Borrelia burgdorferi *bba66* Gene Inactivation Results in Attenuated Mouse Infection by Tick Transmission

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The impact of the *Borrelia burgdorferi* surface-localized immunogenic lipoprotein BBA66 on vector and host infection was evaluated by inactivating the encoding gene, *bba66*, and characterizing the mutant phenotype throughout the natural mouse-tick-mouse cycle. The BBA66-deficient mutant isolate, Bb^{ΔA66}, remained infectious in mice by needle inoculation of cultured organisms, but differences in spirochete burden and pathology in the tibiotarsal joint were observed relative to the parental wild-type (WT) strain. *Ixodes scapularis* larvae successfully acquired Bb^{ΔA66} following feeding on infected mice, and the organisms persisted in these ticks through the molt to nymphs. A series of tick transmission experiments ($n = 7$) demonstrated that the ability of Bb^{ΔA66}-infected nymphs to infect laboratory mice was significantly impaired compared to that of mice fed upon by WT-infected ticks. *trans*-complementation of Bb^{ΔA66} with an intact copy of *bba66* restored the WT infectious phenotype in mice via tick transmission. These results suggest a role for BBA66 in facilitating *B. burgdorferi* dissemination and transmission from the tick vector to the mammalian host as part of the disease process for Lyme borreliosis.

Identifying gene products necessary for *Borrelia burgdorferi*, the tick-borne spirochete that causes Lyme borreliosis, to successfully navigate the complex environmental conditions encountered during the natural tick-to-mammalian host cycle has been of considerable recent interest. Work by several investigators has identified *B. burgdorferi* genes encoding outer membrane lipoproteins located on the 54-kb linear plasmid (lp54) that are functionally important in both the tick vector and mammalian hosts, e.g., *ospA*, *ospB*, *cspA*, *dbpAB*, and *bba07* (1–6). A series of lp54 genes, designated *bba64*, -65, -66, and -73, has been of considerable interest because of properties suggestive of roles in tick-host infectivity. For example, these genes are among the most highly upregulated when *B. burgdorferi* is subjected to mammal- and tick-like conditions *in vitro* (7–12). Additionally, experimentally infected mice and Lyme disease patients develop humoral responses to many of the proteins encoded by these genes, indicating active synthesis and antigen processing by the host's immune system during infection (13–16). Accordingly, longitudinal studies of persistently infected mice found expression of the genes in *B. burgdorferi* localized to various tissues (13, 14, 17).

Consequently, investigating the functional role of these gene products in establishing *B. burgdorferi* infection has yielded some significant findings. Recently, we identified the outer surface lipoprotein BBA64 as a critical component in facilitating *B. burgdorferi* infection in mice strictly through inoculation by tick bite transmission (18, 19). Another membrane-associated protein, BBA66, has shown characteristics parallel to those of BBA64, suggesting a similar role in *B. burgdorferi* pathogenesis in ticks and/or in mammals. For example, BBA66 production by cultured *B. burgdorferi* was shown to be influenced by changes in pH and temperature, such as those that occur when an unfed tick consumes a blood meal on a host (8, 10, 16). BBA66 is surface localized and elicits an antibody response during infection of mice and in Lyme disease patients (13, 15, 16, 20). Additionally, *bba66* is expressed during persistent mouse infection, suggestive of a maintenance function for *B. burgdorferi* in a reservoir host or as a needed component to colonize host tissues (14, 17). Studies by Anguita et al. suggested

that *bba66* may play a role in Lyme arthritis and carditis as assessed in the C3H/HeN mouse model (21), and Antonara et al. found evidence for BBA66 adhesion to murine heart tissue by phage display assay (22). Like other genes in this complex, notably *bba64*, *bba66* is controlled by the Rrp2-RpoN-RpoS global regulation pathway, which regulates a subset of genes both in the tick transition portion of the enzootic cycle and during mammalian infection (7, 13, 16, 23–26). *B. burgdorferi sensu lato* strains harbor BBA66 gene orthologs with a high level of conservation, and relapsing fever strains of *Borrelia* exhibit genes with lesser homology (13), but database searches do not reveal *bba66* homologs in other procaryotes or eucaryotes.

Based on these biological properties, we hypothesized that BBA66 may fulfill an important function related to pathogen persistence and/or dissemination in tick and mammalian hosts. To test this hypothesis, we inactivated the *bba66* gene and subjected the mutant isolate to the infectious stages of the natural tick-mouse enzootic cycle. We report that the mutant was attenuated in its ability to infect mice when delivered by tick bite, suggesting that BBA66 is a cofactor involved in borrelial tick maintenance pathways that mediate mammalian infection.

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MATERIALS AND METHODS

Bacterial strains, ticks, and mice. *B. burgdorferi* wild-type (WT) clonal infectious strain B31-A3 (Bb^{WT}) (27) was used as the parental strain for the generation of strain Bb^{ΔA66}. *B. burgdorferi* cultures were grown in Barbour-Stoenner-Kelly II (BSK-II) complete culture medium at 34°C in sealed tubes, with kanamycin and gentamicin used at 200 μg/ml and 50 μg/ml, respectively, when appropriate. *B. burgdorferi* isolates were maintained as low-passage (<2) frozen stocks in 30% glycerol at –80°C and maintained the full complement of plasmids, except for cp9. Male or female 6- to 8-week-old CD-1 mice were from a specific-pathogen-free colony maintained at the Division of Vector-Borne Diseases, Centers for Disease Control and Prevention (Fort Collins, CO) or purchased from Charles River Laboratories (Wilmington, MA). Female C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Infected *Ixodes scapularis* tick colonies were generated via xenodiagnosis by feeding uninfected *I. scapularis* larvae on CD-1 outbred mice infected via needle inoculation with 1×10^4 cells of Bb^{WT}, Bb^{ΔA66}, or Bb^{ΔA66comp} isolate as described previously (18).

Nymphal feeds were performed by anesthetizing mice by intraperitoneal injection with a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture. Bb^{WT}-, Bb^{ΔA66}-, or Bb^{ΔA66comp}-infected nymphs were placed dorsally on mice between the scapulae and allowed to feed to repletion (approximately 4 days). For the time course feeds, mice were anesthetized with isoflurane, and Bb^{WT}-infected nymphs were gently removed with fine-tip forceps at 33, 48, 57, and 72 h postinfection. Mice were assayed for infection 21 days following the nymphal feeding by serology (immunoblotting against whole-cell *B. burgdorferi* lysates) and culture of ear biopsy specimens in BSK-II supplemented with antibiotics and fungizone as described previously (28). Experimental protocols involving mice were approved by the Division of Vector-borne Diseases and by the University of Pittsburgh Animal Care and Use Committees.

RNA isolation from ticks and qRT-PCR for *bba66* expression. RNA was isolated from unfed, replete, and actively feeding (33, 48, 57, and 72 h) Bb^{WT}-infected nymphs. Four nymphs were collected and pooled from each time point. Nymphs were homogenized with a glass Tenbroek grinder in 500 μl RNeasy lysis buffer (Qiagen, Austin, TX) and stored at –80°C. Total RNA extraction and quantitative reverse transcription-PCR (qRT-PCR) were performed as described previously (19) with *bba66* TaqMan primers (14). Absolute expression levels of *bba66* and *flaB* were determined according to known standard curves with transcript copies of *bba66* measured as a ratio to copies of *flaB*. Data were calculated from 2 or 3 independent nymphal feedings for each time point (each technically replicated in triplicate).

Plasmid construction for insertional inactivation of *bba66*. A 3,262-bp region encompassing *bba66* and partial sequences of upstream and downstream genes (*bba65* and *bba68*) was amplified by PCR from *B. burgdorferi* isolate B31 genomic DNA using primers bba65RT.R (TCAAGCAAAGAGAAATCATAGTA) and bba68RT.F (CTAAAGCAATTGGTAAGGAAGT). The resulting product was cloned into pBAD-TOPO (Invitrogen, Carlsbad, CA). An internal region of *bba66* (757 bp) was deleted by digestion with BglII and NheI, and the 1,243-bp kanamycin resistance cassette (29) was ligated into this site to yield pBADΔ*bba66*::Kan. All plasmids were transformed into TOP10 *Escherichia coli* (Invitrogen), and clones were confirmed by PCR, restriction endonuclease digestion, and DNA sequencing. PCR primers used in this analysis were as follows: *bba66* FL.1 (TTGAAAATCAAACCATTAATAC), *bba66* FL.6 (T TACATTATACTAATGTATGCTTCAAG), 3' Kan (CGAAAACTCATC GAGCATCAA), and Gent.RT.R (CACTACGCGGCTGCTCAAACC).

Construction of plasmid for complementing the loss of *bba66* expression. The gene *bba66* and its native promoter were amplified by PCR using primers proA66.1 (CTTGTCGCAAAAATAGAG) and *bba66*FL.6 and cloned into pCR8/GW/TOPO (Invitrogen). The shuttle vector pBSV2G (30) was converted into a Gateway destination vector using cassette RfB following the manufacturer's protocol (Invitrogen). pCR8-

bba66comp and pBSV2G-GDV-B were recombined using the Gateway system LP recombination reaction producing pBSV2G-*bba66comp*.

Electroporation of *B. burgdorferi*. Plasmids were electroporated into either Bb^{WT} or Bb^{ΔA66} and plated into semisolid BSK media supplemented with antibiotics (31) with modification as described previously (32). Colonies appeared within 8 to 15 days and were cultivated in BSK-H Medium Complete, lot number 045K8412 or lot number 057K8413 (Sigma, St. Louis, MO) with antibiotics.

Mouse pathology studies. Female C3H/HeJ mice (6 to 8 weeks old) were inoculated subcutaneously with 10^4 Bb^{WT}, Bb^{ΔA66}, or Bb^{ΔA66comp} cells. Tibiotarsal measurements were taken biweekly along with weekly blood collections. To determine the change in relative joint diameter in mice at each time point post-needle inoculation, age-matched mock-infected mice injected with BSK medium alone were measured alongside challenged mice. The change in joint diameter was determined by subtracting the measurements of mock-infected mice from those obtained with infected mice. After either 21 days or 35 days postinfection, mice were sacrificed, and heart, joint, and ear samples were collected for DNA isolation and/or outgrowth analysis.

DNA isolation and qPCR analysis. DNA was isolated from heart, joint, and ear tissue as previously described (33). Tissues were incubated in a 0.1% collagenase A solution for 4 h at 37°C. An equal volume of 0.2 mg/ml of proteinase K was added and incubated at 55°C overnight, followed by a series of phenol-chloroform extractions and ethanol precipitations. qPCRs were performed in triplicate using iQ SYBR green Supermix (Bio-Rad) containing 0.5 μM concentrations of each *β-actin* primer (forward, AGAGGGAAATCGTGCGTGAC; reverse, CAATAGTGATGACCTGGCCGT) and 1.0 μM each *ospC* primer (forward, TACGGATTCTAATGCGGTTTAC; reverse, GTGATTATTTTCGGTATCCAAACCA). DNA was added to a final amount of 100 ng per reaction mixture. Reaction conditions were as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. Melting curves were generated by a cycle of 95°C for 1 min, 50°C for 1 min, and 80 cycles of 50°C for 10 s with 0.5°C increments. Standard curves for *ospC* and *β-actin* were prepared using 10-fold serial dilutions of known quantities of *B. burgdorferi* or mouse DNA, respectively. Results are presented as copies of *ospC* per 10^6 copies of *β-actin*.

qPCR of *B. burgdorferi* in individual nymphs infected with Bb^{WT} or Bb^{ΔA66} was performed as previously described (19) with TaqMan Universal PCR Master Mix and *flaB* TaqMan probe and primers (14).

PCR of *B. burgdorferi* plasmids. Plasmid contents for *B. burgdorferi* isolates were validated by performing multiplex PCR as described by Bunikis et al. (34).

IFAs, ELISAs, and antibodies. Indirect immunofluorescence assays (IFAs) were performed on feeding nymphs removed at 72 h postattachment dissected onto a silane-coated slide, air dried, and fixed in acetone for 10 min using chicken anti-BBA66 IgG (1:50) (Aves Laboratories, Tigard, OR) as described previously (19). Enzyme-linked immunosorbent assays (ELISAs) were performed as follows. Two hundred nanograms of recombinant BBA66 in 100 μl binding buffer (0.1 M NaHCO₃, pH 8.6) was applied onto 96-well plates at 4°C overnight. After two washes in distilled water (dH₂O), wells were blocked for 1 h at 25°C with blocking buffer (5% dry milk in phosphate-buffered saline plus 0.1% Tween 20 [PBS-T20]). Primary mouse sera, diluted by 2-fold serial dilution in blocking buffer, were incubated for 1 h at 25°C. After 3 washes with PBS-T20, 100 μl alkaline phosphatase-conjugated anti-mouse IgG (1:20,000) in blocking buffer was incubated for 1 h at 25°C. Wells were washed with PBS-T20 (3×), and 100 μl 1-Step PNPP reagent (Pierce, Rockford, IL) was incubated for 30 min at 25°C. Reactions were stopped with addition of 50 μl 2 N NaOH, and absorbance at 405 nm was measured. Primary antibodies for Western blot analysis included polyclonal mouse anti-*B. burgdorferi* sera collected from needle-inoculated mice and were used at a 1:1,000 dilution. Polyclonal antibody to OppAI (1:1,000) was provided by Linden Hu, Tufts Medical Center, Boston, MA.

FIG 1 *bba66* expression in feeding nymphal ticks by qRT-PCR. (A) RNA was isolated from pooled ticks from independent feedings (denoted by the open circles, filled circles, and triangles) at the designated times postattachment. Absolute *bba66* transcript copies were measured as a ratio of copies per *flaB*. Horizontal bars represent the means of the samples. (B) Agarose gel image of *bba66* amplicons following qRT-PCR. Lanes: 1, unfed nymphs; 2 to 5, nymphs at 33, 48, 57, and 72 to 80 h postinfection, respectively; 6, replete nymphs; 7, no template DNA control; 8, Bb^{WT} genomic DNA positive control.

Statistical analysis. Statistical analysis was performed using GraphPad software with one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test for assessment of changes in joint swelling and spirochete burden in tissues. When comparisons of 2 conditions were performed, a two-way *t* test was used. Statistical analysis of infected ticks per experimental mouse feeds was performed using one-way ANOVA for independent samples.

RESULTS

***B. burgdorferi bba66* is expressed in feeding nymphal-stage ticks.** Total RNA was purified from *B. burgdorferi* wild-type (Bb^{WT})-infected nymphal ticks detached from mice at times postattachment during feeding. Expression of *bba66* in unfed ticks, at 33, 48, 57, and 72 h, and at repletion was determined by qRT-PCR and measured relative to absolute copies of *flaB* expression. *bba66* expression was not detected in unfed nymphs but was detected beginning at 33 h postattachment, with continuous expression observed during the 3- to 4-day tick-feeding process. A spike in expression was seen in replete ticks collected approximately 4 to 20 h after dropoff (Fig. 1A). The *bba66* amplicons from the qRT-PCRs were also visualized by agarose gel electrophoresis (Fig. 1B).

After demonstrating *bba66* transcription during nymphal tick engorgement, we observed BBA66 production by immunofluo-

rescence in a population of the organisms in midguts taken approximately 72 h postattachment (Fig. 2). These results show that *bba66* is turned on and the gene product synthesized in the feeding tick.

Generation of *bba66* mutation in *B. burgdorferi* and complementation in trans. A *bba66* knockout mutant was generated by insertional mutagenesis to assess the putative role of BBA66 during the tick-mouse enzootic cycle. Following cloning of the WT *bba66* gene plus upstream and downstream flanking regions into the *E. coli* plasmid vector pBAD-TOPO, the *bba66* coding sequence was disrupted by insertion of a kanamycin resistance cassette under the control of the *B. burgdorferi* flagellin gene (*flgB*) promoter used as a selection marker (29). This construct, pBAD-*bba66*::Kan, was electroporated into *B. burgdorferi* B31-A3 and through homologous recombination resulted in the *bba66*-inactivated mutant isolate, Bb^{ΔA66} (Fig. 3A). The mutant was trans-complemented by electroporation of pBSV2G-*bba66*comp, which contained a WT copy of the *bba66* gene, resulting in isolate Bb^{ΔA66}comp (Fig. 3B). PCR analyses of the mutant and the complemented isolate showed proper insertion and orientation of the electroporated plasmids (Fig. 3C). Western blot analyses demonstrated the inactivation of BBA66 production in the mutant and restoration of BBA66 in the complemented isolate (Fig. 3D). Moreover, BBA66 production in Bb^{ΔA66}comp responded to alterations in pH in a manner similar to what has been observed in WT infectious isolates (Fig. 3D). Finally, the production of BBA64 protein was not compromised in Bb^{ΔA66} (data not shown).

Bb^{ΔA66} is infectious by needle administration of cultured organisms. C3H/HeJ mice were needle inoculated subcutaneously with 10⁴ *in vitro*-cultivated Bb^{WT}, Bb^{ΔA66}, Bb^{ΔA66}comp cells and euthanized after 21 or 35 days postinfection (dpi). All mice became infected, (except for one mouse challenged with the Bb^{ΔA66}comp isolate), as determined by culture of ear, heart, bladder, and joint tissues and by serological reactivity to *Borrelia* cell lysates (Table 1).

Phenotypic analysis of Bb^{ΔA66} in the C3H/HeJ mouse model of Lyme arthritis after needle inoculation. We measured the change in joint diameter of Bb^{WT}, Bb^{ΔA66}, or Bb^{ΔA66}comp needle-challenged C3H/HeJ mice during the course of infection as an indicator of joint inflammation common in the mouse model of Lyme arthritis. Measurements of both hind-limb joints were taken and compared to age-matched mock-infected mice as a baseline at 18, 21, 25, 28, 32, and 35 dpi (Fig. 4A). In mice inoculated with Bb^{WT}, joint diameter increased and peaked at 28 dpi (mean, 0.883 mm) and then began to decline. Likewise, mice infected with Bb^{ΔA66} demonstrated a similar peak in swelling at 28 dpi (mean, 0.561 mm), but the mean swelling was significantly reduced (*P* < 0.05) at 18, 21, 25, 28, and 32 dpi relative to mice infected with Bb^{WT}. Oddly, mice challenged with Bb^{ΔA66}comp also presented with a significant reduction in mean joint diameter relative to Bb^{WT}-infected mice at all dpi monitored.

To investigate the inability of Bb^{ΔA66}comp to restore the WT phenotype in joint manifestations, qPCR was performed on joint tissue samples at 21 and 35 dpi to determine the spirochete load from mice needle inoculated with Bb^{WT}, Bb^{ΔA66}, or Bb^{ΔA66}comp. Analysis of the spirochete load in the joints of mice at 21 dpi found that mice challenged with Bb^{WT} had a mean of 2,010 copies *ospC*/10⁶ copies of the mouse actin gene, yet mice infected with Bb^{ΔA66} or Bb^{ΔA66}comp had a significant reduction (*P* < 0.05) in the number of spirochetes, with means of 739 and 822 copies *ospC*/10⁶

FIG 2 IFA of tick midgut approximately 72 h postattachment. (A) Field stained with anti-*B. burgdorferi*; (B) same field as in panel A, stained with anti-BBA66; (C) merged image of panels A and B. Arrows point to BBA66-stained organisms.

copies of the mouse actin gene, respectively (Fig. 4B). This difference was not seen at 35 dpi and was likely due to the reduction in relative spirochete load as a function of time in the joints of mice infected with Bb^{WT}, while the Bb^{ΔA66} and Bb^{ΔA66comp} numbers remained constant from 21 to 35 dpi. There was no difference in spirochete burdens between strains detected in other tissues tested, i.e., ears and hearts (Fig. 4B). The reduced burden of Bb^{ΔA66comp} in joints may have been the reason for the observed decreased swelling by inflammation, and an experiment was performed to determine its cause.

The lack of restoration of WT joint swelling phenotype in Bb^{ΔA66comp}-infected mice was not due to loss of the WT gene and subsequent reversion to mutant status, as determined by PCR analysis that showed WT *bba66* was present in tissue reisolates of the infecting complemented organisms (data not shown). Therefore, we postulated that the multicopy nature of the complementing vector, pBSV2G (35), might have resulted in boosted production of BBA66 with a concomitant elevation in anti-BBA66 antibodies that could provide a mechanism for borrelial clearance. By Western blotting, we observed an increased amount of BBA66 produced *in vitro* in the Bb^{ΔA66comp} relative to WT (Fig. 3D), and qRT-PCR of RNA from cultured isolates indicated 4-fold more *bba66* transcript in Bb^{ΔA66comp} relative to Bb^{WT} (data not shown). To determine if more BBA66 production *in vivo* with Bb^{ΔA66comp} resulted in an enhanced antibody response, we determined the endpoint titers against recombinant BBA66 in sera from mice infected with Bb^{WT} and Bb^{ΔA66comp} at 21 dpi and 35 dpi (Fig. 4C). Although the spirochete loads in the joint were 2.4-fold lower at 21 dpi in mice infected with Bb^{ΔA66comp} than in mice infected with Bb^{WT}, we observed an 8-fold-higher anti-BBA66 specific mean titer in these mice relative to WT controls (1,600 relative to 200 reciprocal endpoint titer, $P = 0.0004$). This discrepancy in the

anti-BBA66 specific mean endpoint titer grew to 16-fold at 35 dpi (12,800 relative to 800 reciprocal endpoint titer, $P = 0.0003$) and suggested that BBA66 was produced at considerably higher levels in Bb^{ΔA66comp}-infected mice than in Bb^{WT} controls, leading to a more robust BBA66-specific antibody response. The higher-than-expected anti-BBA66 response could explain the clearing of the Bb^{ΔA66comp} isolate from joint tissue, effectively hindering the rescue of the phenotype seen with the loss of *bba66*.

Bb^{ΔA66} is acquired by larvae following feeding on infected mice and persists transstadially to the nymphal stage. Steps in the experimental design to assess phenotypic differences between the BBA66 mutant and WT *B. burgdorferi* during the tick-mouse infectious cycle are shown in Fig. 5. Larval acquisition of Bb^{ΔA66} was as efficient as Bb^{WT} acquisition based on several experiments and colony feeds during the course of this study. The acquisition rate by larvae for Bb^{WT}, Bb^{ΔA66}, and Bb^{ΔA66comp} were typically >80% (data not shown) as determined by culturing 20 to 30 individual replete larvae/cohort in BSK medium. Positive culture growth demonstrated that the mutant was viable following larval feeding, and PCR analysis showed that all plasmids were present in the WT and the Bb^{ΔA66} reisolates (data not shown). Bb^{ΔA66}-infected unfed nymphs were cultured following the molt and prior to feeding. In each cohort, the mutant-infected nymphs were culture positive at the same percentage as following the larval acquisition, indicating that Bb^{ΔA66} was able to survive transstadially (data not shown).

Quantitative PCR was performed on individual nymphs to determine the number of spirochetes present. The quantity of Bb^{ΔA66} was not significantly different from that of Bb^{WT} in the molted unfed nymphs (Fig. 6). Furthermore, PCR analysis revealed that no plasmids were lost in Bb^{ΔA66}-infected unfed nymph culture reisolates (data not shown). These results demonstrated

FIG 3 Insertional inactivation of *bba66* in infectious *B. burgdorferi*, complementation, and confirmation *in vitro*. (A) Schematic depicting the mutagenesis of *Bb*^{WT} *bba66* by insertion of the *pflg-kan* cassette on *lp54* by homologous recombination with *pBADbba66::kan*. N and B indicate *NheI* and *BglII* restriction endonuclease sites utilized for insertion of the resistance marker. (B) Plasmid construct *pBSV2G-bba66comp*, used to complement the loss of *bba66* in *Bb*^{ΔA66}. The intact *bba66* gene with its promoter (*bba66* + *prom*) provides *trans*-complementation under gentamicin selection (*Gent*^R). Open reading frame 1 (orf 1), orf 2, orf 3, and origin of replication (*ori*) of *pBSV2* are depicted. Numbers with arrowheads in panels A and B indicate primers (defined in panel B) and direction of amplification. (C) Agarose gel of PCR amplicons using the 3 primer sets indicated to verify kanamycin resistance marker insertion in *Bb*^{ΔA66}. Reactions a, b, and c on the right depict control PCR with plasmid templates to verify correct amplicon sizes. Markers are indicated to the left in kilobases (kb). (D) SDS-PAGE separation and silver staining of equal amounts of cell protein lysates from *Bb*^{WT}, *Bb*^{ΔA66}, and *Bb*^{ΔA66comp} grown at pH 7 or pH 8. BBA66 is not visible by silver staining, but *OspA* and *OspC* are indicated by arrows for reference. Below the stained gel is an immunoblot of these cell lysates with anti-BBA66 demonstrating the loss of BBA66 in *Bb*^{ΔA66} and the pH regulation of *bba66* in *Bb*^{WT} and *Bb*^{ΔA66comp}. The immunoblot with anti-*OppA1*, which is not pH regulated *in vitro*, served as a loading control.

TABLE 1 BBA66 is not required for infectivity by needle inoculation of C3H/HeJ mice

Time postinfection and strain used	No. infected/no. inoculated					No. positive by serology/no. inoculated
	Heart	Bladder	Joint	Ear	Total	
Day 21						
Bb ^{WT}	ND ^b	8/8	ND	ND	8/8	8/8
Bb ^{Δbba66}	ND	7/7	ND	ND	7/7	7/7
Bb ^{Δbba66 comp}	ND	8/8	ND	ND	8/8	8/8
Day 35						
Bb ^{WT}	8/8	7/8	5/8 ^a	4/8	8/8	8/8
Bb ^{Δbba66}	7/8	8/8	6/8	3/8	8/8	8/8
Bb ^{Δbba66 comp}	7/8	7/8	6/8	3/8	7/8	7/8

^a Three cultures were contaminated.
^b ND, not determined.

that *Bb*^{ΔA66} was phenotypically identical to *Bb*^{WT} in the ability to be acquired by feeding larvae and in persistence through the molt to nymphs.

***Bb*^{ΔA66} is attenuated for mouse infection by nymphal tick transmission.** Following the larval molt, nymphs infected with the *Bb*^{WT} and *Bb*^{ΔA66} strains were fed on naive mice. Three weeks after tick detachment, the mice were checked for their susceptibility to the tick challenge infection by ear biopsy specimen culture and serology. The cumulative results of 7 individual experiments showed a statistically significant reduction in mouse infection following feeding by *Bb*^{ΔA66}-infected ticks (Table 2). Collectively, the experiments revealed that 15/33 mice (45%) became infected after being fed upon by *Bb*^{ΔA66}-infected nymphs, compared to 17/20 mice (85%) that became infected after *Bb*^{WT}-infected nymphal infestation (*P* value, 0.0082). All naive mice fed upon by nymphs colonized with *Bb*^{ΔA66comp} (*n* = 5) became infected (Table 2, ex-

FIG 4 Phenotypic analyses of C3H/HeJ mice inoculated with Bb^{WT} , $Bb^{\Delta A66}$, and $Bb^{\Delta A66comp}$. (A) Change in hind-limb joint diameter in millimeters (mm) was monitored 18, 21, 25, 28, 32, and 35 days postinfection. Reported changes are relative to age-matched, mock-challenged mice. One-way ANOVA with Dunnett's posttest was performed: *, $P = 0.01$ to 0.05 ; **, $P = 0.001$ to 0.01 ; ***, $P = <0.001$ relative to Bb^{WT} samples. (B) Joint tissues from mice inoculated with Bb^{WT} , $Bb^{\Delta A66}$, or $Bb^{\Delta A66comp}$ were analyzed by qPCR to determine the relative differences in spirochete burden during infection. Borreliae were quantified by copies of *ospC*/ 10^6 copies of the mouse β -actin gene. *, $P = 0.01$ to 0.05 . (C) ELISA results demonstrating immunoreactivity of infected mice to recombinant BBA66. Endpoint titers are presented as the reciprocal in $\log_2$. A two-way t test was performed to compare Bb^{WT} and $Bb^{\Delta A66comp}$ at either 21 dpi or 35 dpi; ***, $P = <0.001$. Data points in panels B and C denote individual mice, with the mean denoted by a horizontal line and vertical lines with bars indicating the standard error of the mean.

FIG 5 Flow chart of the experimental design to assess borrelial phenotypes through the mouse-tick-mouse natural cycle of infection.

periment number 5), with reisolates from mouse tissues harboring the WT *bba66* (as detected by PCR), demonstrating restoration of mouse infectivity by tick transmission to WT levels. To validate that a reduction in transmission was the result of the *bba66* mutation, the following parameters were examined in each of the 7 experiments: (i) fed mutant-infected nymphs were cultured from mice that remained uninfected to ascertain the presence of viable borreliae; (ii) the culture reisolates from these fed nymphs were assessed for their plasmid content to ensure that the isolates did not lose plasmids essential for mammalian infectivity (i.e., lp25, lp28-1, lp36, and cp26); and (iii) reisolates were assessed by PCR for the kanamycin resistance cassette insertion within *bba66*. In each instance, mice that remained uninfected following a Bb^{ΔA66}-infected nymphal feed demonstrated fed ticks harboring viable mutant borreliae containing all the same plasmids as the parental strain. These results demonstrated the genetic stability of the mutant throughout each stage of the culture-mouse-tick-mouse infectious cycle.

Reisolated Bb^{ΔA66} cultures from ticks that fed on mice refractory to infection were subsequently needle inoculated into individual naive mice ($n = 3$). All mice became infected, thus demonstrating that Bb^{ΔA66} retained its original phenotype of mouse infectivity by needle administration despite having traveled through the *in vivo* experimental cycle for many months. Together, these findings demonstrate that inactivation of *bba66* contributes to an attenuation of mouse infectivity when the organisms are administered by tick bite.

However, individual experiments showed variations in the percentage of mice infected by the mutant (Table 2). We assessed assorted experimental parameters that might have accounted for the observed variation including (i) mouse gender, (ii) mouse strain, (iii) age of nymphs, and (iv) number of ticks that fed to repletion. The one parameter that had a measurable effect on the experimental outcome was the number of Bb^{ΔA66}-infected nymphs that fed to repletion. Statistical evaluation of the number of replete Bb^{ΔA66}-infected nymphs collected from each mouse

FIG 6 qPCR enumerating Bb^{WT} and Bb^{ΔA66} isolates in individual ticks at times during mouse feeding. Bars indicate copies of *flaB* in unfed (flat) nymphs, in nymphs collected at 48 and 72 h postattachment, and in replete nymphs collected between 4 and 20 h after dropoff.

showed a significant difference between the number of nymphs that fed to completion and whether or not the mouse became infected (Fig. 7). The average number of infected nymphs that fed per infected mouse was 5.6. The average number of infected nymphs that fed per uninfected mouse was 3.6; a difference of 2 nymphs/mouse (*P* value = 0.017). The data indicated that mice fed upon by Bb^{ΔA66}-infected nymphs had a better chance to become infected if 4 or more ticks completed the feeding process.

FIG 7 Number of Bb^{ΔA66}-infected ticks feeding to repletion as a correlation to mouse infectivity. Solid circles represent individual mice that became infected following feeding by Bb^{ΔA66}-infected ticks; solid squares represent individual mice that did not become infected following feeding by Bb^{ΔA66}-infected ticks. The mean value for each sample set is shown by the horizontal lines with the standard error of the mean denoted by the vertical lines. *P* value (0.017) determined by one-way ANOVA.

Bb^{ΔA66} replicates normally in ticks. We evaluated whether the attenuation of mammalian infectivity was due to a defect in the mutant's ability to replicate normally within the tick during feeding. Borreliae were enumerated by qPCR in Bb^{WT}- and Bb^{ΔA66}-infected nymphs taken throughout the 4-day engorgement

TABLE 2 Results of mouse challenge by tick transmission

Experiment no.	<i>B. burgdorferi</i> strain infecting tick	No. of mice infected/no. of mice challenged	No. of nymphs placed/mouse	No. of replete infected nymphs ^a collected per:	
				Infected mouse	Uninfected mouse
1	Bb ^{WT}	1/1	5	5	
	Bb ^{ΔA66}	0/4	5		1,1,3,3
2	Bb ^{WT}	3/4	5,15 ^b ,15 ^b ,15 ^b	2,2,7	4
	Bb ^{ΔA66}	0/4	5,5,15 ^b ,15 ^b		3,3,3,1
3	Bb ^{WT}	1/1	8	8	
	Bb ^{ΔA66}	0/3	10		8,5,6
4	Bb ^{WT}	4/5	15 ^b	4,3,6,5	6
	Bb ^{ΔA66}	2/5	15 ^b	1,6	2,3,5
5	Bb ^{WT}	3/3	10	8,10,9	
	Bb ^{ΔA66}	5/7	10	10,10,6,8,8	6,7
	Bb ^{ΔA66comp}	5/5	10	6,6,6,3,7	
6	Bb ^{WT}	4/5	10	8,5,5,3	1
	Bb ^{ΔA66}	4/5	10	4,8,5,3	3
7	Bb ^{WT}	1/1	10	4	
	Bb ^{ΔA66}	4/5	10	3,5,4,4	1
Total ^c	Bb ^{WT}	17/20			
	Bb ^{ΔA66}	15/33			

^a Data also presented in Fig. 7.
^b Extra nymphs were placed on these mice for removal during the feed for time course PCR analyses.
^c *P* value = 0.0082 for Bb^{WT} versus Bb^{ΔA66}.

period. The data demonstrated no significant differences in spirochete numbers between the two isolates measured through the feeding process (all P values were <0.05) (Fig. 6). This result indicates that Bb^{ΔA66} replicated normally and that attenuation of mouse infectivity was not due to a reduced density of organisms necessary for infection within the tick. Additionally, Bb^{ΔA66} growth curves were no different from those of Bb^{WT} when cultivated in BSK media (data not shown).

Quantification of Bb^{ΔA66} in infected mouse tissues following tick challenge. The spirochete burden was measured by qPCR from tissues of necropsied mice that became infected by Bb^{ΔA66} and compared to Bb^{WT}-infected tissues. Ears, spleens, heart, and bladder tissues were assessed 6 to 8 weeks following tick feeding. Although the mean Bb^{ΔA66} density in tissues was lower in ear, bladder, and spleen tissues than the density in Bb^{WT}-infected tissues, there was not a statistically significant difference (data not shown). Therefore, BBA66-deficient organisms did not appreciably affect spirochete dissemination or replication in these tissues.

DISCUSSION

The mechanisms by which *B. burgdorferi* navigates its way from reservoir hosts to vector ticks and back again, incidentally infecting humans in this cycle, have been a subject of keen interest. The transmission of *B. burgdorferi* from vector ticks to humans signifies the first stage in Lyme disease causation. Defining the borrelial gene products instrumental in this process identifies potential targets for disruption of the cycle necessary for maintenance of the Lyme borreliosis agent in nature. In this study, we showed that *bba66* was expressed in the nymphal tick stage during consumption of the host blood meal and, correspondingly, that the BBA66 protein was produced by spirochetes in the midgut. The upregulation of *bba66* at this critical juncture of the enzootic transmission cycle suggested that BBA66 functions to prepare *B. burgdorferi* for transmission from vector to host and/or other host-pathogen interactions.

Following construction of a BBA66-deficient isolate, we began by assessing the ability of the mutant to infect mice by needle inoculation of cultured organisms. Bb^{ΔA66} proved infectious via this route of introduction, as has been demonstrated by other investigators using a triple deletion mutant lacking *bba64*, *bba65*, and *bba66* (36). The one pathological phenotypic change in C3H/HeJ mice infected with Bb^{ΔA66} relative to mice infected with Bb^{WT} was a reduction in joint swelling at 21 dpi, a sign of reduced inflammation, suggesting that BBA66 is involved in joint inflammation and colonization in the mouse model of arthritis. Unfortunately, we were unable to restore the WT joint phenotype by complementation of the loss of *bba66*. We attributed this to the abnormally high BBA66 antibody titers that were generated in mice infected with Bb^{ΔA66comp}. This result suggested that the reduced joint inflammation in Bb^{ΔA66comp}-infected mice was a function of decreased spirochete numbers in the joint due to clearance by elevated anti-BBA66 antibodies, but apparently the elevated anti-BBA66 had little effect on spirochete numbers in other tissues assayed (Fig. 4B). One interpretation is that BBA66 may be more highly produced or more accessible on the cell surface in joint tissue or that organisms in joints may be more exposed to antibody attack relative to other tissues, thereby rendering spirochetes in the joint more susceptible to clearance. The evidence that BBA66 is involved in joint tissue colonization and that high levels

of anti-BBA66 antibodies may help eliminate *B. burgdorferi* is a concept that warrants further investigation.

We subjected the BBA66-deficient mutant to the conditions of the natural mammalian host-vector cycle. Inactivation of *bba66* did not prove detrimental for *B. burgdorferi*'s ability to (i) be acquired by clean larvae after feeding on infected mice and (ii) persist transstadially from larva to nymph. However, the *bba66* mutant was impaired in its ability for infecting mice when introduced by nymphal tick bite.

We observed variability in the tick transmission results, as the first 4 experiments demonstrated a pronounced phenotypic effect by the mutant whereas the subsequent 3 experiments showed no differences from WT controls. We addressed this concern by analyzing several experimental variables that could possibly affect the outcomes of tick feeding challenges. We determined that the strain and gender of host mice and age of feeding nymphs were nonfactors. The one parameter that appeared to affect transmission was the number of ticks allowed to infest mice for the challenge. Although a single WT-infected tick can infect a host, our lab routinely uses 5 to 10 ticks per mouse because it is uncommon to recover all ticks after the 4-day feed due to animal grooming or failure of individual ticks to attach. Moreover, not every nymph in a cohort is infected, as colony infectivity rates are not 100%. Therefore, we use multiple ticks in a feed to ensure recovery and documentation of at least one infected tick per animal to validate the final infection status of the mouse. In two experiments, we infested some mice with 15 nymphs for reasons of detaching the extra ticks prior to repletion for use in PCR analyses, leaving 8 to 10 ticks to remain feeding. The interruption of tick feeding by removal of a few from the animal did not have a discernible effect on the eventual host infectivity status. Although it cannot be proven with certainty, it does not seem likely that removal of some ticks would affect the feeding and borrelial transmission from the other ticks that fed to repletion.

We found a statistically significant difference between the numbers of Bb^{ΔA66}-infected nymphs that completed the feed per infected mouse versus uninfected mouse. These data suggest that there was a greater probability of mice becoming infected with Bb^{ΔA66} when more ticks fed to completion and may explain the results of the latter trials whereby mutant-infected mice usually had 4 or more ticks collected at the end of feeding. Bestor et al. observed a similar phenomenon in experiments whereby an isolate lacking the genes *bba01-07* was infectious by tick bite when 20 ticks per mouse were used, but not when the infestation was reduced to 3 ticks per mouse (37, 38).

By this data analysis, we propose a model whereby BBA66 functions via a (currently undefined) mechanism to prepare *B. burgdorferi* for transport from tick to host during the nymph feeding period. Inactivation of *bba66* retards the normal dissemination of organisms in the tick whereby a suboptimal number of cells are eventually transmitted to the host, i.e., at a level below the 50% infectious dose (ID₅₀). The few BBA66-deficient borreliae that are distributed from the tick and deposited into the host skin are subsequently destroyed by the host's innate immune response, resulting in clearance of infection as measured 2 to 3 weeks following tick challenge. However, when several mutant-infected ticks are allowed to complete the feeding process, the result may be a collective inoculum of Bb^{ΔA66} that exceeds the ID₅₀, subsequently causing a breakthrough host infection. Because it has been clearly established that a single infected tick feeding on a host can

cause Lyme borreliosis, it may be prudent to conduct future experiments allowing a feeding of 1 to 3 Bb^{ΔA66} (and other mutant)-infected ticks/mouse when studying the mechanisms involved in pathogen transmission.

Collectively, our findings support two general hypotheses: (i) BBA66 plays a role in joint pathology; and (ii) BBA66 functions within the tick for borrelial dissemination, affecting transmission to and infectivity of the host. Moreover, the results reinforce an important point made in several studies, that tick-borne *B. burgdorferi* possesses biological properties distinct from those of *in vitro*-grown organisms, as evidenced by the infectious phenotype of Bb^{ΔA66} when needle inoculated, in contrast to what is seen with inoculation by tick bite.

Recent research investigating borrelia-vector interactions have focused on inactivation of global regulators, individual genes, and replicons to advance our understanding of *B. burgdorferi* tick colonization and persistence and vector transmission mechanisms (1, 4, 7, 18, 37–54). The loss of BBA66 activity did not completely abolish the ability of *B. burgdorferi* to invade the host by tick bite (at least when more than 4 ticks/mouse fed to repletion), but mouse infectivity was significantly impaired. This phenotype was observed solely by tick bite inoculation and not by needle inoculation of culture-grown organisms; therefore, we conclude that BBA66 functions in the tick vector to facilitate host infection in the natural cycle of Lyme borreliosis. Undoubtedly, an intricate mechanism exists that incorporates elements of the spirochete, vector, and mammal for successful passage from one host to another to complete the infectious cycle. BBA66 is another component of this pathway and adds to our current knowledge of the biological processes involved in vector-borne diseases.

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