

# Role of the Surface Lipoprotein BBA07 in the Enzootic Cycle of *Borrelia burgdorferi*<sup>∇</sup>

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***Borrelia burgdorferi*, the Lyme disease pathogen, dramatically alters its protein profile when it is transmitted 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 an elevated temperature and a 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 of an infectious strain of *B. burgdorferi*, we demonstrated that although BBA07-deficient spirochetes were capable of infecting mice via needle inoculation and surviving in ticks, they were defective in infection of mammals via 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 concluded that the surface lipoprotein BBA07 is produced during tick feeding and facilitates optimal transmission of *B. burgdorferi* from the tick vector to a mammalian host.**

*Borrelia burgdorferi*, a spirochete pathogen that causes Lyme disease, has a complex enzootic life cycle involving ticks (e.g., *Ixodes scapularis*) and various vertebrate hosts (e.g., *Peromyscus leucopus*) (6, 44, 47). This spirochete adapts to these two disparate environments by altering its gene expression (39, 43, 46). Many of the genes that are differentially regulated encode known or predicted lipoproteins, some of which, including OspA, OspC, DbpB/A, BBK32, BptA, BB0365, BBA64, 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 for understanding 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 at an elevated temperature (37°C) and a lower pH (pH 6.8) (surrogate conditions for the midgut of feeding ticks), *bba07* expression increased more than 10-fold compared to the expression in spirochetes grown at a lower temperature (23°C) and normal culture pH (pH 7.5) (*in vitro* conditions mimicking the conditions in unfed ticks) (37). More recently, microarray analyses conducted by us and other workers showed that *bba07* is one of the candidate genes under control of the Rrp2-RpoN-RpoS pathway (2, 8, 30). The Rrp2-RpoN-RpoS pathway, also called the  $\sigma^{54}$ - $\sigma^S$  sigma factor cascade, is a central pathway that is activated at the onset of nymphal tick feeding and is indispensable for transmission of spirochetes from ticks to mice, as well as for mammalian infection (2, 7, 12, 30). In this

pathway, a two-component response regulator and  $\sigma^{54}$ -dependent transcriptional activator, Rrp2, controls transcriptional activation of *rpoS* from a typical  $\sigma^{54}$ -type -24/-12 promoter. RpoS ( $\sigma^S$ ), a global regulator, then modulates expression of more than 100 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 7- to 138-fold decreases in the number of *bba07* transcripts (2, 7, 30).

Given the importance of the Rrp2-RpoN-RpoS pathway in the enzootic cycle of *B. burgdorferi*, we have focused on elucidating the functions of the Rrp2-RpoN-RpoS pathway-controlled lipoproteins as a strategy to identify new virulence factors in *B. burgdorferi*. In this study, we showed that *bba07* is indeed an Rrp2-RpoN-RpoS-dependent gene and that its expression is induced by an elevated temperature and a reduced culture pH. Furthermore, we found that *bba07* expression is induced during nymphal tick feeding and plays a role in the transmission 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), which contains all of the plasmids described for parental strain B31 MI except cp9, was kindly provided by 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 isogenic *rrp2* [*rrp2(G239C)*] and *rpoS* mutants mutants have been 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% CO<sub>2</sub> unless indicated otherwise. Relevant antibiotics were added to the cultures at the following final concentrations: 100  $\mu$ g/ml streptomycin and 50 ng/ml erythromycin. The suicide vectors that were constructed were maintained in *Escherichia coli* strain TOP10.

**Construction of the *bba07* mutant.** To construct a suicide vector for generating the *bba07* mutant by homologous recombination, the 1.5-kb upstream region and the 1.5-kb downstream region of *bba07* were PCR amplified from strain B31 clone A3 genomic DNA with primer pairs *bba7*-UF/*bba7*-UR and *bba7*-DF/*bba7*-DR (Table 1), respectively. The resulting DNA fragments were cloned

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TABLE 1. Primers used in this study

Primer	Sequence (5' → 3')	Use
bba07-DF	GGATCCATCTGCTTCGTTTTAACTTTT	Amplify <i>bba07</i> downstream region
bba07-DR	GGTACCGATTACGTGATTTCTCCATT	Amplify <i>bba07</i> downstream region
bba07-UF	GCGGCCGCTGTAATCAAGCTCATTGCAAAT	Amplify <i>bba07</i> upstream region
bba07-UR	CTGCAGTTCATACGTCTCCACACACAT	Amplify <i>bba07</i> upstream region
Combba07-F	GCGGCCGAGTAAAAGCTGTAGTCAAGCCCAT	Amplify <i>bba07</i> complement region
Combba07-R	AGATCTAAAAAACAAAAACAAGCACAAACG	Amplify <i>bba07</i> complement region
Qbba07-F	ATGGTGATCAAAATAAAGAACTTA	qRT-PCR primer for <i>bba07</i>
Qbba07-R	GGATTGCTATCAAGAACTACCTGT	qRT-PCR primer for <i>bba07</i>
Bba07-cDNA-R	AGAGCCATTTTAGCCTTCTTTTG	cDNA synthesis for <i>bba07</i>
QflaB-F	ACCAGCATCACTTTCAGGGTCTCA	qRT-PCR primer for <i>flaB</i>
QflaB-R	CAGCAATAGCTTCATCTTGGTTTG	qRT-PCR primer for <i>flaB</i>
qFlaB-cDNA-R	GATTCAAGTCTATTTTGGAAAGCACC	cDNA synthesis for <i>flaB</i>

upstream and downstream of an erythromycin resistance cassette, respectively, in the pCR-XL-TOPO cloning vector (Invitrogen). The resulting suicide vector was confirmed by sequencing and was designated pHX07. pHX07 plasmid DNA was then transformed into *B. burgdorferi* strain B31 clone A3 using a previously described protocol (38, 56). Erythromycin-resistant transformants were analyzed by reverse transcription-PCR (RT-PCR) and immunoblotting to confirm the loss of *bba07*. Plasmid profiles of the *bba07* mutant clones were determined by performing PCR with 21 pairs of primers specific for the endogenous plasmids (10, 23, 28, 35).

**Complementation of the *bba07* mutant.** The *bba07* mutant was transcomplemented by inserting a wild-type copy of *bba07* into the intergenic region of the *bb0445* and *bb0446* chromosomal genes, a strategy that was developed previously by Li et al. (25). The pXLF14601 suicide vector, which contains a streptomycin resistance cassette (*aadA*) inserted in the intergenic region between *bb0445* and *bb0446* along with ~3 kb of flanking regions, was kindly provided by 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 suicide vector constructed was then transformed into the *bba07* mutant. Streptomycin- and erythromycin-resistant transformants were selected and subjected to PCR and Western blot analysis to confirm that *bba07* expression was restored in these clones.

**Mouse infection via needle inoculation.** Groups of 3- to 4-week-old immunocompetent C3H/HeN mice (Harlan, Indianapolis, IN) were subcutaneously inoculated with wild-type *B. burgdorferi* strain B31 clone A3, the *bba07* mutant, or the complemented *bba07* mutant strain at a dose of either  $1 \times 10^3$  or  $1 \times 10^5$  spirochetes per mouse. Two weeks postinoculation, ear punch biopsy specimens were collected and cultured in BSK-H medium supplemented with an antibiotic mixture specific for *Borrelia* growth (Sigma, St. Louis, MO). Four weeks postinoculation, mice were sacrificed, and mouse tissues (ear, skin, heart, and joint) were collected and cultured for spirochete 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 clone A3, *bba07* mutant, or complemented *bba07* mutant spirochetes ( $1 \times 10^5$  spirochetes per mouse). Two weeks postinoculation, mouse infection was confirmed by cultivation of ear punch biopsy specimens for spirochete growth. Then unfed larvae were placed on the infected mice (150 to 200 larvae/mouse). The ticks were allowed to feed, and fed ticks were collected within 24 h after repletion. Ten fed larvae from each group were subjected to immunofluorescence assay (IFA) analysis. 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 h after repletion and subjected to IFA analysis. Two and 4 weeks after tick infestation, mouse tissues were collected and cultivated for spirochete growth as described above.

**IFA.** The 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-*B. burgdorferi* 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 the IFA.

**Quantitative RT-PCR (qRT-PCR).** RNA samples were extracted from either *B. burgdorferi* cultures or ticks using an RNeasy mini kit (Qiagen, Vanelcia, CA) according to the manufacturer's protocols. For RNA analysis of *in vitro*-cultivated spirochetes, three independent culture samples were used for each strain. For RNA analysis of tick samples, 10 groups of fed larvae (three ticks/group), three groups of unfed nymphs (40 ticks/group), or 10 groups of fed nymphs (one tick/group) were utilized. Mouse tissue RNAs isolated from *Borrelia*-infected mice were kindly provided by F. T. Liang (Louisiana State University, Baton Rouge, LA) (51). For RNA analysis of spirochetes in mouse tissues, four RNA samples from four mice were used. Contaminating genomic DNA in RNA samples was digested using RNase-free DNase I (Promega, Madison, WI), and the absence of DNA was confirmed by PCR amplification using primers specific for the *B. burgdorferi* *flaB* gene. For cDNA synthesis, SuperScript III reverse transcriptase and random primers (Invitrogen, Carlsbad, CA) were used for RNA samples isolated from *in vitro* cultures. Because of the low levels of *B. burgdorferi* RNA that were present in tick and mouse samples, cDNA was synthesized using a mixture of primers specific for *bba07* (bba7-cDNA-R) and *flaB* (qFlaB-cDNA-R) (Table 1). Absolute quantitative PCR was performed in triplicate using the ABI 7000 sequence detection system and SYBR Green PCR master mixture (ABI, Pleasanton, CA). Briefly, a cloning vector containing an unrelated DNA sequence was used as the standard template. A series of 10-fold dilutions of the standard template (concentrations,  $10^0$  to  $10^7$  copies per  $\mu$ l) was prepared. qPCR was performed using 1  $\mu$ l of the standard template in each reaction mixture. A standard curve was then generated by plotting the number of copies of the standard template against the cycle threshold ( $C_T$ ) values. The numbers of copies of the *bba07* and *flaB* genes were extrapolated from the standard curve based on the  $C_T$  values obtained for each reaction.

**Proteinase K accessibility experiment.** A proteinase K accessibility experiment was performed as previously described (5, 50). Briefly, wild-type *B. burgdorferi* B31 clone A3 was cultivated at 37°C and harvested at a density of  $1 \times 10^8$  spirochetes per ml. The sample was divided into two parts; one part was treated with 200  $\mu$ g proteinase K (PK) (Sigma, St. Louis, MO) for 1 h at room temperature, and the other part was incubated with  $1 \times$  phosphate-buffered saline (PBS) as a control. Samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

**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) have also been described previously (53). A polyclonal rat antibody against BBA07 was kindly provided by J. Blevis and M. Norgard (University of Texas Southwestern Medical Center, Dallas, TX). To determine serological responses of mice infected with various strains of *B. burgdorferi* ("seroconversion" test) (20), serum was collected from each C3H/HeN mouse at 4 weeks after tick infestation, and each mouse serum was used for immunoblotting against whole-cell lysates of wild-type *B. burgdorferi* B31 clone A3 to determine seroreactivity.

**Statistical analyses.** Data were analyzed by Fisher's exact test (for infectivity) or by an unpaired *t* test (for qRT-PCR and numbers of spirochetes). The *P* values for the tests are indicated below.

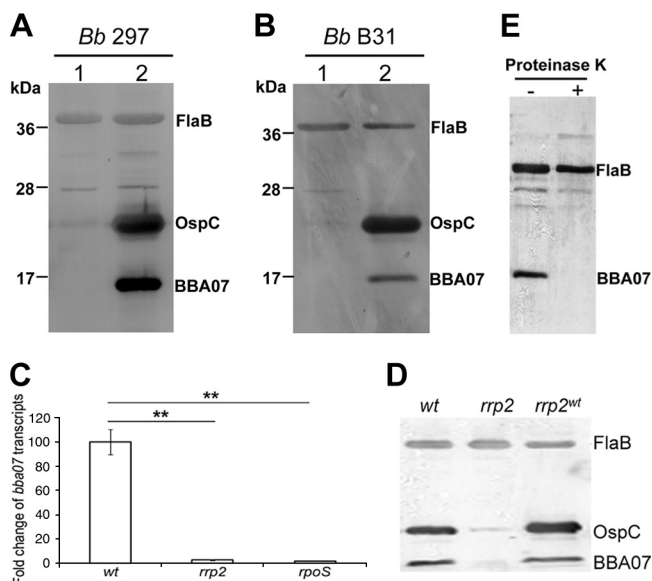


FIG. 1. Regulation of BBA07 expression under *in vitro* cultivation conditions. (A and B) BBA07 expression is induced by elevated temperature and lower pH. Either *B. burgdorferi* 297 (*Bb* 297) (A) or B31 clone A3 (*Bb* B31) (B) was cultivated in BSK-H medium at 23°C and pH 7.5 (lane 1) or at 37°C and pH 7.0 (lane 2). Spirochetes were harvested at the late logarithmic 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 positions of proteins are indicated on the right. (C and D) Control of *bba07* expression by the Rrp2-RpoN-RpoS pathway. Wild-type *B. burgdorferi* 297 (*wt*), an isogenic *rrp2* mutant (*rrp2*), a complemented *rrp2* strain (*rrp2<sup>wt</sup>*), and an *rpoS* mutant (*rpoS*) were cultivated at 37°C in BSK-H medium (pH 7.5), harvested at the late logarithmic growth phase, and subjected to either qRT-PCR analysis (C) or Western blot analysis 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 determined. The data represent the results for three independent culture samples. \*\*,  $P < 0.01$ . (E) Surface localization of BBA07. Wild-type *B. burgdorferi* strain B31 clone A3 was incubated with either PBS alone (lane -) or PBS containing proteinase K (lane +). The treated cells were then subjected to immunoblot analysis using a combination of antibodies against FlaB and BBA07. FlaB was used as a negative control.

## RESULTS

**Characterization of *bba07* expression under *in vitro* cultivation conditions.** Workers have described several microarray analyses in which more than 100 differentially expressed *B. burgdorferi* genes were identified 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 study identified *bba07* as a differentially regulated gene (37); Revel et al. showed that *bba07* expression is upregulated more than 10-fold by an elevated culture temperature and lower pH. Therefore, we first investigated whether the culture temperature and pH affect BBA07 production. Wild-type *B. burgdorferi* 297 was cultivated either at 23°C and pH 7.5 or at 37°C and pH 7.0. Cells were harvested at late logarithmic phase ( $5 \times 10^7$  cells per ml) and then subjected to Western blot analysis. As shown in Fig. 1A, similar to OspC expression,

BBA07 expression was induced by an elevated temperature and reduced culture pH, a result that is consistent with previous microarray results described by Revel et al. (37). The same experiment was performed using another *B. burgdorferi* strain, strain B31 clone A3, and the results were similar to the results obtained for strain 297 (Fig. 1B), suggesting that the effect of temperature and pH on BBA07 production is not strain specific.

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

Sequence analysis predicted that BBA07 is a putative lipoprotein (14). To determine whether BBA07 is surface exposed, wild-type *B. burgdorferi* B31 clone A3 was treated with proteinase K and subjected to immunoblot analysis. As shown in Fig. 1E, BBA07 was sensitive to proteinase K treatment, whereas the periplasmic protein FlaB remained intact upon 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 examined *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 B31 clone A3. Infected fed larvae were allowed to molt to nymphs. One month after molting, unfed nymphs were allowed to feed on naïve mice in order to produce fed nymphs. Fed larvae, unfed nymphs, and fed nymphs were collected and subjected to qRT-PCR analysis. As shown in Fig. 2A, the *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 are transmitted from ticks to mammals. This observation is consistent with the 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) obtained from mice that had been needle inoculated with wild-type spirochetes (4 weeks postinoculation). As shown in Fig. 2B, *bba07* transcripts were readily detected in heart and joint tissues but were not detected in skin samples. The undetectable level of *bba07* transcripts in the skin samples was not due to low numbers of spirochetes in the samples, as *flaB* transcripts were readily detected in the same samples. These results suggest that the *bba07* gene is expressed in spirochetes while they are replicating in the mammalian host, at least in some tissues.

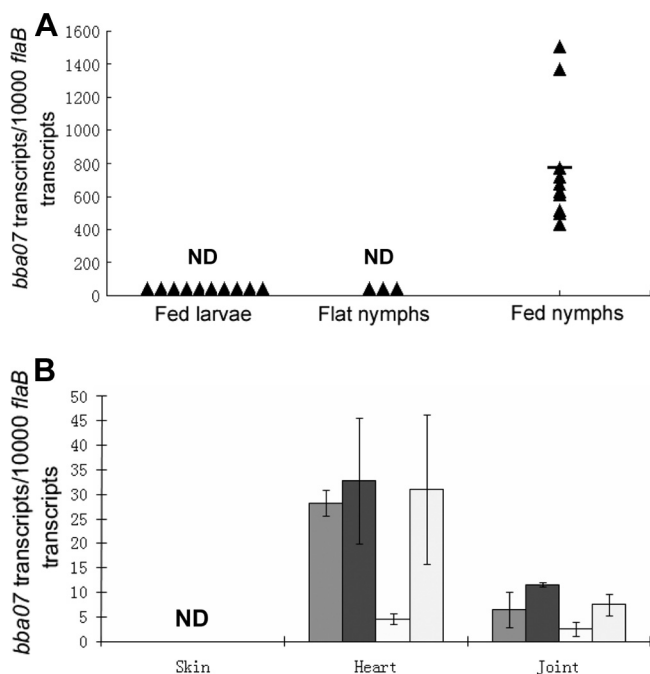


FIG. 2. Expression of *bba07* in ticks and mice. (A) Relative levels of the *bba07* transcript in fed larvae, unfed nymphs, and fed nymphs determined by qRT-PCR analysis and expressed as the number of *bba07* transcripts per 10,000 copies of the *flaB* transcript. The triangles indicate the values for data points. For fed larvae, each data point was generated using three fed larvae (a total of 30 ticks for 10 data points); for flat nymphs, each data point was generated using 40 flat nymphs (a total of 120 ticks for three data points); and for fed nymphs, each data point was generated using one tick (a total of 10 ticks for 10 data points). The bar indicates the mean. (B) Relative levels of the *bba07* transcript in joint, heart, and skin tissues harvested from mice that were needle infected with *B. burgdorferi*. Four mice were examined for each group. Each bar indicates the data for one tissue sample harvested from one mouse. For each RNA sample, three replicates were used for qRT-PCR analysis, and the error bars represent standard deviations for the three reactions. ND, not detected.

**Construction of the *bba07* mutant and 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 shown in Fig. 3A. Briefly, suicide vector pHX07 carrying a disrupted *bba07* gene along with intact surrounding regions was transformed into strain B31 clone A3. The lack of *bba07* expression in the transformant clones was confirmed at both the RNA and protein levels (Fig. 3B and 3C). Disruption of *bba07* did not appear to affect the cell morphology or growth kinetics of spirochetes in BSK-H medium (data not shown). Further plasmid profile analyses using PCR identified one *bba07* mutant clone that contained all of the endogenous plasmids present in the parental wild-type strain; this clone was selected for complementation. However, as described below, this clone lost lp28-4 during storage.

To complement the *bba07* mutant, we employed a strategy previously described by Li et al. (25), in which a wild-type copy of *bba07* (driven by its native promoter) was inserted into the intergenic region between chromosomal genes *bb0445* and *bb0446* (Fig. 3A). Restoration of *bba07* expression in the complemented clones was confirmed by RT-PCR and immunoblot

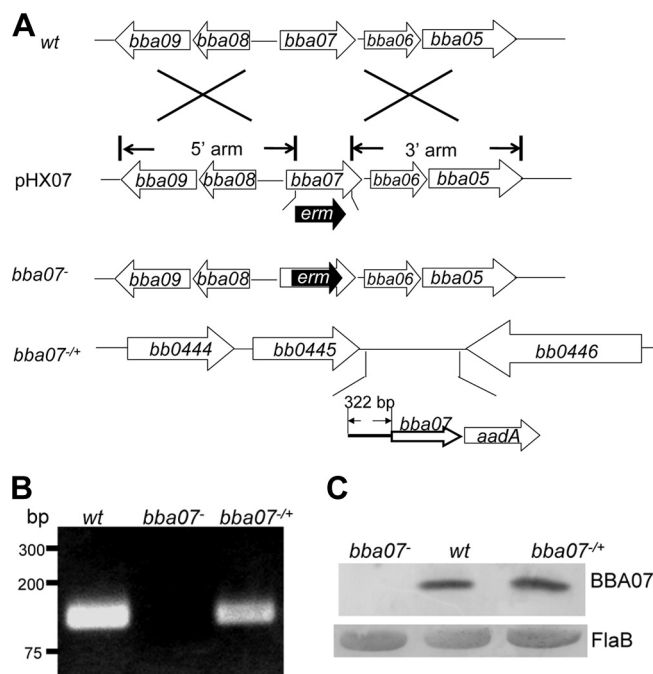


FIG. 3. Construction of the *bba07* mutant and the complemented strain. (A) Strategy used to construct the *bba07* mutant and the complemented strain. *wt*, genomic structure of *bba07* and the surrounding regions in wild-type *B. burgdorferi* B31 clone A3. Plasmid pHX07 was the suicide construct used for generating the *bba07* mutant. Only the relevant portion of the plasmid is shown. The 5' arm and the 3' arm are the two PCR fragments used for cloning upstream and downstream of the erythromycin resistance (*erm*) gene. *bba07*<sup>-</sup>, genomic structure of the *bba07* mutant; *bba07*<sup>-/+</sup>, *bb0445*-*bb0446* region in the complemented *bba07* mutant 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 analysis (B) and Western blot analysis (C) of the wild type (*wt*), the *bba07* mutant (*bba07*<sup>-</sup>), and the complemented strain (*bba07*<sup>-/+</sup>). Spirochetes were cultured at 35°C and harvested at the late logarithmic growth phase. RT-PCRs were performed using the primers specific for *bba07*, and Western blot analysis was performed using rat polyclonal antibody against BBA07. FlaB was used as a loading control.

analysis (Fig. 3B and 3C). Plasmid profile analysis of three randomly picked complemented clones showed that none of them contained lp28-4. Subsequent analysis revealed that this was due to loss of lp28-4 in the *bba07* mutant clone used for complementation during storage. lp28-4 is one of the 21 endogenous plasmids that are present in the *B. burgdorferi* genome. Previous studies using clones in which there had been spontaneous loss of lp28-4 showed that this plasmid is not essential for infection of mice by *B. burgdorferi* via needle inoculation, but it partially contributes to infection of mammals since loss of lp28-4 leads to an increase in the 50% infective dose (ID<sub>50</sub>) (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 contains all of the endogenous plasmids have not been successful. Thus, we chose a *bba07* mutant clone and a complemented strain that both lack lp28-4 for further study. Our rationale was that comparison of the *bba07* mutant with its complemented strain should allow us to assess the

TABLE 2. Infectivity of the *bba07* mutant for mice via needle inoculation

Time (days postinoculation)	Strain	Inoculum (spirochetes/ml)	No. of cultures positive/total no. of cultures					No. of mice positive/total no. of mice
			Ear <sup>a</sup>	Skin	Joint	Heart	All sites	
28	B31 clone A3	10 <sup>5</sup>	5/5	5/5	5/5	5/5	15/15	5/5
		10 <sup>3</sup>	9/10	9/10	9/10	9/10	27/30	9/10
	<i>bba07</i> mutant	10 <sup>5</sup>	5/5	5/5	5/5	5/5	15/15	5/5
		10 <sup>3</sup>	3/10	3/10	3/10	3/10	9/30	3/10
		Complemented <i>bba07</i> mutant	10 <sup>5</sup>	5/5	5/5	5/5	15/15	5/5
180	B31 clone A3	10 <sup>5</sup>	2/2	2/2	2/2	2/2	6/6	2/2
		10 <sup>3</sup>	2/2	2/2	2/2	2/2	6/6	2/2
	<i>bba07</i> mutant	10 <sup>5</sup>	2/2	2/2	2/2	2/2	6/6	2/2
		10 <sup>3</sup>	2/2	2/2	2/2	2/2	6/6	2/2
		Complemented <i>bba07</i> mutant	10 <sup>5</sup>	2/2	2/2	2/2	6/6	2/2

<sup>a</sup> Ear punch biopsy specimens obtained 14 days postinoculation were also examined, and the results were identical to those for specimens obtained 28 days postinoculation.

impact of inactivation of *bba07* on spirochete behavior. In addition, we believed that a comparison of the complemented strain lacking lp28-4 and the wild-type strain should provide insight into the role of lp28-4 in the enzootic cycle of *B. burgdorferi*.

**The *bba07* mutant is capable of infecting mice and causing a persistent infection when needle inoculation is used.** We first examined the *bba07* mutant's ability to establish an infection in mice when needle inoculation was used. Groups of five immunocompetent C3H/HeN mice were inoculated with a high dose ( $1 \times 10^5$  spirochetes per mouse) or a low dose ( $1 \times 10^3$  spirochetes per mouse) of the wild-type, *bba07* mutant, or complemented strain. Four weeks postinoculation, mice were sacrificed, and various mouse tissues (skin, heart, and joint) were collected and cultured for spirochete growth. When the high dose was used, all of the mice inoculated with wild-type, *bba07* mutant, or complemented spirochetes were infected (Table 2), suggesting that BBA07 is not necessary for *B. burgdorferi* to establish an infection in mice when needle inoculation is used. When the low dose was used, both the *bba07* mutant and the complemented strain showed reduced infectivity, which is consistent with the previous observation that lp28-4 contributes to infection of mammals (23, 35). Since the infectivities of the *bba07* mutant and the complemented strain were virtually identical ( $P = 1$ ), this result suggests that inactivation of *bba07* did not affect mouse infection via needle inoculation.

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

**The *bba07* mutant can colonize ticks, but it does not 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, *bba07* mutant, or 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 the numbers of spirochetes in each group of ticks (data not shown). To determine the ability of spirochetes to survive during tick develop-

ment (ecdysis), fed larvae were kept in a tick incubator and allowed to molt to nymphs (4 to 5 weeks). Flat (unfed) nymphs were then fed on groups of naive C3H/HeN mice. Twenty-four hours after repletion, fed nymphs were collected and subjected to IFA analysis. Spirochetes were detected in all fed nymphs examined (Fig. 4A, B, and C). Thus, neither BBA07 nor lp28-4 is essential for spirochete colonization of ticks and survival during 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 the number of spirochetes between the *bba07* mutant and the complemented strain (Fig. 4D). The absence or presence of *bba07* expression in the *bba07* mutant- or complemented strain-infected nymphs was confirmed by RT-PCR analysis (Fig. 4E). This result suggests that lp28-4, but not *bba07*, is important for optimal survival of spirochetes in ticks.

To further examine the *bba07* mutant's ability to infect mice via tick infestation, tissue samples from mice that were bitten by ticks in the experiment described above were collected and cultured to determine the presence of spirochetes. All mice infested with ticks carrying wild-type spirochetes were infected (Table 3). In contrast, none of the 18 mice infested with ticks harboring the *bba07* mutant were infected. Complementation of the *bba07* mutant with the wild-type *bba07* gene partially restored the noninfectious phenotype of the mutant when tick infestation was used; 3 of 10 mice in this group were culture positive for spirochete growth and positive for seroconversion (Fig. 5). The partial restoration of infectivity in the complemented strain was due to loss of lp28-4. This result suggests that inactivation of *bba07* did not affect infection of mammals by needle inoculation, but it impaired the ability of spirochetes to infect mice via tick transmission. As the *bba07* mutant does not have a defect in colonization of ticks, BBA07 likely plays a role in transmission of spirochetes 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 spirochete transmission and infection of mammals (2, 7, 8, 12, 30), this pathway must control expression of borrelial factors important for the enzo-

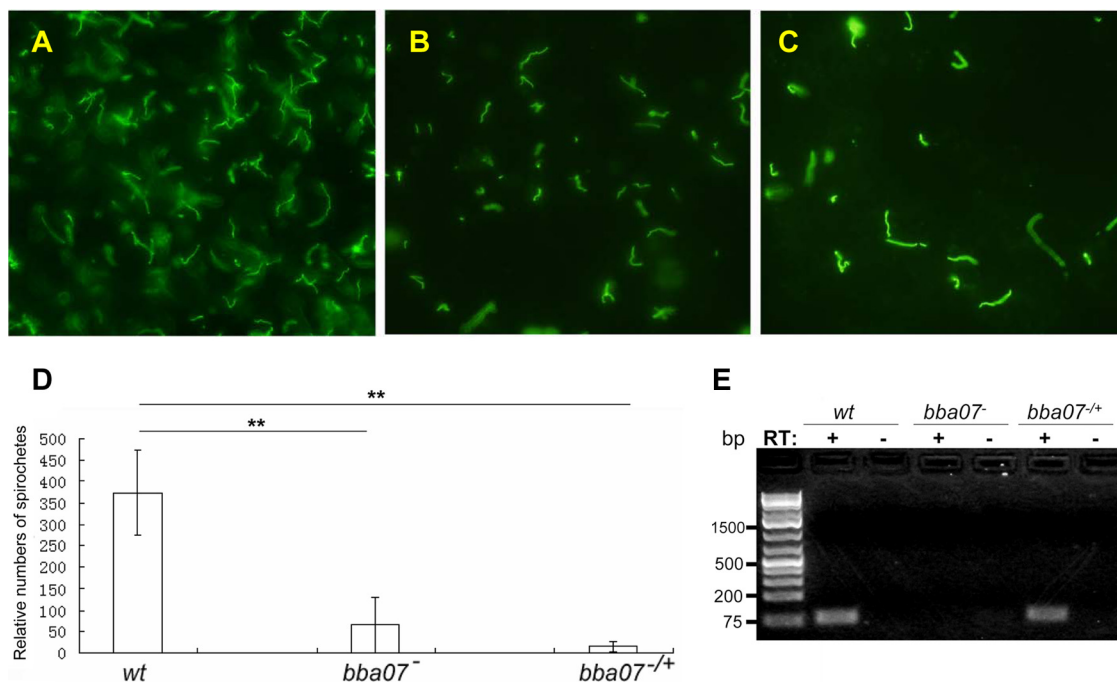


FIG. 4. IFA and RT-PCR analyses of spirochetes in fed nymphs. Unfed *I. scapularis* nymphs harboring either the wild type (*wt*) (A), the *bba07* mutant (*bba07*<sup>-</sup>) (B), or the complemented *bba07* mutant strain (*bba07*<sup>-/+</sup>) (C) were fed on naive C3H/HeN mice, and engorged nymphs were collected within 24 h and subjected to IFA analysis using fluorescein isothiocyanate-labeled anti-*B. burgdorferi* antibody. Seven ticks were examined for each group, and a representative image for each group of ticks is shown. (D) Relative number of spirochetes in each group of ticks. Spirochetes were counted with a fluorescence microscope. The error bars represent standard deviations for seven randomly picked fed nymphs. \*\*, *P* < 0.01. (E) Confirmation of *bba07* expression in fed nymphs. RNAs were extracted from six fed nymphs harboring wild-type, *bba07* mutant, or complemented *bba07* mutant strain spirochetes and subjected to RT-PCR in the presence (lanes +) or absence (lanes -) of reverse transcriptase (RT).

otic 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 that apparently plays a role in transmission of spirochetes from ticks to mammals.

The microarray analysis described by Revel et al. showed that *bba07* is 1 of 79 *B. burgdorferi* genes whose expression is influenced by culture temperature and pH (37). The results of this study confirmed the finding of Revel et al. for *bba07*. We further demonstrated that expression of *bba07* is under control of the Rrp2-RpoN-RpoS pathway. The pattern of *bba07* expression *in vitro* correlated well with the subsequent *in vivo*

TABLE 3. Infectivity of the *bba07* mutant for mice via tick transmission

Strain	No. of cultures positive/total no. of cultures					No. of mice positive/total no. of mice
	Ear	Skin	Joint	Heart	All sites	
B31 clone A3	5/5	5/5	5/5	5/5	15/15	5/5
<i>bba07</i> mutant	0/18	0/18	0/18	0/18	0/54	0/18 <sup>a</sup>
Complemented <i>bba07</i> mutant	3/10	3/10	3/10	3/10	9/30	3/10 <sup>b</sup>

<sup>a</sup> The *P* value for a comparison of the *bba07* mutant and the complemented strain is <0.05, as calculated using Fisher's two-tailed exact test.

<sup>b</sup> The *P* value for a comparison of strain B31 clone A3 and the complemented strain is <0.05, as calculated using Fisher's two-tailed exact test.

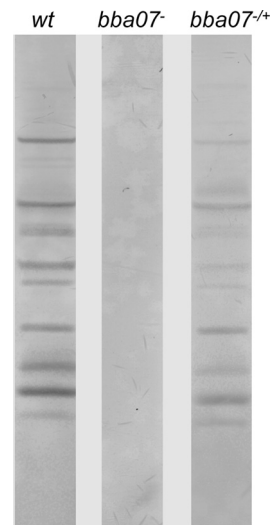


FIG. 5. Serological responses of mice infected with various strains of *B. burgdorferi*. Serum was collected from each C3H/HeN mouse 4 weeks after infestation with ticks harboring wild-type spirochetes (lane *wt*), *bba07* mutant spirochetes (lane *bba07*<sup>-</sup>), or complemented spirochetes (lane *bba07*<sup>-/+</sup>). Positive seroconversion for each mouse was determined by immunoblotting with the serum from each mouse (1:300 dilution) against whole-cell lysates of wild-type *B. burgdorferi* B31 clone A3. A representative immunoblot for each group is shown. For the group of mice infected with the complemented strain, an immunoblot for only one of the three infected mice is shown.

observation that the *bba07* transcripts were upregulated exclusively 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 infection of mammals. Interestingly, *bba07* transcripts were not detectable in mouse skin samples, suggesting that there is a possible tissue tropism for *bba07* expression.

*B. burgdorferi* has a segmented genome that is composed of a small linear chromosome that is approximately 900 kb long and at least 21 different linear and circular plasmids (9, 14). This 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). Three of these 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 contained all of the important plasmids listed above, but they did not contain lp28-4. Unlike lp25, lp28-1, or lp36, lp28-4 is not essential for infection of mammals via needle inoculation of a medium or high dose of spirochetes ( $>1 \times 10^4$  bacteria per mouse) (3, 21, 23, 35, 52). However, lp28-4 partially contributes to infection of mammals since the spirochetes that lack lp28-4 have reduced infectivity when a small inoculum ( $\leq 1 \times 10^3$  bacteria per mouse) is used (3, 23). Our finding that the complemented *bba07* mutant strain (lacking lp28-4) exhibited reduced infectivity for mammals in this study is consistent with this conclusion. There are conflicting reports about the contribution of lp28-4 in the tick vector. Strother et al. reported that loss of lp28-4 dramatically reduced the percentage of ticks infected by spirochetes when ticks were fed using the artificial capillary feeding method (45). However, in another study Elias et al. showed that when ticks were fed on infected mice (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 of the ticks examined. This suggests that loss of lp28-4 does not affect the rate of infection of ticks by spirochetes. However, there was a reduced spirochete load in the ticks infected with spirochetes without lp28-4, suggesting that lp28-4 contributes to optimal survival of *B. burgdorferi* in the tick vector.

Despite the absence 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*. BBA07 does not appear to play a role in infection of mammals, since the infectivity of the *bba07* mutant was similar to that of the isogenic complemented strain at either high or low doses (Table 2). In the tick vector, the *bba07* mutant and the complemented strain colonize and survive similarly. However, the outcomes for infection of mice via tick infestation were significantly different for the *bba07* mutant and the isogenic complemented strain. The *bba07* mutant was not able to infect mice via tick infestation, while the complemented strain was partially infectious. Since both strains lacked lp28-4, the absence of lp28-4 could not fully account for the noninfectious phenotype observed for 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 that of a re-

cently reported *bba52* mutant; while the *bba52* mutant infects mice normally when needle inoculation is used, it is not infectious via tick bites because its transmission from the tick midgut to salivary glands is impaired (22). Since the *bba07* mutant is capable of colonizing ticks and establishing an infection in mice via needle inoculation, its inability to infect mice via tick transmission suggests that BBA07 likely plays a role in the transmission from the tick midgut to the mammalian host. Future work to pinpoint the *bba07* mutant's defect in transmission, such as a defect in its ability to interact with tick gut cells, migrate to the hemolymph, survive in the hemolymph, or enter the salivary glands, is warranted.

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 information about the putative function BBA07 since it does not share significant homology with any other protein. Curiously, BBA07 was annotated as ChpAI in the TIGR *B. burgdorferi* genome database (14). However, this assignment was based solely on the low level of homology (e value,  $>1$ ) of the N-terminal portion of BBA07 to *E. coli* ChpAI (MazE, ChpR), an antitoxin. Along with the 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 evidence argue against annotation of BBA07 as a ChpAI homologue. First, the level of homology between BBA07 and ChpAI is low. Second, a BLAST search did not identify putative ChpAK genes 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 to elucidate the physiological function of this surface lipoprotein is warranted.

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