA4-com). The values of the *oppA4* mRNA were normalized with the *flaB* mRNA level.

**Fig. 2. Endogenous plasmid profile analysis.** WT, wild-type strain 5A18NP1. OppA4-Mut, the *oppA4* mutant. OppA4-Com, the *oppA4* mutant complemented with a shuttle vector carrying a wild-type copy of *oppA4*. cp, circular plasmid; lp, linear plasmid.

**Fig. 3. Increased level of OspC production in the *oppA4* mutant.** Strains of WT, OppA4-Mut, and OppA4-Com were cultured in standard BSK-II medium at 37°C. Spirochetes were collected at mid-log (M, 10<sup>7</sup> spirochetes/ml) or stationary phase (S, 5 X 10<sup>8</sup> spirochetes/ml) and subjected to SDS-PAGE analysis. The band corresponding to OspC was labeled and indicated by arrow.

**Fig. 4. Increased RpoS and BosR levels of the *oppA4* mutant.** WT, OppA4-Mut and OppA4-Com were cultured in standard BSK-II medium at 37°C. Spirochetes were collected at mid-log (M, 10<sup>7</sup> spirochetes/ml) or stationary phase (S, 5 X 10<sup>8</sup> spirochetes/ml) and subjected to immunoblot analysis using antibodies specific for FlaB, RpoS, OspC, BosR or BadR. For immunoblotting against OspC, 50-fold less amount of samples were loaded.

**Fig 5. Increased levels of *rpoS*, *bosR* and *ospC* mRNA in the *oppA4* mutant.** WT, OppA4-Mut and OppA4-Com were cultured in standard BSK-II medium at 37°C, and were collected at mid-log phase (M,



$10^7$  spirochetes/ml). RNAs were isolated and then subjected to qRT-PCR analyses. Levels of each gene transcript were normalized with *flaB*. The bars represent the mean values of three independent experiments, and the error bars represent the SD. \*,  $P < 0.05$ .



