The role of the surface lipoprotein BBA07 in the enzootic cycle of *Borrelia burgdorferi*

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ABSTRACT

*Borrelia burgdorferi*, the Lyme disease pathogen, dramatically alters its protein profiles as it transmits between ticks and mammals. Several differentially expressed proteins have been shown to be critical for the enzootic cycle of *B. burgdorferi*. In this study, we demonstrated that expression of the surface lipoprotein-encoding gene *bba07* is induced by elevated temperature and reduced pH during *in vitro* cultivation as well as during nymphal tick feeding. Expression of *bba07* is regulated by the Rrp2-RpoN-RpoS pathway, a central regulatory network that is activated during nymphal feeding. By generating a *bba07* mutant in an infectious strain of *B. burgdorferi*, we demonstrate that although BBA07-deficient spirochetes were capable of infecting mice by needle inoculation and capable of surviving in ticks, they were defective in mammalian infection through tick transmission. Complementation of the *bba07* mutant with a wild-type copy of *bba07* partially restored the transmission defect of the *bba07* mutant. Based on these findings, we conclude that the surface lipoprotein BBA07 is produced during tick feeding and facilitates the optimal transmission of *B. burgdorferi* from the tick vector to the mammalian host.
INTRODUCTION

*Borrelia burgdorferi*, a spirochetal pathogen that causes Lyme disease, is in nature by a complex enzootic cycle involving ticks (e.g., *Ixodes scapularis*) and various vertebrate hosts (e.g., *Peromyscus leucopus*) (6,44,47). The spirochete adapts to these two disparate environments by accordingly altering its gene expression programs (39,43,46). Many of these differentially regulated genes encode known or predicted lipoproteins, some of which, including OspA, OspC, DbpB/A, BBK32, BptA, BB0365, BB064, and lp6.6, have been shown to be important to the enzootic cycle of *B. burgdorferi* (1,11,18,26,31,32,34,36,41,42,56,57). Thus, elucidating the functions of differentially expressed lipoproteins is important to our understanding of the infectious cycle of *B. burgdorferi*.

*bba07* is one of the putative lipoprotein genes located on plasmid lp54 of *B. burgdorferi*. Previous microarray analyses by Revel et al. identified *bba07* as a highly differentially expressed gene. When *B. burgdorferi* was cultivated under the conditions of elevated temperature (37°C) and lowered pH (pH 6.8) (a surrogate condition for the midgut of feeding ticks), *bba07* expression increases more than 10-fold in comparison to that of spirochetes grown at lowered temperature (23°C) and normal culture pH (pH 7.5) (an *in vitro* condition mimicking the unfed ticks) (37). More recently, microarray analyses conducted by us and others showed that *bba07* is one of the candidate genes under the control of the Rrp2-RpoN-RpoS pathway (2,8,30). The Rrp2-RpoN-RpoS pathway, also called the σ^34-σ^S^ sigma factor cascade, is a central pathway that is activated at the onset of nymphaal tick feeding and is indispensable for *B. burgdorferi*'s
transmission from ticks to mice as well as for mammalian infection (2,7,12,30). In this pathway, a two-component response regulator and $\sigma^A$-dependent transcriptional activator Rrp2, controls transcriptional activation of $rpoS$ from a typical $\sigma^A$-type -24/-12 promoter. RpoS ($\sigma^S$), a global regulator, then modulates expression of more than one hundred genes including the two major virulence genes $ospA$ and $ospC$ (2,7,12,15,19,30,55). Global gene expression analyses showed that mutations in $rrp2$, $rpoN$, or $rpoS$ resulted in a 7- to 138-fold decreases in $bba07$ transcripts (2,7,30).

Given the importance of the Rrp2-RpoN-RpoS pathway in the enzootic cycle of $B. burgdorferi$, we have been focusing on elucidating the functions of the Rrp2-RpoN-RpoS pathway-controlled lipoproteins as one of the strategies of identifying new virulence factors in $B. burgdorferi$. In this study, we showed that $bba07$ is indeed an Rrp2-RpoN-RpoS-dependent gene and its expression is induced by elevated temperature and reduced culture pH. Furthermore, we found that $bba07$ expression is induced during nymphal tick feeding and plays a role in the transmission process of $B. burgdorferi$ from ticks to mammals.
MATERIALS AND METHODS

Bacterial strains and plasmids. Low–passage, virulent *B. burgdorferi* strain B31 clone A3 (B31 A3) that contains all plasmids described in the parental strains MI B31 except for cp9, was kindly provided by Dr. P. Rosa, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health (10). Low–passage, virulent *B. burgdorferi* strain 297 and its isogenic *rrp2 [rrp2(G239C)]* and *rpoS* mutants (*rpoS*) were described previously (19,56). Borreliae were cultivated in Barbour-Stoenner-Kelly (BSK-H) medium (Sigma, St. Louis, MO) supplemented with 6% normal rabbit serum (Pel Freez Biologicals, Rogers, AR) at 23°C or 37°C with 5% CO₂ unless indicated otherwise. Relevant antibiotics were added to the cultures with the following final concentrations: 100 μg/ml for streptomycin and 50 ng/ml for erythromycin. The constructed suicide vectors were maintained in *E. coli* strain TOP10.

Construction of the *bba07* mutant. To construct a suicide vector for generating the *bba07* mutant via homologous recombination, the 1.5 kb upstream and the 1.5 kb downstream regions of *bba07* were PCR amplified from B31 A3 genomic DNA with primer pair *bba7-UF/bba7-UR* and *bba7-DF/bba7-DR* (*Table 1*), respectively. The resulting DNA fragments were cloned into upstream and downstream of an erythromycin-resistant cassette, respectively, within the pCR-XL-TOPO cloning vector (Invitrogen). The resulting suicide vector was confirmed by sequencing and designated as pHX7. The pHX7 plasmid DNA was then transformed into *B. burgdorferi* strain B31 A3 with a protocol described previously (38,56). Erythromycin-resistant
transformants were analyzed by RT-PCR and immunoblotting analyses to confirm the loss of bba07. Plasmid profiles of the bba07 mutant clones were determined by PCR analyses with twenty-one pairs of primers specific for each of the endogenous plasmids (10,23,28,35).

Complementation of the bba07 mutant. The bba07 mutant was trans-complemented by inserting a wild-type copy of bba07 into the intergenic region of the chromosomal genes bb0445 and bb0446, a strategy that was developed previously by Li et al. (25). The suicide vector pXLF14601 that contains a streptomycin-resistant cassette (aadA) inserted in the intergenic region between bb0445 and bb0446 along with ~3 kb of flanking regions, was kindly provided by Drs. X. Li and E. Fikrig (Yale university school of medicine) (13,25). A wild-type copy of bba07 along with its putative native promoter region (322 bp) was then cloned upstream of the aadA marker in pXLF14601. The constructed suicide vector was then transformed into the bba07 mutant. Streptomycin- and erythromycin-resistant transformants were selected and subjected to PCR and Western blot analyses to confirm restoration of bba07 expression in these clones.

Mouse infection via needle inoculation. Groups of 3-4 weeks old immunocompetent C3H/HeN mice (Harlan, Indianapolis, IN) were subcutaneously inoculated with wild-type B. burgdorferi strain B31 A3, the bba07 mutant, or the bba07 mutant-complemented strain at a dose of either 1 x 10^3 or 1 x 10^5 spirochetes per mouse. Two weeks post-inoculation, ear punch biopsies were collected and cultured in BSK-H medium supplemented with an antibiotic mixture specific for Borrelia growth (Sigma, Saint Louis, MO). Four weeks post-inoculation, mice were sacrificed.
and mouse tissues (ear, skin, heart, and joint) were collected and cultured for spirochetal growth. A single growth-positive culture was used as the criterion for infection of each mouse. All animal experiments and tick protocols described below were approved by the Institutional Animal Care and Use Committee at Indiana University.

**Tick-mouse cycle of B. burgdorferi.** Pathogen-free *Ixodes scapularis* larvae were purchased from the Tick-Rearing Center at Oklahoma State University, Stillwater, OK. The tick-mouse experiments were conducted in the Vector-borne Diseases Laboratory at Indiana University School of Medicine, Indianapolis, IN. Groups of two C3H/HeN mice were first needle-infected with wild-type strain B31 A3, the *bba07* mutant, or the *bba07* mutant-complemented spirochetes (1×10⁵ spirochetes per mouse). Two weeks post-inoculation, mouse infection was confirmed by cultivation of ear punch biopsies for spirochetal growth. Then unfed larvae were placed on these infected mice (150-200 larva/mouse). Ticks were allowed to feed and fed ticks were collected within 24 hrs after repletion. Ten fed larvae in each group were subjected to Immunofluorescence assay (IFA). The rest of the fed larvae were maintained in the tick incubator and allowed to molt to the nymphal stage (about 5 weeks). One month after molting, unfed nymphs were then allowed to feed on naïve C3H/HeN mice (12 ticks/mouse). Fully engorged nymphal ticks were collected within 24 hrs of repletion and subjected to IFA analyses. Two and four weeks post-tick infestation, mouse tissues were collected and cultivated for spirochetal growth as described above.

**Immunofluorescence assay (IFA).** IFA was performed as described previously (2). Briefly, the
entire contents of a fed tick were smeared and fixed on a silylated microscope slide (CEL Associates, Pearland, TX). The slides were incubated with BacTrace fluorescein isothiocyanate-conjugated goat anti-<i>B. burgdorferi</i> antibody (Kirkegaard and Perry Laboratories Gaithersburg, MD) at 37°C. Samples were observed using an Olympus BX50 fluorescence microscope. Ten ticks from each group were examined by IFA.

**Quantitative RT-PCR (qRT-PCR).** RNA samples were extracted from either <i>B. burgdorferi</i> cultures or ticks using the RNeasy<sup>®</sup> mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocols. For RNA analyses of <i>in vitro</i> cultivated spirochetes, three independent culture samples were used for each strain. For RNA analyses of tick samples, ten groups of fed larvae (three ticks/group), three groups of unfed nymphs (40 ticks/group), or ten groups of fed nymphs (one tick/group) were utilized. Mouse tissue RNAs isolated from Borrelia-infected mice were kindly provided by Dr. Liang (Louisiana State University, Baton Rouge, LA) (51). For RNA analyses of spirochetes in mouse tissues, four RNA samples from four mice were used. Digestion of contaminating genomic DNA in RNA samples was performed using RNase-free DNase I (Promega, Madison, WI), and removal of DNA was confirmed by PCR amplification using primers specific for the <i>B. burgdorferi</i> flaB gene. For cDNA synthesis, SuperScript III reverse transcriptase with random primers (Invitrogen, Carlsbad, CA) was used for RNA samples isolated from <i>in vitro</i> cultures. Because of the low levels of <i>B. burgdorferi</i> RNA present in tick and mouse samples, cDNA was synthesized using the primer mix specific for <i>bba07</i> (bba7-cDNA-R) and <i>flaB</i> (qFlaB-cDNA-R) (**Table 1**). Absolute quantitative PCR were performed in triplicate using the
ABI 7000 Sequence Detection System and GREEN PCR Master Mix (ABI, Pleasanton, CA). Briefly, a cloning vector containing an unrelated DNA sequence serves as standard template. A series of ten-fold dilutions of the standard template at the concentration of $10^0$ to $10^7$ copies per microliter was prepared. qPCR was performed using one microliter of the standard template for each reaction. A standard curve was then generated by plotting the copies of the standard template against the Ct values. The copy numbers of the bba07 and flaB were extrapolated from the Standard Curve plot based on their Ct values obtained from each reaction.

**Proteinase K accessibility experiments.** The proteinase K accessibility experiment was performed as previous reported (5,50). Briefly, wild-type *B. burgdorferi* B31 A3 was cultivated at 37°C and harvested at the cell density of $1 \times 10^8$ spirochetes per ml. The sample was divided into two parts; one part was treated with 200 µg proteinase K (PK) (Sigma, Saint Louis, MO) for 1 hour at room temperature, and the other part was incubated with 1x PBS as a control. Samples were then subjected to SDS-PAGE and immunoblotting analyses.

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.** SDS-PAGE and immunoblotting were performed as previously described (54). Monoclonal antibodies directed against OspC (182-105-D2) and FlaB (8H3-33) were also described previously (53). The polyclonal rat antibody against BBA07 was kindly provided by Drs. Blevins and Norgard (University of Texas Southwestern Medical Center, Dallas, Texas). For determining serological responses of mice infected with various strains of *B. burgdorferi*...
(“seroconversion” test) (20), sera were collected from each C3H/HeN mice at four weeks post tick infestation and each mouse serum was used for immunoblotting against whole cell lysates of wild-type *B. burgdorferi* B31 A3 for seroreactivity.

5 **Statistical analyses.** Data were analyzed by Fisher's exact test (for infectivity) or an unpaired *t* test (for qRT-PCR and spirochetes numbers). The *P* values for each test were indicated in the Table or the corresponding figure legends.
RESULTS

Characterization of bba07 expression under the in vitro cultivation conditions. Several microarray analyses have been published which identified more than one hundred differentially expressed B. burgdorferi genes by cultivating spirochetes under various temperature or pH conditions, in the presence of host blood, or in dialysis membrane chambers transplanted into peritoneal cavities of rats (4,29,37,48). Remarkably, only one report among these studies identified bba07 as a differentially regulated gene (37); Revel et al. showed that bba07 expression is upregulated more than 10-fold by elevated culture temperature and lowered pH. Therefore, we first sought to investigate whether the culture temperature and pH affect BBA07 production. Wild-type B. burgdorferi 297 was cultivated at either 23°C at pH 7.5 or 37°C at pH 7.0. Cells were harvested at late logarithmic phase (5 x 10^7 per ml) and then subjected to Western blot analysis. As shown in Fig. 1A, similar to OspC, BBA07 was induced by elevated temperature and reduced culture pH, a result that is consistent with a previous microarray result published by Revel et al. (37). The same experiment also was performed using another B. burgdorferi strain B31 A3, which yielded a similar result as to strain 297 (Fig. 1B), suggesting that influence of BBA07 production by temperature and pH is not a strain-specific phenomenon.

The Rrp2-RpoN-RpoS pathway is a central pathway modulating differential gene expression in B. burgdorferi. Recent microarray analyses published by us and others indicated that bba07 is one of the genes whose expression is under the control of the Rrp2-RpoN-RpoS pathway (2,8,30). To validate this microarray result, wild-type B. burgdorferi 297, the isogenic rrp2(G239C) mutant (55),
and the rpoS mutant (19) were cultivated in BSK-H medium to late phase at 37°C. RNAs were
isolated from these spirochetes and subjected to qRT-PCR analyses. Similar to ospC expression,
mutation in rrp2 or inactivation of rpoS virtually abolished bba07 expression at both transcription and
protein levels (Fig. 1C and 1D). Thus, BBA07 is an Rrp2-RpoN-RpoS pathway-dependent protein.

Sequence analysis predicts that BBA07 is a putative lipoprotein (14). To determine
whether BBA07 is surface exposed, wild-type B. burgdorferi B31 A3 was treated with proteinase
K and subjected to immunoblotting analysis. As shown in Fig. 1E, BBA07 was sensitive to the
proteinase K treatment, whereas the periplasmic protein FlaB remained intact upon the treatment,
suggesting that BBA07 is a surface exposed lipoprotein.

Expression of bba07 is induced during nymphal tick feeding. Given that bba07 is differentially
expressed, we sought to examine bba07 expression in the enzootic cycle of B. burgdorferi.
Pathogen-free I. scapularis larvae were fed on C3H/HeN mice infected with wild-type B. burgdorferi
strain B31A3. Infected fed larvae were allowed to molt to nymphs. One month after
molting, unfed nymphs were allowed to feed on naïve mice to produce fed nymphs. Fed larvae,
unfed nymphs, and fed nymphs were collected and subjected to qRT-PCR analyses. As shown in Fig.
2A, bba07 transcript was not detected in either fed larvae or unfed nymphs. In contrast, bba07
expression was drastically induced in fed nymphs, the stage when spirochetes transmit from ticks to
mammals. This observation is consistent with the above observation that bba07 expression is
controlled by the Rrp2-RpoN-RpoS pathway, the pathway that is activated at the onset of nymphal
tick feeding (8).
To determine the expression profile of bba07 during mammalian infection, qRT-PCR analyses were performed with RNAs extracted from various mouse tissues (skin, heart, and joint) isolated from needle inoculated mice with wild-type spirochetes (four weeks post inoculation). As shown in Fig. 2B, the bba07 transcripts were readily detected in heart and joint tissues, but not detected in skin samples. Undetectable level of bba07 transcript in the skin sample was not due to a low spirochetal number in the sample, as flaB transcripts were readily detected in the same sample. These results suggest that the bba07 remains expressed in spirochetes while replicating in the mammalian host, at least in some tissues.

Construction the bba07 mutant and the complemented strains. To investigate the role of BBA07 in the enzootic cycle of B. burgdorferi, we constructed a bba07-deficient mutant by the allele exchange method as depicted in Fig. 3A. Briefly, a suicide vector pHX07 carrying a disrupted bba07 gene along its intact surrounding regions was transformed into strain B31 A3. Loss of bba07 expression in the transformant clones was confirmed at both RNA and protein levels (Fig. 3B and 3C). Disruption of bba07 did not appear to affect cell morphology or growth kinetics of spirochetes in the BSK-H medium (data not shown). Further plasmid profile analyses by PCR identified one bba07 mutant clone that retained all the endogenous plasmids present in the parental wild-type strain; this clone was then selected for complementation. However, as described below, this clone lost lp28-4 during the process of storage.

To complement the bba07 mutant, we employed a strategy previously described by Li et al. (25), by inserting a wild-type copy of bba07 (driven by its native promoter) into the intergenic space.
region between the chromosomal genes bb0445 and bb0446 (Fig. 3A). Restoration of bba07 expression in the complemented clones was confirmed by RT-PCR and the immunoblot analyses (Fig. 3B and 3C). Plasmid profile analyses on three randomly picked complemented clones showed that they all lost lp28-4. Subsequent analysis revealed that this was due to the loss of lp28-4 in the bba07 mutant clone used for complementation during the process of storage. lp28-4 is one of the twenty-one endogenous plasmids present in the B. burgdorferi genome. Previous studies using clones with spontaneous loss of lp28-4 show that this plasmid is not essential for B. burgdorferi to infect mice upon needle inoculation, but it partially contributes to mammalian infection since loss of lp28-4 leads to an order increase of the ID₅₀ value (23,35). The role of lp28-4 in the tick-mouse cycle has not been fully examined.

Multiple efforts to obtain a bba07 mutant or a complemented strain that retains all endogenous plasmids have not been successful. Thus, we chose the bba07 mutant clone and the complemented strain both lacking lp28-4 for further study. The rationale is that by comparing the bba07 mutant with its complemented strain, it should allow us to assess the impact of inactivation of bba07 on spirochetal behavior. In addition, a comparison between the complemented strain lacking lp28-4 and the wild-type strain would also provide insight into the role of lp28-4 in the enzootic cycle of B. burgdorferi.

The bba07 mutant is capable of infecting mice and persisting infection through needle inoculation. We first examined the bba07 mutant’s ability to establish infection in mice by needle inoculation. Groups of five immunocompetent C3H/HeN mice were inoculated with a high dose
(1×10⁵ spirochetes per mouse) or low dose (1×10³ spirochetes per mouse) of wild-type, the bba07 mutant, or the complemented strain. Four weeks post inoculation, mice were sacrificed and various mouse tissues (skin, heart, and joint) were collected and cultured for spirochetal growth. At the high dose of inoculum, all mice inoculated with wild-type, the bba07 mutant, or the complemented spirochetes were infected (Table 2), suggesting that BBA07 is not essential for B. burgdorferi to establish infection in mice by needle inoculation. At the low dose of inoculum, both the bba07 mutant and the complemented strain showed reduced infectivity, which is consistent with the previous observations that lp28-4 partially contributes to mammalian infection (23,35). Since the infectivity between the bba07 mutant and the complemented strain was virtually identical (p = 1), this result suggests that inactivation of bba07 did not affect mouse infection via the route of needle inoculation.

To further examine the bba07 mutant’s ability to cause persistent infection in mice, groups of two infected mice were kept for 180 days and then examined for infectivity; all mouse tissues remained culture positive for spirochetal growth. This result suggests that neither BBA07 nor lp28-4 is required for maintaining persistent infection in mice.

The bba07 mutant can colonize in ticks, but failed to infect mice via tick infestation. To examine whether BBA07 plays a role in the tick-mouse cycle of B. burgdorferi, larval ticks were allowed to feed on mice carrying wild-type, the bba07 mutant, or the complemented strain spirochetes. IFA analyses of fed larvae showed that spirochetes were present in all tick midguts examined and there was no significant difference in spirochetal numbers among each group of
ticks (data not shown). To determine spirochetal ability to survive through the process of tick development (ecdysis), fed larvae were kept in a tick incubator and allowed to molt to nymphs (4-5 weeks). Flat (unfed) nymphs were then fed on groups of naïve C3H/HeN mice. Twenty-four hrs after repletion, fed nymphs were collected and subjected to IFA analyses. Spirochetes were readily detected among all fed nymphs examined (Fig. 4ABC). Thus, neither BBA07 nor lp28-4 is essential for spirochetal colonization in ticks and survival through the process of tick ecdysis. However, significantly reduced numbers of spirochetes were observed in ticks harboring either the bba07 mutant or the complemented strain, but there was no significant difference in spirochetal numbers between the bba07 mutant and the complemented strain (Fig. 4D). Absence or presence of bba07 expression in the bba07 mutant- or the complemented strain-infected nymphs was confirmed by RT-PCR analyses (Fig. 4E). This result suggests that lp28-4, not bba07, is important to the optimal spirochetal survival in ticks.

To further examine the bba07 mutant’s ability to infect mice via the route of tick infestation, tissue samples from mice that received tick bites in the experiment described above were collected and cultured for the presence of spirochetes. All mice infested with ticks carrying wild-type spirochetes were infected (Table 3). In contrast, none of the eighteen mice infested with ticks harboring the bba07 mutant were infected. Complementation of the bba07 mutant with wild-type bba07 partially restored the non-infectious phenotype of the mutant via tick infestation: three out of ten mice in this group were culture positive for spirochetal growth and positive for seroconversion (Fig. 5). Partial restoration of infectivity in the complemented strain was due to the loss of the lp28-4. This result suggests that inactivation of bba07 did not affect mammalian
infection by needle inoculation, but it impaired the spirochetal ability to infect mice through the route of tick transmission. As the \textit{bba07} mutant has no defect in tick colonization, BBA07 likely plays a role in spirochetal transmission from ticks to mammals.
DISCUSSION

The Rrp2-RpoN-RpoS pathway is a central regulatory pathway that modulates differential gene expression in *B. burgdorferi*. Since it is indispensable for spirochetal transmission and mammalian infection (2,7,8,12,30), this pathway must control expression of borrelial factors important to the enzootic cycle of *B. burgdorferi*. To date, only a few RpoS-dependent virulence factors have been identified, including OspC, DbpB/A, and BBK32 (1,18,32,41,42,49). In this study, we showed that BBA07 is an Rrp2-RpoN-RpoS pathway-controlled surface lipoprotein and appears to play a role in the process of spirochetal transmission from ticks to mammals.

The microarray analysis published by Revel et al. showed that *bba07* is among the 79 *B. burgdorferi* genes whose expression is influenced by culture temperature and pH (37). The results from this study confirmed the finding by Revel et al. for *bba07*. We further demonstrated that expression of *bba07* is under the control of the Rrp2-RpoN-RpoS pathway. The pattern of *bba07* expression *in vitro* correlated well with the subsequent *in vivo* observation that the *bba07* transcripts were exclusively up-regulated during nymphal feeding, the period when RpoS is induced (8,40). In addition, *bba07* transcripts were also expressed in mouse heart and joint tissues during mammalian infection. Interestingly, *bba07* transcripts were not detectable in mouse skin samples, suggesting a possible tissue tropism for *bba07* expression.

*B. burgdorferi* has a segmented genome that is composed of a small linear chromosome of approximately 900 kb and at least 21 different linear and circular plasmids (9,14). Such an unusual genome structure presents a major challenge for studying virulence determinants in *B. burgdorferi*. Some plasmids are prone to loss during *in vitro* manipulation (11,23,24,35,52). Among them,
plasmids lp25, lp28-1, and lp36 have been shown to be critical for B. burgdorferi to establish infection and cause disease in mice (16,17,20,21,23,35,52). In this study, the bba07 mutant and the complemented strain retained all important plasmids listed above, but they lost lp28-4. Unlike lp25, lp28-1, or lp36, lp28-4 is not essential for mammalian infection upon needle inoculation with a medium or high dose of spirochetes (>1 x 10^4 bacteria per mouse) (3,21,23,35,52). However, lp28-4 partially contributes to mammalian infection since the lp28-4-lacking spirochetes have a reduced infectivity with a low inoculum (≤1 x 10^3 bacteria per mouse or lower) (3,23). Our finding that the bba07 mutant-complemented strain (lacking lp28-4) had reduced mammalian infectivity in this study is consistent with this notion. In the tick vector, there are conflicting reports of the contribution of lp28-4. Strother et al. reported that loss of lp28-4 dramatically reduced the percentage of ticks being infected by spirochetes when ticks were fed using the artificial capillary feeding method (45). However, another report by Elias et al. showed that when ticks were fed on infected mice (i.e., via the natural route of acquisition), a B31 clone lacking lp28-4 infected ticks normally (10). In this study, we found that spirochetes lacking lp28-4 (both the bba07 mutant and the complemented strain) infected all the ticks examined. This suggests that loss of lp28-4 does not affect the infection rate of ticks by spirochetes. However, there was a reduced spirochetal load in the ticks infected with lp28-4-minus spirochetes, suggesting that lp28-4 contributes to the optimal survival of B. burgdorferi in the tick vector.

Despite the loss of lp28-4 in the bba07 mutant and the complemented strain, our studies provide important insights into the contribution of BBA07 to the enzootic cycle of B. burgdorferi. For mammalian infection, BBA07 does not appear to play a role, since the infectivity of the bba07
mutant is similar to that of the isogenic complemented strain at either high or low doses of inoculum (Table 2). In the tick vector, the bba07 mutant and the complemented strain colonize and survive similarly. However, the outcome of mouse infection via tick infestation was significantly different between the bba07 mutant and the isogenic complemented strain. The bba07 mutant was no longer able to infect mice through the route of tick infestation, while the complemented strain remained partially infectious. Since both strains lacked lp28-4, the loss of lp28-4 could not fully account for the non-infectious phenotype observed in the bba07 mutant. Thus, abrogation of bba07 expression in the bba07 mutant must contribute to this phenotype. The phenotype of the bba07 mutant is similar to a recently reported bba52 mutant, while the bba52 mutant infects mice normally upon needle inoculation, it is no longer infectious via tick bites as the result of an impaired ability to transmit from the tick midgut to salivary glands (22). Since the bba07 mutant is capable of colonizing ticks and establishing infection in mice by needle inoculation, its inability to infect mice via tick transmission suggests that BBA07 likely plays a role in the transmission process from the tick midgut to the mammalian host. Future work is warranted to pinpoint the defect of the bba07 mutant in the process of transmission such as the mutant’s ability to interact with tick gut cells, migrate to the hemolymph, survive in the hemolymph, or enter into salivary glands.

BBA07 is one of the few plasmid-encoded lipoproteins that do not belong to a paralogous family in B. burgdorferi (9,14). BLAST analyses revealed that the BBA07 is highly conserved among Lyme disease Borrelia species (B. burgdorferi sensu stricto, B. garinii, and B. afzelii) (data not shown). However, sequence analysis did not provide any putative function about BBA07
since it does not share significant homology with any other proteins. Curiously, BBA07 was annotated as ChpAI in the TIGR *B. burgdorferi* genome database (14). However, this assignment was solely based on a low homology (e value > 1) of the N-terminal portion of BBA07 to *E. coli* ChpAI (MazE, ChpR), an antitoxin. Along with toxin ChpAK, ChpAI and ChpAK constitute a ChpAIK (MazEF) toxin-antitoxin system (27,33). ChpAK kills cells by inhibiting translation and replication under nutritional stress conditions (bacterial programmed cell death). ChpAI counteracts ChpAK-mediated inhibition of translation and replication. However, several lines of evidences argue against the annotation of BBA07 as a ChpAI homologue. First, the homology between BBA07 and ChpAI is low. Second, BLAST search did not identify a putative ChpAK in the *B. burgdorferi* genome. Third, unlike the cytosolic location of ChpAI, BBA07 is localized on the cell surface (Fig. 1E). Given that BBA07 plays a role in the enzootic cycle of *B. burgdorferi*, further study is warranted to elucidate the physiological function of this surface lipoprotein.

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Reference List


FIGURE LEGENDS

Fig. 1. Regulation of BBA07 expression under the in vitro cultivation conditions. (A) and (B), BBA07 expression is induced by elevated temperature and lowered pH. Either *B. burgdorferi* 297 (A) or B31 A3 (B) strain was cultivated in BSK-H medium at 23°C (pH 7.5) or 37°C (pH 7.0). Spirochetes were harvested at the late growth phase, and whole-cell lysates were separated by SDS-PAGE and subjected to Western blot analysis using a mixture of antibodies against FlaB, OspC, and BBA07. The bands corresponding to each protein were labeled on the right. (C) and (D), Control of bba07 expression by the Rpo2-RpoN-RpoS pathway. Wild-type *B. burgdorferi* 297 (wt), the isogenic *rrp2* mutant (*rrp2*), the *rrp2* complemented strain (*rrp2*), or the *rpoS* mutant (*rpoS*) were cultivated at 37°C in BSK-H medium (pH 7.5), harvested at late-logarithmic growth, and subjected to either qRT-PCR analyses (C) or Western blot analyses using a combination of antibodies against FlaB, OspC, and BBA07 (D). Levels of the *bba07* transcripts were normalized with the *flaB* transcript in each sample and relative levels of *bba07* expression were reported. Data represents three independent culture samples. **, *p* value < 0.01. (E), Surface localization of BBA07. Wild-type *B. burgdorferi* strain B31 A3 was incubated with either PBS alone (-) or PBS containing proteinase K (+). Treated cells were then subjected to immunoblot analysis using a combination of antibodies against FlaB and BBA07. FlaB served as a negative control.

Fig. 2. Expression of *bba07* in ticks and mice. (A), Relative levels of *bba07* transcript in fed larvae, unfed nymphs, or fed nymphs were determined by qRT-PCR analyses and reported as the
numbers of the bba07 transcripts per 10,000 copies of the flaB transcripts. Black triangles represent values from each data point. For fed larvae, each data point was generated from three fed larvae (total of 30 ticks for ten data points); for flat nymphs, each data point was generated from forty flat nymphs (total of 120 ticks for three data points); for fed nymphs, each data point was generated from one tick (total of 10 ticks for ten data points). The horizontal bar represents the mean value. (B), Relative level of bba07 transcripts in joint, heart, and skin tissues harvested from mice needle-infected with B. burgdorferi. Four mice were examined for each group. Each column represents one tissue sample harvested from one mouse. For each RNA sample, three replicates were performed for qRT-PCR analyses and the error bar represents the data variation among the three reactions. ND, undetectable.

Fig. 3. Construction of the bba07 mutant and the complement strain. (A), Strategy for constructing the bba07 mutant and the complemented strain. wt, genomic structure of bba07 and the surrounding regions in wild-type B. burgdorferi B31 A3. Plasmid pHX07 represents the suicide construct used for generating the bba07 mutant. Only the relevant portion of the plasmid was shown. The 5′ arm and 3′ arm represent the two PCR fragments used for cloning into the upstream and downstream of the erythromycin-resistant (erm) gene. bba07, the diagram showing the genomic structure of the bba07 mutant. bba07−/+, the diagram showing the region of bb0445-bb0446 in the bba07 mutant-complemented strain, where a wild-type copy of bba07 along its 322 bp upstream putative promoter region and streptomycin-resistance gene (aadA) were inserted into the intergenic region between bb0445 and bb0446. (B) and (C), RT-PCR (B) and Western blot analyses (C) of wild-type (wt), the bba07 mutant (bba07), and the complemented strain (bba07−/+). Spirochetes were
cultured at 35°C and harvested at late logarithmic growth. RT-PCR reactions were performed using the primers specific for bba07, and Western blot analysis was performed using rat polyclonal antibody against BBA07. The FlaB was used as a loading control.

**FIG. 4. IFA and RT-PCR analyses of spirochetes in fed nymphs.** Unfed *I. scapularis* nymphs harboring either wild-type (wt) (A), the bba07 mutant (bba07) (B) or the bba07 mutant-complement strain (bba07+) (C) were fed on naive C3H/HeN mice, and engorged nymphs were collected within 24 hrs and subjected to IFA analyses using fluorescein isothiocyanate-labeled anti-*B. burgdorferi* antibody. Seven ticks were examined in each group and a representative image for each group of ticks is shown. (D), Relative numbers of spirochetes in each group of ticks. Numbers of spirochetes were counted under the fluorescent microscope. The error bars represent data from seven randomly picked fed nymphs. **p value < 0.01. (E), Confirmation of bba07 expression in Fed nymphs. RNAs were extracted from six fed nymphs harboring wt, bba07, or bba07+ spirochetes, and subjected to RT-PCR in the presence (+) or absence (-) of reverse transcriptase (RT).

Fig. 5. Serological responses of mice infected with various strains of *B. burgdorferi*. The serum was collected from each C3H/HeN mouse four weeks post infestation with ticks harboring wild-type (wt), the bba07 mutant (bba07), or the complemented spirochetes (bba07+). Positivity of seroconversion for each mouse was determined by immunoblotting analyses with the serum from each individual mouse (1:300 dilution) against whole cell lysates from wild-type *B. burgdorferi* B31 A3. A representative immunoblotting result was shown for each group. For the
group of mice infected with the complemented strain, only the immunoblotting result from one of the three infected mice was shown.
Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>bba07-DF</td>
<td>GGATCCATCTGCTCGTTTTAACCTTTT</td>
<td>amplify bba07 downstream region</td>
</tr>
<tr>
<td>bba07-DR</td>
<td>GGTACCGATTTACGTGATTTCTCCATT</td>
<td>same as above</td>
</tr>
<tr>
<td>bba07-UF</td>
<td>GCGGCCGCTGTAATCAAGCTTGGCAAAT</td>
<td>amplify bba07 upstream region</td>
</tr>
<tr>
<td>bba07-UR</td>
<td>CTGCAGTTCATACGTCTCCCACACACAT</td>
<td>same as above</td>
</tr>
<tr>
<td>Combba07-F</td>
<td>GCGGCCGCAGTAAAAAGCTTAGAAGCCCAT</td>
<td>amplify bba07 complement region</td>
</tr>
<tr>
<td>Combba07-R</td>
<td>AGATCTAAAAAACAACAAACAGCACAAACG</td>
<td>same as above</td>
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<tr>
<td>Qbba07-F</td>
<td>ATGGTGCATCAAATAAGAAGCTTA</td>
<td>qRT-PCR primer for bba07</td>
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<tr>
<td>Qbba07-R</td>
<td>GGATTGCTATCAAGAAGCTACCTGT</td>
<td>same as above</td>
</tr>
<tr>
<td>Bba07-cDNA-R</td>
<td>AGAGCCATTCTTAGCTTTCTTTTTG</td>
<td>cDNA synthesis for bba07</td>
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<td>QflaB-F</td>
<td>ACCAGCATCTTTCAGGGTCTCA</td>
<td>qRT-PCR primer for flaB</td>
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<td>QflaB-R</td>
<td>CAGCAATAGCTTCAAGCTTGTTTG</td>
<td>same as above</td>
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<td>qFlaB-cDNA-R</td>
<td>GATTCAAGTCTATTTTGGAAAGCACC</td>
<td>cDNA synthesis for flaB</td>
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Table 2. Mouse infectivity of the *bba07* mutant by needle inoculation

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of cultures positive/total No.</th>
<th>No. of mice positive/total No. of mice</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Ear*</td>
<td>Skin</td>
</tr>
<tr>
<td>B31 A3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^3</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^3</td>
<td>9/10</td>
<td>9/10</td>
</tr>
<tr>
<td><em>bba07</em></td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^3</td>
<td>3/10</td>
<td>3/10</td>
</tr>
<tr>
<td><em>bba07</em>+</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^3</td>
<td>2/5</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B31 A3</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td><em>bba07</em></td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td><em>bba07</em>+</td>
<td>2/2</td>
<td>2/2</td>
</tr>
</tbody>
</table>

* Ear punch biopsies at 14 days post inoculation were also examined and the results were identical to that of 28 days post inoculation.
Table 3. Mouse infectivity of the *bba07* mutant by tick transmission

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of cultures positive/total No.</th>
<th>No. of mice positive/total No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ear</td>
<td>Skin</td>
</tr>
<tr>
<td>B31 A3</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td><em>bba07</em></td>
<td>0/18</td>
<td>0/18</td>
</tr>
<tr>
<td><em>bba07&lt;sup&gt;+/−&lt;/sup&gt;</em></td>
<td>3/10</td>
<td>3/10</td>
</tr>
</tbody>
</table>

<sup>a</sup>The *p* value between the *bba07* mutant and the complemented strain is <0.05 calculated using Fisher’s exact two-tail test.

<sup>b</sup>The *p* value between B31 A3 and the complemented strain is <0.05 calculated using Fisher’s exact two-tail test.
Xu, et al., Fig.1CD
FlaB
BBA07
-+
Proteinase K
Xu, et al., Fig. 1E
Xu, et al., Fig. 2B

B

ND

Skin
Heart
Joint

bba07 transcripts/10000 faB transcripts

0
5
10
15
20
25
30
35
40
45
50

ND
Relative numbers of spirochetes

Xu, et al., Fig. 4ABCD

A                                           B                   C
D

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