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BBA52 facilitates *Borrelia burgdorferi* transmission from feeding ticks to murine hosts

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Abstract

Borrelia burgdorferi, the pathogen of Lyme borreliosis, persists in nature through a tick-rodent transmission cycle. A selective assessment of the microbial transcriptome, limited to gene encoding putative membrane proteins, reveals that *bba52* transcription *in vivo* is strictly confined to the vector-specific portion of microbial life cycle with highest expression levels in feeding ticks and swift downregulation in mice. *bba52* deletion did not affect murine disease as assessed by the genesis of arthritis and carditis or long-term pathogen persistence in mice or ticks. However, *bba52* deficiency did impair microbial transitions between hosts and vector, defects that could be fully rescued when *bba52* expression was genetically restored to the original genomic locus. These studies establish that BBA52 facilitates vector-host transitions by the pathogen and as such, is a potential antigenic target for interference with *B. burgdorferi* transmission from ticks to mammalian hosts.

Keywords

Borrelia burgdorferi; Lyme disease; pathogen transmission

INTRODUCTION

Lyme borreliosis, caused by *Borrelia burgdorferi*, is a vector-borne zoonosis prevalent in North America and Europe [1]. When feeding on an infected host, usually wild rodents, immature *Ixodes* ticks acquire the pathogen, transstadially maintain the infection and, during a subsequent blood meal, transmit the pathogen to mammals. Once infected with *B. burgdorferi*, humans develop a wide array of clinical complications including the characteristic skin rash *erythema migrans*, arthritis, carditis and a variety of neurological disorders [2]. As wild rodents are the natural reservoir hosts of *B. burgdorferi*, certain inbred mice, such as C3H mice are considered excellent models of pathogenesis and are use to study transmission cycle of spirochetes [3]. Genome sequencing of *B. burgdorferi* [4,5], studies on the expression and regulation of borrelial genes [6-17] and advances in genetic manipulation techniques [18] have all greatly contributed to our understanding of the unique biology and enzootic infection cycle of this spirochete. However, a human vaccine against *B. burgdorferi* is currently unavailable, and thus, the development of effective preventive measures remains one of the major focuses of Lyme disease research.

B. burgdorferi may persist in a host or vector for months to years, shuffling between locations during short episodes of tick feeding [19]. During migration from an infected tick to a host, *B.*

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burgdorferi invades salivary glands and transmits along with tick saliva [19]. Many salivary gland proteins (Salps) are feeding-induced, soluble and can influence the spirochete transition between vector and host [20,21]. A few *I. scapularis* Salps have been identified [22-24] that play important roles in spirochete infection cycle. Further characterization of the interactions of Salps with borrelial antigens and their contributions to infectivity will aid in our understanding of poorly-understood aspects of borrelial transmission.

It is clear that the identification of borrelial antigens that play important roles in spirochete survival in ticks or enable vector-host transitions is the key to blocking pathogen transmission. As extracellularly exposed membrane proteins may directly interact with different environments during transmission or dissemination thus contributing to pathogen adaptation, we sought to assess the expression of selected putative membrane proteins in feeding ticks and mice. We further studied one of these genes, *bba52*, which displayed vector-specific expression. BBA52, annotated as an outer membrane protein of no assigned function [4,5], is encoded by the linear plasmid (lp) lp54, which is a stable extra-chromosomal element and is considered to be a necessary part of the spirochete genome [25]. We show that BBA52 facilitates vector-host transitions of *B. burgdorferi* and is a potential antigenic target to interfere with transmission of Lyme borreliosis.

METERIALS AND METHODS

Mice, *Borrelia* and ticks

An infectious isolate of *Borrelia burgdorferi* B31, clone A3, was used throughout the study [26]. For *in vitro* studies, spirochetes were harvested from the log phase of growth (10^7 cells/ml). Six- to eight-week-old female C3H/HeN mice were purchased from the National Institutes of Health. The ticks used in this study were reared in the laboratory as described [27]. All animal experiments were performed according to the guidelines of the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee.

PCR

Following *B. burgdorferi* target genes were selected based on their predicted localization to the spirochete membrane according to database annotation (www.tigr.org) and PSORT *in silico* analysis as recently described [28]: bb0019, bb0027, bb0028, bb0108, bb0144, bb0155, bb0213, bb0258, bb0262, bb0298, bb0319, bb0323, bb0328, bb0353, bb0379, bb0381, bb0469, bb0586, bb0656, bb0678, bb0679, bb0735, bb0744, bb0769, bb0843, bba03, bba33, bba52, bba62, bba64, bba73, bba74, bbb27, bbe31, bbg01, bbi16, bbj23, bbj27, bbk33, bbk45, bbl23, bbm38, bbn26, bbn38, bbn39. Nucleotide sequences of each of the gene-specific primers will be available on request. For gene expression analysis, groups of C3H mice (3 animals/group) were infected with *B. burgdorferi* (10^5 cells/mouse), and following two weeks of infection, dermal samples were collected. For analysis of gene expression in feeding ticks during transmission, naturally infected nymphs (25 ticks/mouse) were allowed to engorge on naïve mice, and were collected at 24, 48 and 96 hours after attachment and pooled together. Two independent experiments used the same parameters of gene expression analysis to ensure the reproducibility of the assay. For analysis of *bba52* expression, RNA was isolated from mice (10 animals/group) at 1, 2 and 3 weeks after infection, from ticks that parasitized on infected mice (25 ticks/mice, 3 animals/group) and from infected nymphs (10 nymphs/mouse, 3 animals/group) that engorged on naïve mice. Total RNA was isolated from murine and tick samples, and RT-PCR or quantitative RT-PCR (qRT-PCR) analysis was performed as described previously [28].

Generation of recombinant proteins and antisera

The primers used for amplification of *bba52* without the respective signal peptides, are shown in Table 1. Purification of recombinant BBA52 and generation of murine BBA52 antisera were performed as described [28]. In addition, using a commercial source (GenScript Corporation), affinity-purified polyclonal antibodies against a BBA52 peptide sequence (EFLDDPSQESDELEC) of predicted immunogenicity was generated in rabbits. Generated BBA52 antibodies specifically detected 33-kDa native *B. burgdorferi* BBA52 and did not cross-react with other spirochete proteins.

Confocal immunofluorescence microscopy

Confocal immunofluorescence of tick salivary glands was performed using LSM-510 laser scanning microscope (Zeiss) as detailed earlier [29,30]. Samples for each time point of analysis were dissected from a minimum of 5 ticks, and whole organs were scanned at 0.6 μm intervals through the full tissue thickness. Spirochetes were detected using FITC-labeled anti-*B. burgdorferi* goat IgG (KPL), whereas tick salivary glands were labeled with Texas Red-phalloidin (Invitrogen), respectively.

Genetic manipulation of *B. burgdorferi*

BBA52-deficient *B. burgdorferi* was generated via homologous recombination by replacing the entire *bba52* open reading frame (ORF) with a *flaBp-kan* cassette as described [28], using the oligonucleotide primers as detailed in Table 1. For genetic complementation, initial efforts to restore *bba52* expression used our published strategies [28]; however, transformation strategies using either a DNA insert representing the upstream of the *bba52* ORF with the intergenic region (40 nucleotides) or *flaB* promoter, failed to yield any transformants. We therefore, devised a new strategy to accomplish *bba52* complementation *in cis* by re-inserting a wild-type copy of *bba52* ORF into the original gene locus in plasmid lp54. To achieve this, we amplified and assembled two DNA inserts, one using P1-P11 encompassing *bba52* ORF and the other using the primers P12 and P13 that included the *aadA* cassette [31], which confers resistance to streptomycin used for the selection of transformants, and the *flgB* promoter (figure 5*A*). The insert carrying P1-P13 amplicon was replaced with the P1-P2 flanking region of the original mutagenic construct pXLF-P1P4 to obtain *bba52* complemented construct. The construct was checked for identity and 25μg DNA was transformed into *bba52* mutant. Twelve clones grew in the presence of both kanamycin (350μg/ml) and streptomycin (100μg/ml). One of the clones that restored *bba52* mRNA and protein, and also retained a comparable plasmid profile to the mutant, except for loss of the non-essential plasmid lp5 [32], was used for further studies.

Phenotypic analysis of genetically-manipulated *B. burgdorferi*

To ascertain the phenotype, the mutants and wild-type spirochetes (10^5 cells/mouse) were separately inoculated into groups of mice (15 animals/group). Skin, heart, joint and bladder, samples were isolated at 1, 2, 3, 4 and 12 weeks following infection. For each time point, samples from 3 mice were pooled by the tissue type and pathogen burdens were assessed by qRT-PCR analysis of *flaB* mRNA and normalized against murine or *β-actin* gene as described [28,33]. Three micrograms of RNA per tissue sample was used for qRT-PCR analysis. At time of euthanasia, mice (3 animal/group) were assessed for swelling of the tibiotarsal joints. Histopathology of joints and hearts collected at weeks 0, 2, 3 and 4 following infection were performed as detailed [28,34]. Portions of heart and spleen were also cultured in BSK-H medium. For acquisition studies, mice were infected (10^5 spirochetes/mouse, 3 animals/group). After two weeks, nymphs (25 ticks/mouse) were allowed to engorge on the mice and collected either during 24 and 48 hours of feeding or as repleted ticks. For transmission studies, infected nymphs were generated by allowing larva to feed on wild-type or genetically-manipulated *B.*

burgdorferi-infected mice as described [29]. Infected nymphs (10 ticks/mouse, 3 animals/group) were allowed to fed on naïve mice and *B. burgdorferi* burdens in partially fed ticks (48 and 60h) or engorged ticks were determined by qRT-PCR. As one tick was sufficient in transmitting the infection to mice [35], single infected nymphs were allowed to feed on separate groups of naïve mouse (3 mice/group). At day 7 following tick feeding, all the mice were sacrificed, and the skin, joints, heart and bladder tissues were isolated and assessed for spirochete burden. Portions of heart and spleen tissues were cultured in BSK-H medium.

Statistical analysis

Results are expressed as the mean ± standard error (SEM). The significance of the difference between the mean values of the groups was evaluated by two-tailed Student's *t*-test.

RESULTS

B. burgdorferi genes upregulated during transmission

B. burgdorferi faces new environments during host-vector transitions and presumably alters antigenic expression to complete the transition and persist in the new environment. In order to identify microbial genes that are differentially expressed in feeding ticks during transmission, the transcript levels of selected spirochete genes in nymphs and murine dermis were compared using quantitative RT-PCR (qRT-PCR) analysis. The genes were selected due to their putative membrane localization as determined by database annotation and *in silico* analysis for extracellular exposure. Naïve mice (5 animals/group) were infested with *B. burgdorferi*-infected nymphs (25 ticks/group), and ticks were collected at 24-96 hours of feeding and pooled together. Dermis samples were collected from groups of mice (5 animals/group) two weeks after infection. Both murine and tick samples were subjected to qRT-PCR analysis as detailed in the Materials and Methods section. The results are represented as fold increase in individual gene transcript levels relative to *flaB* expression. *bba52*, along with a few other *B. burgdorferi* genes, is highly expressed in feeding ticks during transmission, in comparison to its transcript levels in mice (figure 1). We choose to focus on *bba52* based on previous studies showing likely tick-specific expression [14,36], its annotation as a non-paralogous and outer membrane protein of unknown function [4,5] and unique genomic location as an insertion into the stable plasmid lp54, which otherwise contains many redundant sequences [4].

bba52 is upregulated during spirochete entry and exit through ticks

We assessed *bba52* expression in detail during representative phases of the *B. burgdorferi* infection cycle. Mice (10 animals/group) were infected with *B. burgdorferi* (10^5 cells/mouse). Total RNA was isolated from skin, joint, heart and bladder samples at 1, 2 and 3 weeks post-infection and pooled by the tissue type. Ticks were parasitized on parallel groups of two-week-old-infected mice (25 nymphs or 30 larvae/mouse), and engorged ticks were isolated. Fed intermolt nymphs were analyzed at 25 days after feeding while larvae were allowed to molt to nymphs. Newly molted infected nymphs were allowed to feed on naïve mice (10 ticks/mice), and were collected following 12-48 hours of feeding. Skin samples were also collected from mice following 5 days of tick engorgement. Total RNA was prepared and subjected to qRT-PCR analysis to measure *bba52* transcripts and normalized against *flaB*. The results showed that *bba52* transcripts were undetectable in mice during persistent infection or early tick-borne infection, but were obvious at all tested stages of *B. burgdorferi* infection in ticks, with the highest levels during tick feeding (figure 2).

Construction of *bba52* mutant *B. burgdorferi*

To understand the role of BBA52 in spirochete infectious cycle, we sought to create BBA52-deficient *B. burgdorferi* using an infectious clone. A suicide plasmid was created to replace

the entire open reading frame of *bba52* with an antibiotic resistance gene using homologous recombination (figure 3*A*). The construct was transformed into an infectious clone of *B. burgdorferi*, and a clone with intended recombination event was selected (figure 3*B*). The isolated mutant retained the same set of plasmids as in the wild-type spirochetes, but failed to produce *bba52* mRNA (figure 3*C*) or protein (figure 3*D*). The genetic manipulation did not introduce unwanted polar effects as the mutant expressed the immediately neighboring genes, *bba51* and *bba53*, at similar levels as the wild-type isolate (figure 3*C*). Deletion of *bba52* did not alter *B. burgdorferi* growth kinetics *in vitro* (figure 3*E*).

BBA52-deficient *B. burgdorferi* remains infectious in mice

To determine whether *bba52* deletion interferes with the ability of *B. burgdorferi* to persist in mice and induce inflammation, C3H mice (15 animals/group) were injected intradermally with the wild-type spirochetes or *bba52* mutants (10^5 cells/mouse). Mice were sacrificed at weeks 1, 2, 3, 4 and 12 following infection. Pathogen burdens were assessed in isolated skin, heart, joint and bladder samples by qRT-PCR analysis using *flaB* as a surrogate marker, and normalized against murine *β-actin* levels. The results showed that both parental and *bba52* mutants persisted at similar levels in all time points and tissues, and data from weeks 2, 3 and 12 are presented (figure 4*A*). Both *bba52* mutant and wild-type spirochetes could be cultured from spleen tissues collected following one week of infection (data not shown). In agreement with the comparable pathogen loads, mice infected with either wild type or *bba52* mutant *B. burgdorferi* developed similar disease, as evaluated by the development of ankle swelling (figure 4*B*) and histopathological observation of arthritis and carditis (data not shown). Taken together, these results suggest that BBA52 is not essential for persistence and virulence of *B. burgdorferi* in mice.

bba52 mutant *B. burgdorferi* displays significant defects during transition between mice and ticks

Although both wild-type spirochetes and *bba52* mutants persisted at similar levels in the murine dermis throughout the infection (figure 4*A*), the mutant was significantly impaired in its ability to transmit to naïve ticks. To ensure the specificity of the result, we sought to complement the mutants with a wild-type copy of the *bba52* gene *in cis*, and use this isolate in mouse-tick transmission studies. The native promoter for *bba52* is undefined and heterologous *flaB* promoter failed to drive *bba52* expression in *B. burgdorferi*. We therefore, devised a new strategy (figure 5*A*) to re-insert *bba52* in the original gene locus using homologous recombination, as detailed in the Material and Methods section. The complemented construct was transformed into mutants, and isolates were selected using antibiotics. PCR analysis confirmed that one of the *bba52*-complemented isolates retained all endogenous, except for the loss of the non-essential plasmid, lp5 (data not shown). The *bba52*-complemented isolate produced both *bba52* mRNA (figure 5*B*, upper panel) and BBA52 protein (figure 5*C*). As expected, the genetic manipulation process did not introduce polar effects in complemented isolates as assessed by the transcription of surrounding genes, *bba51* and *bba53* (figure 5*B*, lower panel).

We then assessed whether BBA52 is required for *B. burgdorferi* entry, persistence and transmission through ticks. To examine the effect of the *bba52* deletion on spirochete acquisition by ticks, larval and naïve nymphal ticks were allowed to parasitize mice that had been infected with wild type, *bba52* mutant or *bba52*-complemented isolates. Partially-fed nymphs were forcibly removed 24 and 48 hours after the onset of feeding and parallel groups of larvae or nymphs were collected as fully-engorged ticks. The spirochete burden was assessed by qRT-PCR analysis of *flaB* normalized against tick *β-actin* levels. Compared to wild type or *bba52*-complemented isolates, the levels of *bba52* mutants were significantly lower in feeding ticks analyzed at 24 hours ($P < 0.002$) and 48 hours ($P < 0.02$) of host attachment (figure

6*A*). However, analysis of fully engorged larva or nymphs at 7 and 25 days post-feeding (figure 6*B*) showed similar burdens of wild type and mutant spirochetes, suggesting *bba52* deletion only transiently affected *B. burgdorferi* acquisition by ticks, without significant influence on microbial persistence in the ticks. We then compared the ability of the *bba52* mutants to transmit back from infected ticks to naïve mice. Separate groups of nymphs naturally infected with wild type or mutant isolates were allowed to feed on naïve C3H mice (9 animals/group) and were collected as partially-fed (48 and 60 hours) or fully-engorged ticks. Spirochete burdens in ticks were assessed by qRT-PCR and confocal immunofluorescence analyses. Following 7 days of feeding, mouse infection was assessed by culture analysis of the heart and spleen samples and qRT-PCR analysis of skin, heart and bladder tissues. Results indicated that burdens of wild type and *bba52* mutants were similar in fed tick gut (data not shown); however, the *bba52* mutant was highly impaired in its ability to migrate to salivary glands (figure 6*C*) and transmit to mice (figure 6*D*). Both wild type and *bba52* complemented isolates were recovered by culture analysis of murine spleen and heart samples. In contrast, the *bba52* mutant remained undetectable in all of the 6 individual mouse spleens analyzed, but was recovered from the four of the 6 heart samples. This observation indicates a minor proportion of *bba52* mutants that remained untraceable in immunofluorescence and qRT-PCR analyses are still capable of transmission. Collectively, these data establish that BBA52 function is nonessential for *B. burgdorferi* persistence in murine hosts or ticks but facilitates *B. burgdorferi* transitions between hosts and vector.

DISCUSSION

B. burgdorferi undergoes remarkable changes in antigenic composition as it invades and colonizes diverse tissues in arthropods and mammals [10,28,37-41]. These changes, at least in part, are mediated by regulatory networks involving Rrp2-RpoN/RpoS or Rrp1–Hpk1 TCS and c-di-GMP [7,13,17,42,43], in addition to intergenic recombination-based mechanism involving the *vlsE* locus [44,45]. Microarray analyses of transcriptional alterations in cultured spirochetes identified a large number of genes that are differentially expressed, including *bba52*, which responded to physiochemical alterations including variations in temperature, the addition of blood, or growth in a dialysis membrane chamber (DMC) implanted within the murine host [6,7,14,36]. In agreement, our data show that selected *B. burgdorferi* genes are also variably expressed *in vivo* and highly transcribed in feeding ticks during transmission. The expression pattern of many of these genes (*bb0323, bba52, bba62, bba74 and bbe31*) agreed with previous studies involving cultured spirochetes that predicted preferential expression in ticks [6,7,11,14]. Specifically, *bba74* [46] and *bba62* [33] were recently identified as being expressed in feeding ticks. The majority of these genes, however, encoded proteins of unknown functions that are possibly relevant for pathogen transmission from feeding ticks or the establishment of early mammalian infection.

The systematic identification of *B. burgdorferi* gene products important for infectivity is possible due to the seminal discovery of a borrelial genetic transformation process [47] and further progress in the gene manipulation process [18]. However, the unusual organization of the spirochete genome and the lack of promoter information in a large number of spirochete genes poses serious challenges for mutagenesis, especially for genetic complementation, as we encountered for *bba52*. The promoter of *bba52* is undefined and ORF of 14 upstream genes are unidirectional possessing overlapping or short intergenic regions indicating potentially linked expression. The latter speculation is also suggested by their similar temperature regulation, such as enhanced transcript levels in spirochetes grown at 23°C relative to 37°C [36]. *bba52* shares a short (40 base pair) intergenic region with *bba51*; however, use of this intergenic sequence as a native promoter to drive *bba52* expression in *B. burgdorferi* was unsuccessful (data not shown). Although constitutively-active borrelial promoter *flaB* was able to produce BBA52 in *E. coli* (data not shown), the same promoter failed to restore *bba52*

expression in *B. burgdorferi* suggesting that the constitutive expression of *bba52* may be detrimental to spirochetes. Thus, genetic complementation of regulated borrelial genes without a promoter identity remains technically challenging. In this case, we were able to complement *bba52* by replacing the gene at its native *cis* location without polar effects, which fully restored the wild-type phenotype. This strategy could be helpful for the complementation of other regulated borrelial genes, particularly those lacking discernible promoters.

BBA52 is encoded by the linear plasmid (lp) lp54, a core part of the spirochete genome [25] and retains 65-68% amino acid identity to orthologs in *B. afzelii* and *B. garinii*. Our mutagenesis studies suggest that BBA52 is involved in spirochete transmission from ticks to mice. Following tick-borne transmission, *bba52* mutants were undetectable in mice, but sometimes recoverable by culture analysis of murine tissues. Therefore, a basal level of spirochete transmission occurs independent of BBA52, possibly suggesting multiple pathways of transmission available to the spirochete. Alternatively, BBA52, along with other borrelial proteins, such as OspC or Lp6.6 [30,33], could have complementary but non-essential roles in transmission process, as these antigens are all localized in the outer membrane [48,49] and co-expressed in feeding ticks [33,40]. Although the precise function of BBA52 in spirochete biology remains unknown, further characterization of antigenic determinants required for microbial transition between hosts and vectors may contribute to the development of novel transmission-blocking vaccines against vector-borne diseases.

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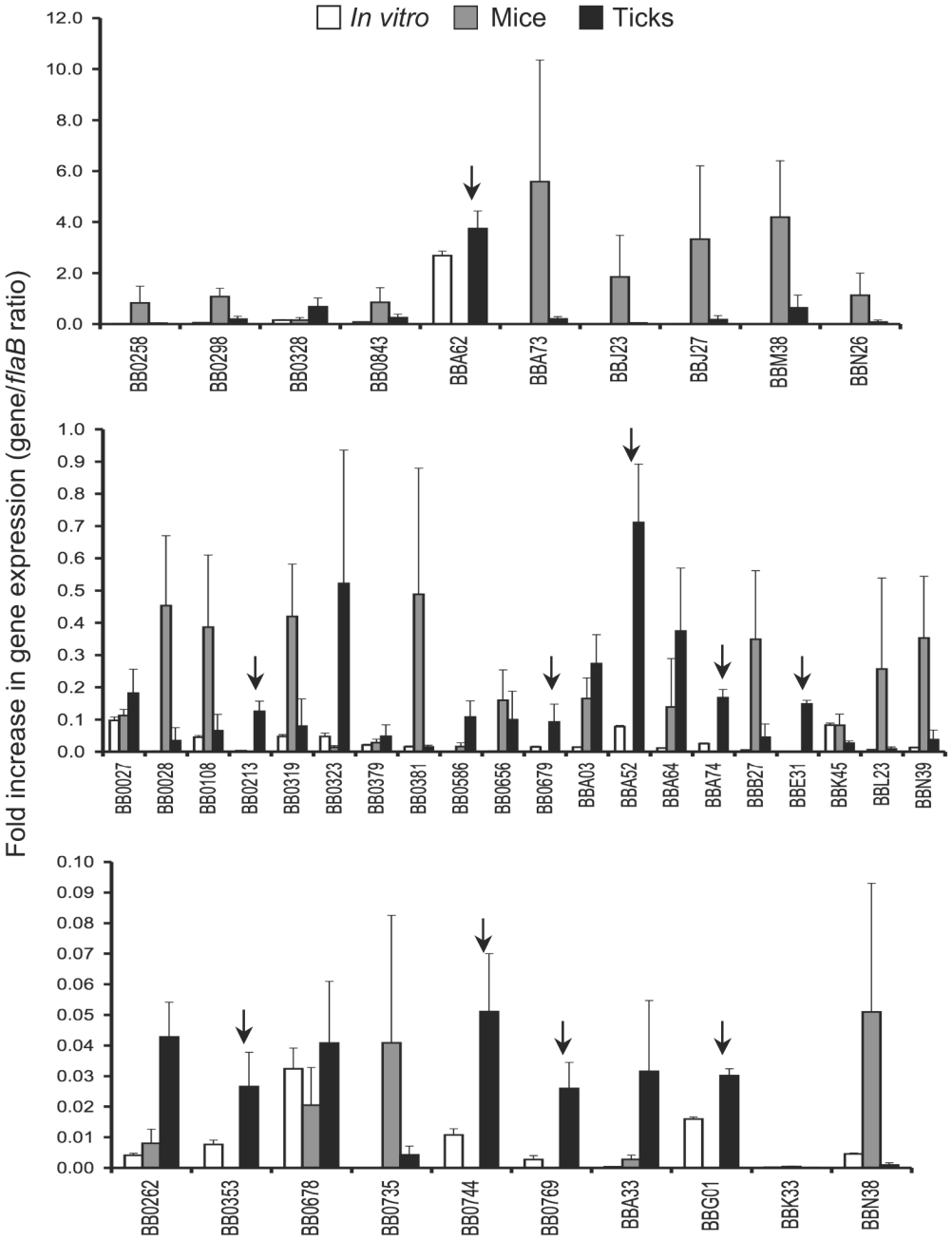

Figure 1. Relative expression levels of selected *B. burgdorferi* genes in feeding ticks during transmission

Total RNA was isolated from cultured spirochetes, from pooled *B. burgdorferi* infected nymphs collected at 24, 48 and 96 hours of feeding on naïve mice and from murine skin following 2 weeks of *B. burgdorferi* infection, and converted to cDNA for measuring gene-specific transcripts using qRT-PCR. Fold increase in the expression of individual genes in each of the tick or murine samples was calculated based on threshold cycle (Ct) values using the $2^{-\Delta\Delta Ct}$ method normalized against *flaB* Ct values. Upper, middle and lower panels represent genes with the highest, moderate and lowest expression ratios relative to that of *flaB*. Bars indicate the mean ± SD from four qRT-PCR analyses of two independent infection

experiments. Arrows indicates genes that are highly expressed in feeding ticks but remain undetectable in the murine dermis.

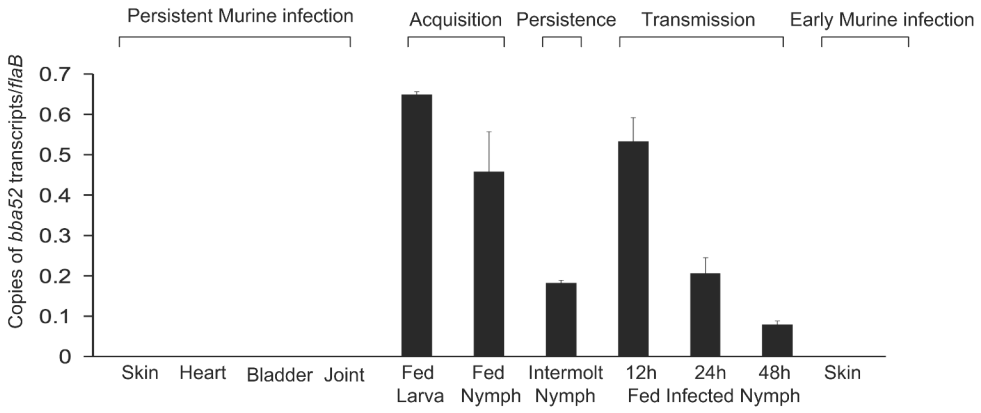

Figure 2. *bba52* expression is vector-specific

bba52 expression is analyzed at various stages of murine and tick infectivity. RNA was isolated from mice (10 animals/group) at 1, 2 and 3 weeks after *B. burgdorferi* infection and pooled by the tissue types (skin, heart, joint and bladder). Naïve larvae and nymphs were allowed to feed on *B. burgdorferi*-infected mice (25 ticks/mice) and collected at 96 hours (fed larva and fed nymph) or 25 days following feeding (intermolt nymph). *B. burgdorferi*-infected nymphs (10 nymphs/mouse) were allowed to feed on naïve mice and collected at 12, 24 and 48 hours of feeding (fed infected nymph). Murine skin samples were collected following 5 days of tick engorgement. RNA samples from murine and tick samples were analyzed by qRT-PCR and presented as copies of *bba52* transcript per copy of *flaB* transcript. Error bars represent the mean ± SEM from four qRT-PCR analyses of two independent murine-tick infection experiments.

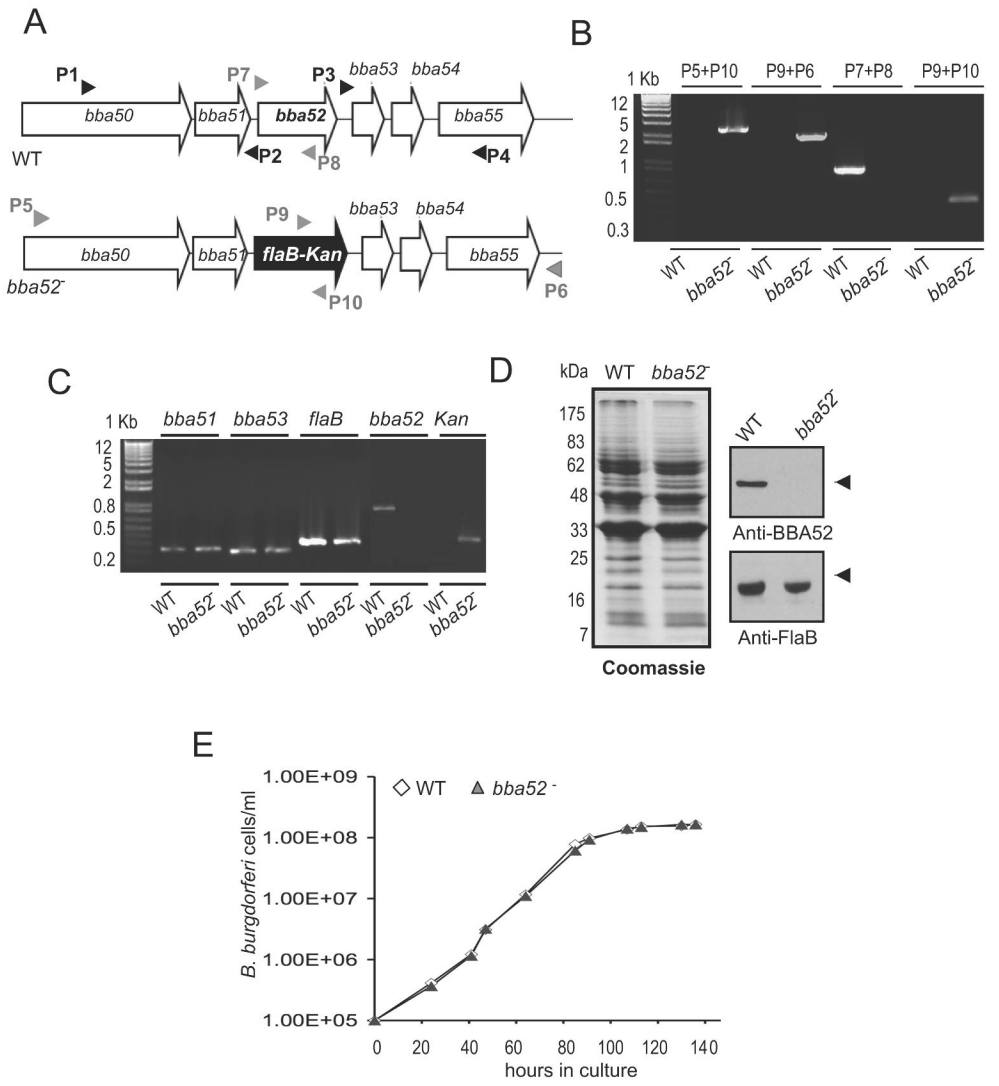

Figure 3. Construction and analysis of *bba52* mutant *B. burgdorferi*

(**A**) Schematic representation of wild type (WT) and *bba52* mutant (*bba52*$^-$) *B. burgdorferi* at the *bba52* locus. Genes *bba50-bba55* (white box arrows) and the kanamycin-resistance cassette driven by the *B. burgdorferi flaB* promoter (*flaB-Kan*, black box arrow) are indicated. The regions up- and down-stream of the *bba52* locus were amplified using primers P1-P4 (black arrow-heads) and ligated on either side of the *flaB-Kan* cassette to obtain the mutagenic construct, as detailed in the text. (**B**) Integration of the mutagenic construct, *flaB-Kan*, in the intended genomic locus. Primers 5-10 (gray arrowheads, positions indicated in figure 3*A*) were used for PCR analysis using isolated DNA from wild type (WT) or mutant *B. burgdorferi* (*bba52*$^-$) and subjected to gel electrophoresis. The combination of primers used for PCR is indicated at the top, and migration of the DNA ladder is shown on the left. (**C**) RT-PCR assessment of *bba52* transcripts and the polar effects of mutagenesis. Total RNA was isolated from wild-type *B. burgdorferi* (WT) and *bba52* mutant (*bba52*$^-$), converted to cDNA and used to amplify regions within *bba52*, *flaB*, kanamycin and genes surrounding the *bba52* locus (*bba51* and *bba53*) and visualized on a gel. (**D**) Protein analysis of wild-type *B. burgdorferi* (WT) and *bba52* mutant (*bba52*$^-$). Equal amounts of protein were separated on an SDS-PAGE

gel, and either stained with Coomassie blue (left panel) or transferred onto a nitrocellulose membrane and probed with BBA52 and FlaB antibodies (right panels). Migration of protein standards is shown to the left in kDa. (**E**) Growth curves for the wild-type and *bba52* mutant *B. burgdorferi*. Spirochetes were diluted to a density of 10^5 cells/ml and grown at 34°C in BSK-H medium. Triplicate samples were counted under a dark-field microscope using a Petroff-Hausser cell counter. Differences between wild type and *bba52* mutant numbers were insignificant at all times of growth ($P > 0.05$).

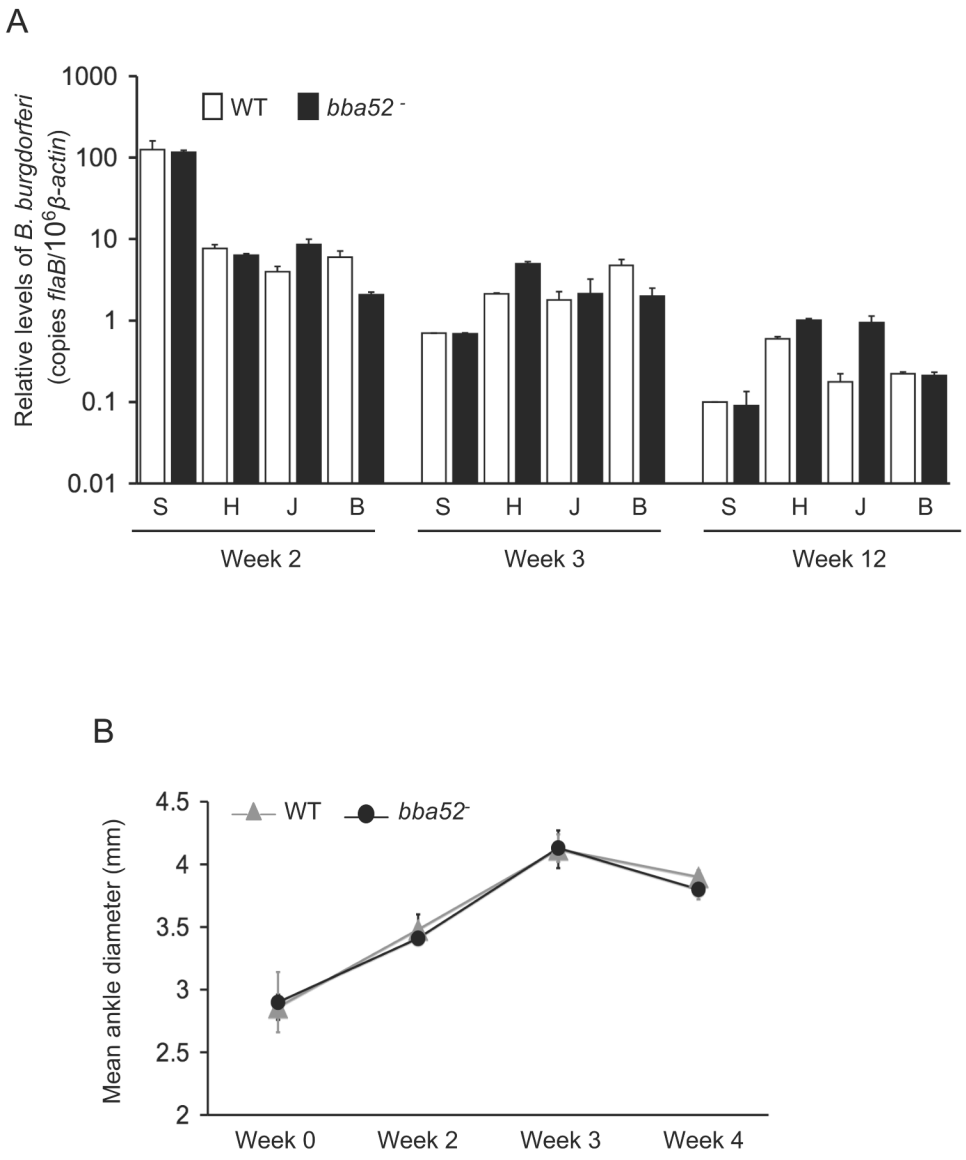

Figure 4. *bba52* mutant *B. burgdorferi* retain full infectivity in mice

(**A**) The pathogen burdens in multiple tissues of infected mice are shown. Mice (15 animals/group) were infected with wild type or the *bba52* mutant isolates and spirochete burdens were analyzed in skin (S), heart (H), joint (J) and bladder (B) samples by measuring copies of *B. burgdorferi flaB* RNA at 2, 3 and 12 weeks of infection. Amounts of murine *β-actin* were determined in each sample and used to normalize the quantities of spirochete RNA. Bars represent the mean measurements ± SEM of qRT-PCR analyses from two independent infection experiments. The difference between wild type and *bba52* mutant levels was statistically insignificant at all time points and tissues ($P > 0.05$). (**B**) Assessment of joint swelling in *B. burgdorferi*-infected mice. Groups of mice (3 animals/group) were infected with wild type or *bba52* mutant and examined for joint swelling, using a digital caliper at 0, 2, 3 and 4 weeks after spirochete challenge. Data represent the mean ± SEM from two independent infection experiments. No difference in the ability of the wild type and *bba52* mutant to induce joint swelling was recorded ($P > 0.05$).

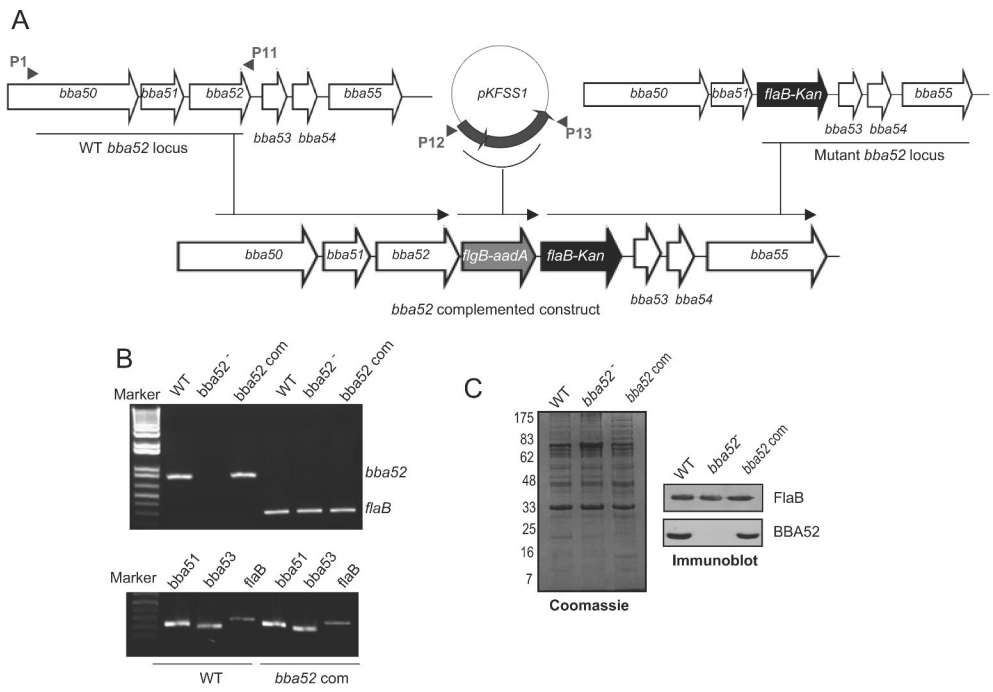

Figure 5. Genetic complementation of *bba52* mutant *B. burgdorferi*

(**A**) Construction of the *bba52*-complemented construct for re-insertion of *bba52 in cis*, in the original gene locus of the lp54 plasmid. A new 5′ arm was generated using primers P1-P11 and P12-P13, which were used to amplify and assemble two DNA inserts surrounding *bba52* and *aadA* cassette with the *flgB* promoter, respectively. The insert representing P1-P13 amplicon (new 5′ arm) was fused with the *flaB-Kan* cassette carrying the old 3′ arm (as generated using P3-P4, figure 3*A*) to obtain *bba52* complemented construct, and integrated in *B. burgdorferi* lp54 locus via homologous recombination. (**B**) RT-PCR analysis of the *bba52* complemented isolate. Total RNA was isolated from either the wild type (WT), *bba52* mutant (*bba52*$^{-}$) or *bba52*-complemented *B. burgdorferi* (*bba52* Com), converted to cDNA, then subjected to PCR analysis with *flaB* and *bba52* primers, and analyzed on a 1.5% agarose gel (upper panel). *bba52* complemented isolates did not display polar effects on the transcription of genes surrounding *bba52* locus (*bba51* and *bba53*) (lower panel). (**C**) Production of BBA52 protein in the complemented *B. burgdorferi*. Spirochete lysates were separated on a SDS-PAGE gel, stained with Coomassie blue (left panel), or transferred to nitrocellulose membrane and probed with BBA52 and FlaB antibodies (right panels).

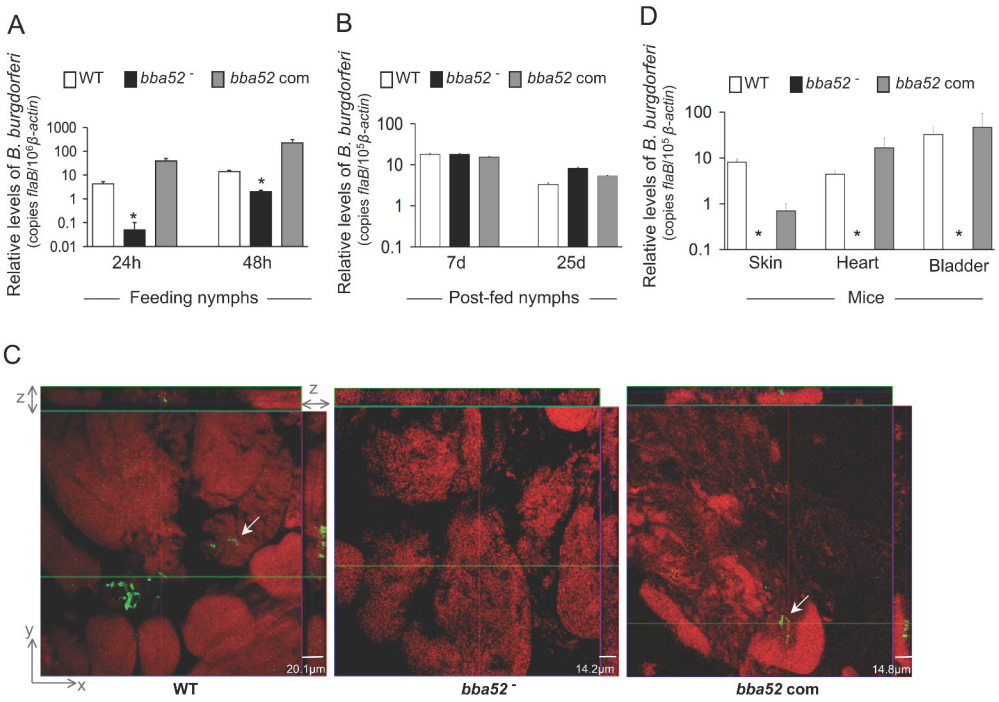

Figure 6. *bba52* mutant *B. burgdorferi* is impaired in its ability to transit between murine hosts and ticks

(**A**) *B. burgdorferi* burdens in ticks during acquisition from infected mice. Mice were infected with *B. burgdorferi* (3 mice/group) and, following 2 weeks of infection, naïve *I. scapularis* larvae or nymphs (25 ticks/mouse) were allowed to feed on mice. *B. burgdorferi* burdens in ticks were analyzed at the indicated time intervals following feeding by measuring copies of the *B. burgdorferi flaB* RNA. Amounts of tick *β-actin* were determined in each sample and used to normalize the quantities of spirochete RNA. Bars represent the mean ± SEM of eight qRT-PCR analyses derived from two independent infection experiments. Differences in the spirochete burdens in ticks infected with *bba52* mutant and those with the *bba52*-complemented isolates or wild-type spirochetes were significant both at 24 and (*$P < 0.002$) 48 hours (*$P < 0.02$). (**B**) *B. burgdorferi* burdens in post-fed ticks. Nymphs were allowed to engorge on infected mice as described in figure 6*A* and *B. burgdorferi* burdens in post-fed ticks were analyzed at the indicated time intervals by measuring copies of the *B. burgdorferi flaB* RNA and normalize against tick *β-actin* RNA. Bars represent the mean ± SEM of eight qRT-PCR analyses derived from two independent infection experiments. Similar burdens of *bba52* mutants, wild type and *bba52*-complemented isolates were evident at day 7 or day 25 ($P > 0.05$). (**C**) *B. burgdorferi* localization in infected salivary glands during transmission. A representative image showing confocal orthogonal display of infected salivary glands in the XZ and YZ axis revealing the distribution of spirochetes through the full thickness of the 60-hour fed salivary glands is shown. The spirochetes (arrow) were labeled with FITC-labeled goat anti-*B. burgdorferi* antibody (shown in green) and gland morphology were revealed by labeling of acinar actin filaments using Texas Red-phalloidin (shown in red). While wild type and complemented *B. burgdorferi* (*bba52* Com) were occasionally observed within the gland, *bba52* mutant (*bba52*$^-$) was consistently undetected. (**D**) *B. burgdorferi* transmission from infected ticks to mice. *B. burgdorferi*-infected nymphs were generated by feeding larvae on mice infected with wild type and genetically-manipulated spirochetes, as described in the text. Newly-molted *B. burgdorferi*-infected nymphs were allowed to feed on naïve mice (1 tick/mouse, 3 animals/group). *B. burgdorferi* burdens were assessed in the indicated murine tissues after one week of tick feeding by measuring copies of the *B. burgdorferi flaB* RNA and

normalized against mouse *β-actin* levels. Bars represent the mean ± SEM of four qRT-PCR analyses derived from two independent animal infection experiments. * *bba52* mutants were undetectable.

Table 1

Oligonucleotide primers used in the current study

Sequence (5′ – 3′)	Purpose
GG*GAGCTC*AAAAGACAAAATCGCTTTGC	Primer P1, 5′ PCR of the left arm for constructing *bba52* mutant. A *SacI* site (italicized) is attached for the purpose of cloning.
AA*GGATCC*AATATTCTCCTAATATTTAGATGT	Primer P2, 3′ PCR of the left arm for constructing *bba52* mutant. A BamHI (italicized) is attached for the purpose of cloning.
GG*GTCGAC*TGATTTGCTTTGGAAGTTTT	Primer P3, 5′ PCR of the right arm for constructing *bba52* mutant. A Sal1 site (italicized) is attached for the purpose of cloning.
GC*GGTACC*TTAATCCTTTTGGCGAGTT	Primer P4, 3′PCR of the right arm for constructing *bba52* mutant. A KpnI site (italicized) is attached for the purpose of cloning.
GGTTGCATTCGATTCCTGTT	Primer P5, upstream 5′ PCR primer for the detection of intended integration of *pflaB-Kan* cassette in *bba52* locus
AAGTAAAATCACCTCATCTTCTGCTGTT	Primer P6, downstream 3′ PCR primer for the detection of intended integration of *pflaB-kan* cassette in *bba52* genomic locus
AGTGTTGCAAGACCATTTGATTTTA	Primer P7, *bba52* forward primer
TTAAATAAACTGATCTTCAAGAGAA	Primer P8, *bba52* reverse primer
ATGAATAAGCAAGAGATTGCGAC	Primer P9, kanamycin internal forward primer
ATTCCGACTCGTCCAACATC	Primer P10, kanamycin internal reverse primer
GTAAGCTCAGCCCGTGCA	Forward primer for RT-PCR of *bba51*
GCTGTAATAAACCCCCAGATTAA	Reverse primer for RT-PCR of *bba51*
TGACGAAGAGATTGCAGTCAA	Forward primer for RT-PCR of *bba53*
CTACCTTTGCTTTTTGGCTTT	Reverse primer for RT-PCR of *bba53*
GCTCAAATAAGAGGTTTGTC	Forward primer for RT-PCR of *flaB*
ATTCCAAGCTCTTCAGCTG	Reverse primer for RT-PCR of *flaB*
TTGCTGATCAAGCTCAATATAACCA	Forward primer for Quantitative RT-PCR of *flaB*
TTGAGACCCTGAAAGTGATGC	Reverse primer for Quantitative RT-PCR of *flaB*
AGAGGGAAATCGTGCGTGAC	Forward primer for Quantitative RT-PCR of mouse β-*actin*
CAATAGTGATGACCTGGCCGT	Reverse primer for Quantitative RT-PCR of mouse β -*actin*:
GGTATCGTGCTCGACTC	Forward primer for Quantitative RT-PCR of tick β –*actin*
ATCAGGTAGTCGGTCAGG	Reverse primer for Quantitative RT-PCR of tick β -*actin*:
CCAAAAGCCCACAAGGTGTA	Forward primer for Quantitative RT-PCR of *bba52*
TCTCTTTCCCCATCATCTGG	Reverse primer for Quantitative RT-PCR of *bba52*
CG*GAATTC*TTAAATAAACTGATCTTCAAGAG	Primer P11, 3′ PCR of the left arm for *bba52* complementation. A EcoRI site (italicized) is attached for cloning.

Sequence (5′ – 3′)	**Purpose**
CG*GAATTC*CGAGCTTCAAGGAAGA	Primer P12, Forward primer for amplification of *flgB-aadA* cassette in pKFSS1 vector. A EcoRI site (italicized) is attached for cloning.
CG*CGGATCC*ATTATTTGCCGACTACC	Primer P13, primer for amplification of *flgB-aadA* cassette in pKFSS1 vector. A BamHI site (italicized) is attached for cloning.
GA*GGATCC*AGTGTTGCAAGACCATTTGATTTTA	Forward primer for recombinant BBA52 production in *E. coli*. A BamHI site (italicized) is attached for cloning.
GG*CTCGAG*TTAAATAAACTGATCTTCAAGAGAA	Reverse primer for recombinant BBA52 production in *E. coli*. A XhoI site (italicized) is attached for cloning.