Published in final edited form as:
J Infect Dis. 2009 October 15; 200(8): 1318–1330. doi:10.1086/605846.

BB0323 function is essential for *Borrelia burgdorferi* virulence and persistence through tick-rodent transmission cycle

Xinyue Zhang, **Xiuli Yang**, **Manish Kumar**, and **Utpal Pal***
Department of Veterinary Medicine, University of Maryland, College Park, MD 20742 and Virginia-Maryland Regional College of Veterinary Medicine, College Park, MD 20742

Abstract

Borrelia burgdorferi bb0323 encodes an immunogenic protein in mammalian hosts including humans. An analysis of *bb0323* expression *in vivo* showed variable transcription throughout the spirochete infection cycle, with elevated expression during tick-mouse transmission. Deletion of *bb0323* in infectious *B. burgdorferi* did not affect microbial survival *in vitro*, despite significant alterations in growth kinetics and cell morphology. *bb0323* mutants were unable to infect either mice or ticks, and were quickly eliminated from immunocompetent and immunodeficient hosts and the vector, within the first few days of inoculation. Chromosomal complementation of the mutant with native *bb0323* and phenotypic analysis *in vivo* indicated the significant restoration of spirochete virulence and persistence throughout the mouse-tick infection cycle. BB0323 may serve an indispensable physiological function that is more pronounced during microbial persistence and transitions between the host and the vector *in vivo*. Strategies to interfere with BB0323 function may interrupt the infectious cycle of spirochetes.

Keywords

Lyme disease; *Borrelia burgdorferi*; microbial pathogenesis

INTRODUCTION

Lyme borreliosis remains an increasing arthropod-borne health threat in humans and animals in many parts of the world [1]. The disease is caused by the tick-borne spirochete *Borrelia burgdorferi*, which survives in a wide range of vertebrate hosts and, remarkably, in many organ locations within a given host. Clinical complications of Lyme borreliosis result from pathogen-induced host inflammatory responses. While the natural hosts for *B. burgdorferi* are asymptomatic, a selective set of incidental hosts, including humans, may develop multi-system diseases including skin rashes, arthritis, carditis, and neuropathy [2,3]. Antibiotic therapy for Lyme disease is usually curative; however, patients with persistent infections often require intense antibiotic treatment and, in some cases, infection continues for months or years after antibiotic therapy. Moreover, a vaccine to prevent the incidence of human Lyme disease is not currently available.

*Corresponding author: Utpal Pal, Department of Veterinary Medicine, Building 795, Room 1341, University of Maryland, College Park, MD 20742. Phone: 301-314-2118, Fax: 301-314-6855, upal@umd.edu.
Correspondence and requests for reprints should be addressed to: Utpal Pal, Department of Veterinary Medicine, Building 795, Room 1341, University of Maryland, College Park, MD 20742. Phone: 301-314-2118, Fax: 301-314-6855, upal@umd.edu

The authors declare that they have no competing financial interests.

The *B. burgdorferi* genome contains approximately 1780 genes, many of which encode hypothetical proteins of undefined functions [4,5] and are differentially produced in the arthropod-mammal infection cycle of the spirochete [6]. The spirochete is known to alter gene expression patterns when exposed to altered growth conditions [7–11] or when encountering a new host environment [12]. Thus, investigators have used gene expression patterns as a surrogate to understand the role of spirochete genes in host infectivity and transmission. Genes that are selectively expressed in specific phases of the *B. burgdorferi* life cycle may encode proteins of functional significance, as shown by recently-developed genetic studies [13] and identified a handful of *B. burgdorferi* genes that support *B. burgdorferi* persistence in a natural infection cycle, such as *pncA* [14], *adeC* [15], *bmpA/B* [16], *ospA/B* [17], *ospC* [18,19] *dbpA/B* [20,21], *bbk32* [22,23], *bptA* [24], *bb0365* [25] and *bb0690* [26]. *B. burgdorferi* genes have also been identified that are differentially expressed *in vivo*, such as *ospD, luxS*, *BbCRASP-2*, and *chbC*, but lack an essential role in the spirochete natural infection cycle [27–31]. Therefore, the development of preventive measures against Lyme disease will be contributed by the continued efforts to identify immunogenic antigens that are essential for microbial virulence and infectivity.

bb0323 encodes a hypothetical lipoprotein [4,5] that has been identified as a membrane-associated immunogenic protein in mammalian hosts including humans [32,33]. An earlier study on genome-wide transposon mutagenesis of non-infectious *B. burgdorferi* yielded a *bb0323* disrupted clone with obvious defects in its outer membrane integrity [34]. Based on the studies in immunogenicity and outer membrane localization of BB0323, we assessed the role of BB0323 in the life cycle of spirochetes and have shown that the antigen is essential for *B. burgdorferi* virulence, persistence and transmission through the enzootic infection cycle of the pathogen.

MATERIALS AND METHODS

Borrelia burgdorferi*, ticks and mice*

B. burgdorferi infectious isolate, B31-A3 [35], was used throughout this study. Four- to six-week-old C3H/HeN mice and NCr-SCID mice were purchased from the National Institutes of Health. *Ixodes scapularis* ticks used in this study originated from a colony that is maintained in the laboratory [27]. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Bio-safety Committee.

PCR

The primers used in specific PCR reactions are indicated in supplementary Table 1. Isolation of RNA and RT-PCR or quantitative RT-PCR (qRT-PCR) analysis was performed as described [27]. *bb0323* transcripts were analyzed by qRT-PCR in groups of 5 C3H mice (10^5 spirochetes/mouse) at 7, 14, 21 or 28 days of infection or in larval and nymphal ticks that fed on 14-day infected mice (20 ticks/mouse) as described [17, 19, 36, 37]. Ticks (whole larva and dissected nymphal gut) were analyzed at days 1, 2 or 3 of feeding or 25 days after feeding. For assessment of *bb0323* expression during transmission, infected nymphs were fed on naïve mice (20 ticks/mice, 3 mice/group), and dissected tissues were analyzed at days 1, 2 or 3 of feeding as described [17, 19, 36, 37]. The levels of *bb0323* transcript were normalized against *flaB* transcripts.

Protein expression, preparation of BB0323 antiserum and immunoblotting

The *bb0323* gene was cloned into pGEX-6P-1 (Amersham-Pharmacia Biotech) using specific primers (supplementary Table 1) and the recombinant protein without the N-terminal leader sequence was produced in *E. coli*. Expression, purification and enzymatic cleavage of the glutathione S-transferase (GST) fusion protein were performed as described previously [16].

Generation of polyclonal murine antiserum against recombinant BB0323 (without the GST tag) and assessment of titer and specificity of the antisera, using ELISA and immunoblotting, were performed as described [27].

Preparation of outer membrane vesicles and Proteinase K accessibility assay

Preparation of outer membrane (OM) vesicles, immuno-detection of membrane proteins and proteinase K accessibility assay were performed as described [27,38] and further detailed in the supplementary text.

Generation of *bb0323* mutant and complemented isolates of *B. burgdorferi*

Genetic manipulation of *B. burgdorferi* was performed according to published procedures [27] using the primers listed in supplementary Table 1, and further detailed in the supplementary text. One of the *bb0323* knockout isolates retaining the same set of plasmids as the wild type isolate [16,27] was selected for further experiments. Wild type and *bb0323* mutants were also processed for transmission electron microscopy as described earlier [36]. Genetic complementation of the *bb0323* mutant was achieved by re-insertion of a wild type copy of the *bb0323* gene in the *B. burgdorferi* chromosome, as described [26].

Phenotypic analysis of *bb0323* mutant and complemented isolates

Spirochete burdens in mice and ticks were assessed using qRT-PCR analysis of *flaB* gene and normalized against murine or tick *β-actin* genes as described [27]. C3H mice were infected with *B. burgdorferi* (10^5 spirochetes/mouse), and sacrificed at days 7, 14, 21 and 28 following inoculation. Groups of SCID mice (3 mice/group) were also similarly infected with spirochetes and analyzed at 14 days after infection. The skin, heart and joint were stored in liquid nitrogen, and aliquots of blood and spleen tissues were cultured in BSK medium to test for the presence of viable spirochetes. Pathogen burdens were assessed in ticks (20 ticks/mouse) that fed on infected mice following two weeks of infection as described [16,19,25]. Naturally-infected or microinjected nymphs were also generated, and spirochete burdens in dissected gut were determined by confocal microscopy as described [17,19]. Infected ticks were fed on naïve mice (5 ticks/mice, 6 mice/group) and *B. burgdorferi* burdens in whole ticks or dissected gut and salivary glands were determined by qRT-PCR at different time points. To assess the capability of spirochetes to transmit from ticks to mice, infected nymphs were allowed to feed to repletion (3 ticks/mouse, 5 mice/group). The engorged ticks were subjected to qRT-PCR analysis to assess the infection. At day 14 following tick feeding, all the mice were sacrificed, and the tissues were isolated and assessed for the spirochete burden by qRT-PCR.

Evaluation of arthritis

B. burgdorferi-infected mice were examined for swelling of the tibiotarsal joints as detailed earlier [16] and further detailed in the supplementary text. Joint sections were assessed for histological parameters of *B. burgdorferi*-induced inflammation, as described [39,40].

Bioinformatics and Statistical analysis

Bioinformatic analysis and gene/protein annotations are detailed in the supplementary text. 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

Expression of *bb0323* throughout the mouse-tick infection cycle of *B. burgdorferi*

To understand the role of BB0323, we first assessed the temporal and spatial expression of *bb0323* in an animal model of *B. burgdorferi* enzootic cycle. C3H mice were infected with *B.*

burgdorferi and skin, joint, heart and bladder samples were collected at days 7, 14, 21 or 28 following infection. Larval and nymphal ticks were fed on parallel groups of 14-day-infected mice (25 ticks/mouse) and engorged ticks were isolated following 1, 2 or 3 days of feeding. One group of fed intermolt larva was collected at day 25 after feeding, while another group was allowed to molt to nymphs. The unfed infected nymphs were allowed to feed on naïve C3H mice (20 ticks/mice), and their gut and salivary glands were isolated following 1, 2 or 3 days of feeding. Quantitative RT-PCR analysis indicated a lower *bb0323* expression in mice, but the gene is highly expressed in infected ticks (figure 1). The highest level of *bb0323* expression was noted during transmission of spirochetes from ticks to the murine host. In agreement with an earlier report [32], infected mice developed antibody response against BB0323 (data not shown).

Generation of *bb0323* mutant *B. burgdorferi*

The gene product of *B. burgdorferi bb0323* is annotated as LysM (Lysin Motif) domain protein. The location of LysM-like domain in BB0323 and its amino acid sequence similarities ($E = 1.8e^{-04}$) with a designated LysM domain (Pfam database accession number PF01476) or similar domains in related spirochetes or other bacteria with closest homologies are shown (figure 2*A*). BB0323 is associated with the spirochete outer membrane (figure 2*B*), however, proteinase K-mediated digestion of *B. burgdorferi* surface proteins (figure 2*C*) and densitometric analysis of the immunoblot (data not shown) indicated that, unlike FlaB, the antigen has a minor (18±1.3% of cellular BB0323 levels) sensitivity to proteinase K treatment. We next created a BB0323-deficient *B. burgdorferi* for direct assessment of its role in the pathogen life cycle. An infectious *B. burgdorferi* isolate was used to create an isogenic mutant by the exchange of the *bb0323* open reading frame with a kanamycin resistance cassette via homologous recombination (figure 2*D*). Out of 12 transformed clones that grew in antibiotic-containing media, PCR analysis further selected one clone with the desired chromosomal integration of the antibiotic cassette (figure 2*E*) and with the same endogenous plasmid profile as the parental isolate (supplementary figure S1*A*). RT-PCR analysis showed that the mutant failed to produce *bb0323* mRNA, and that the mutagenesis did not impose polar effects on the transcription of surrounding genes *bb0322* and *bb0324* (figure 2*F*). The protein profile of the *bb0323* mutant was similar to that of the wild type spirochete (figure 2*G*, left panel), and the mutant failed to produce BB0323 protein (figure 2*G*, upper right panel). Compared to parental isolates, the *bb0323* mutant displayed a slower growth rate *in vitro* and formed unusually large clumps, especially at cell density greater than 10^7 cells/ml (supplementary figure S1*B* and S1*C*). In agreement with an earlier study [34], transmission electron microscopic analysis further confirmed significant morphological deformities of BB0323-deficient spirochetes.

BB0323-deficient *B. burgdorferi* is not infectious to murine host and ticks

To determine whether the lack of BB0323 influences *B. burgdorferi* infectivity *in vivo*, groups of 5 C3H/HeN mice were inoculated intradermally with equal numbers of wild type or *bb0323* mutant *B. burgdorferi* (10^5 spirochetes/mouse). Infection was assessed by quantitative RT-PCR analysis of pathogen burden in skin and blood samples at 3, 5, 7 and 10 days of infection, and by culture of tissue biopsies. The qRT-PCR results indicated that, although wild type spirochetes persisted in mice, *bb0323* mutants were undetectable during murine infection. Similarly, wild type spirochetes were isolated by culture of infected spleen and blood; whereas, attempts to isolate viable *bb0323* mutants remained unsuccessful (data not shown). Mice infected with wild type *B. burgdorferi* developed ankle swelling and histological signs of arthritis, which were absent in mice infected with *bb0323* mutant *B. burgdorferi* (data not shown). When *Ixodes* ticks were allowed to engorge on the mice infected with wild type or *bb0323*-deficient *B. burgdorferi* and fed ticks were subjected to qRT-PCR analyses, mutants remained undetectable in ticks. Similar to immunocompetent mice, *bb0323* mutants remained non-infectious in severe combined immunodeficient (SCID) mice. Groups of SCID mice (3

animals/group) were infected with 10^5 *B. burgdorferi* and infectivity was assessed at 14 days after infection. qRT-PCR analysis of pathogen burdens in the heart, skin, bladder and joint samples, and culture of blood and spleen biopsies, consistently detected the wild type spirochetes; whereas, *bb0323* mutants remained undetectable (data not shown). Collectively, these observations suggest that BB0323 is required for survival of *B. burgdorferi* in the murine host and for acquisition of spirochetes by ticks.

Complementation restores the phenotypic defects including virulence and persistence of BB0323-deficient B. burgdorferi throughout the mouse-tick infection cycle

To ascertain that the observed phenotypic defects of *bb0323* mutant *B. burgdorferi* were due to the loss of *bb0323* gene rather than the result of anomalous effects of genetic manipulation, we complemented the *bb0323* mutant spirochetes with a wild type copy of the *bb0323* gene in the chromosome and used this isolate in murine infection studies. To accomplish this, the open reading frame of *bb0323*, with its upstream promoter, was fused to the streptomycin resistance cassette *aadA* and inserted into pXLF14301, which carries up- and downstream recombination arms for insertion of a DNA construct into *B. burgdorferi* chromosome via allelic exchange (figure 3*A*). The mutant was transformed with the recombinant plasmid pXLF14301-pbb0323, and one of the transformants that produced both *bb0323* transcript (figure 3B) and BB0323 protein (figure 3*C*) was isolated. Quantitative RT-PCR (data not shown) showed that the complemented isolates, however, produced lower (30 ±10%) levels of *bb0323* transcripts during growth *in vitro*, compared to parental isolates. The *bb0323*-complemented isolate contained a similar set of plasmids as *bb0323* mutant or wild type isolates (supplementary figure S1*A*). Unlike *bb0323* mutant, the complemented isolate displayed growth patterns similar to the parental isolate and did not form larger clumps (supplementary figure S1*C*). Transmission electron microscopic studies further confirmed that while the *bb0323* mutant culture is dominated by spirochetes with obvious defects in the organization of outer membrane, presence of numerous vesicular structures or membrane blebs, both wild type and bb0323-complemented spirochetes have normal outer membrane organization without significant presence of membrane blebs (figure 3*D*).

We then examined the virulence and persistence of the *bb0323*-complemented spirochetes throughout the mouse-tick infection cycle. Groups of 5 C3H/HeN mice were intradermally challenged with the wild type spirochete, *bb0323* mutant, or *bb0323*-complemented *B. burgdorferi* (10^5 cells/mouse) and spirochete burden in skin, heart and joint were evaluated at days 7, 14, 21 or 28 of infection. The results showed that while *bb0323* mutants remained undetectable, both *bb0323*-complemented isolates and wild type spirochetes persisted in all murine tissues throughout the infection (figure 3*E*). When larval and nymphal ticks were allowed to engorge on mice following 14 days of *B. burgdorferi* infection, only wild type and *bb0323*-complemented *B. burgdorferi* were able to migrate to larvae (data not shown) and nymphs (figure 3*F*). Fed infected larvae were allowed to molt into nymphs in the laboratory and when fed on naïve mice, both wild type and *bb0323*-complemented *B. burgdorferi* transmitted to the mice (figure 3*G*). As expected, both wild type and *bb0323*-complemented isolates induced significantly higher levels of disease in mice than the *bb0323* mutant, as reflected by the development of swelling in the tibiotarsal joints and the histopathological signs of arthritis (figure 4). These experiments conclusively demonstrate that BB0323 is essential for microbial persistence and virulence in mice.

As the *bb0323* mutant was unable to infect mice, and thus, cannot be naturally acquired by ticks, the role of this gene product for spirochete life cycle in the vector remained inconclusive. Although *bb0323*-complemented isolate was acquired by ticks from infected mice (figure 3*F*), it is possible that *bb0323* function may be required for spirochete entry into the vector, but may be redundant for persistence in ticks or transmission back to mice. We, therefore,

directly introduced *bb0323* mutants into ticks, and studied the ability of the mutants to persist in unfed and fed ticks, and transmit back to naïve mice. A microinjection procedure was used to deliver an equal number of wild type or genetically-manipulated isolates into the tick gut as previously described [17, 19]. Three groups of ticks were infected with the wild type spirochete, *bb0323* mutant or *bb0323*-complemented *B. burgdorferi* (10^3 cells/tick). Following infection, one group of ticks was allowed to remain in the unfed condition for 14 days, while a parallel group was allowed to feed on naïve C3H mice (5 ticks/mouse) for 48 hrs. The spirochete burdens in the unfed ticks, the feeding tick gut and the salivary glands were determined by qRT-PCR. Unlike wild type spirochetes and *bb0323*-complemented isolates, BB0323-deficient spirochetes were unable to survive in unfed ticks (figure 5*A*). At 24 hours of feeding, the burden of *bb0323* mutant into ticks began to decline, and at 48 hours of feeding, the numbers of *bb0323* mutants in the gut or salivary gland remained significantly lower, when compared to *bb0323*-complemented or wild type isolates (figure 5*B*). Following 14 days of feeding, analysis of mouse infection by culture (data not shown) or qRT-PCR analysis (figure 5*C*) indicated that *bb0323* failed to transmit to mice; whereas, both wild type spirochetes and *bb0323*-complemented isolates were transmitted to the host. These data establish that BB0323 is necessary for *B. burgdorferi* persistence in the vector and transmission through feeding ticks to the murine host.

DISCUSSION

Certain *B. burgdorferi* genes encoding membrane antigens alter their expression in new host or vector environments and may be important for pathogen persistence [12,25,30,41]. *bb0323* was previously identified as a differentially-regulated spirochete gene, since its expression induced in spirochetes cultured in environmental parameters that partially mimic fed ticks, such as 37°C and pH 6.8, compared to the expression in unfed tick-like conditions, such as 23°C and pH 7.5 [10]. Here, we show that *bb0323* is variably expressed throughout the spirochete infectious cycle and is indispensable for membrane organization and pathogen survival *in vivo*, regardless of vector and host environments. Such consistent requirement of BB0323 is in contrast with other regulated membrane antigens, which are either necessary in specific phases of the spirochete life cycle [16,17] or functionally redundant [27,29,30]. With the sole exception of a cellular enzyme, nicotinamidase (PncA) [14], BB0323 is possibly the only other antigen required for persistence and transmission of spirochetes throughout the enzootic infection cycle.

BB0323 is highly conserved in the *B. burgdorferi* sensu lato complex that causes human infection. BB0323 is immunogenic in mammals, including humans, and identified as a membrane-associated antigen [32,33]. Deficiency of BB0323 was previously shown to impose severe clumping of cells and the disintegration of outer membranes, often resulting in enormous blebs and few single cells, suggesting the involvement of BB0323 in the fission process [34]. Our data support these observations; however, the specific role of BB0323 in spirochete biology remains unknown. The antigen has an estimated molecular mass of 44 kDa; however, the native protein migrates on SDS-PAGE gels as a 30-kDa protein, which may be the result of aberrant migration of the highly-basic BB0323 or possible proteolytic processing of the immature protein [42]. A major clue to the BB0323 function is the presence of a single LysM (Lysine Motif)-like domain in the carboxyl terminus. LysM domains are found in a variety of prokaryotic and eukaryotic proteins and typically consists of 44–65 amino acids, occurring in single or multiple tandem copies with a poorly conserved central region and relatively high conservation over the first 16 and last 10 amino acids [43]. The BB0323 LysM domain, however, contains poor sequence identity to proteins with defined functions [43], and functions of few proteins with relatively close homologies, including the closest, an outer membrane protein of *Neisseria meningitidis* (Figure 2A), are unknown. In bacteria, LysM domains generally bind peptidoglycan and are found either in various types of enzymes associated with

cell wall degradation or in surface receptors involved in host-pathogen interaction [43] contributing to microbial virulence [44]. *bb0323* mutants display dramatic alterations in their cell shape and organization of membranes, which are structurally supported by peptidoglycan [45]. Major fractions of cellular BB0323 remain as a subsurface antigen, and if localized in the periplasm, BB0323 could interact with peptidoglycan via LysM domain, potentially contributing to the proper organization of the spirochete membrane. Our findings that BB0323 is also detectable in the outer membrane with limited yet detectable surface exposure is puzzling and appears to contradict the existence of a functional LysM domain, as peptidoglycan is not present in the borrelial outer membrane [13]. However, BB0323 might have dual roles in spirochete membrane organization and host-pathogen interaction, as shown for other LysM domain-containing proteins, such as *Staphylococcus aureus* autolysin Aaa [46], which possesses bacteriolytic and host adhesion properties. Nevertheless, as *bb0323* deletion imposes significant growth and morphological defects of spirochetes *in vitro* [34] and results in elimination of spirochetes within the first few days of host or vector infection, this chromosomally-encoded antigen must be involved in a crucial physiological function that is most pronounced during pathogen survival *in vivo*. Understanding of and interference with the function of *B. burgdorferi* antigens that are essential for microbial virulence and persistence through the natural infection cycle of the pathogen could, potentially, contribute to the development of effective therapeutic strategies to combat Lyme borreliosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by funding from the American Heart Association, Arthritis Foundation, and the National Institutes of Health (AR055323 and AI076684).

We thank Patricia Rosa for providing us the *B. burgdorferi* lp28-1-Gm clone. We sincerely thank Carolyn Marks, Adam Coleman, Kamoltip Promnares, Deborah Shroder and John Anderson for their assistance with this study.

References

1. Piesman J, Eisen L. Prevention of Tick-Borne Diseases. Annu Rev Entomol 2008;53:323–343. [PubMed: 17877457]
2. Barthold, SW.; DeSouza, M.; Fikrig, E.; Persing, DH. Lyme borreliosis in the laboratory mouse. In: Schuster, SE., editor. Lyme disease. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1992. p. 223-242.
3. Steere AC, Glickstein L. Elucidation of Lyme arthritis. Nat Rev Immunol 2004;4:143–52. [PubMed: 15040587]
4. Casjens S, Palmer N, van Vugt R, et al. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. Mol Microbiol 2000;35:490–516. [PubMed: 10672174]
5. Fraser CM, Casjens S, Huang WM, et al. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature 1997;390:580–6. [PubMed: 9403685]
6. de Silva AM, Fikrig E. Arthropod- and host-specific gene expression by *Borrelia burgdorferi*. J Clin Invest 1997;99:377–9. [PubMed: 9022068]
7. Brooks CS, Hefty PS, Jolliff SE, Akins DR. Global analysis of *Borrelia burgdorferi* genes regulated by mammalian host-specific signals. Infect Immun 2003;71:3371–83. [PubMed: 12761121]
8. Caimano MJ, Iyer R, Eggers CH, et al. Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle. Mol Microbiol 2007;65:1193–217. [PubMed: 17645733]

9. Ojaimi C, Brooks C, Akins D, et al. *Borrelia burgdorferi* gene expression profiling with membrane-based arrays. Methods Enzymol 2002;358:165–77. [PubMed: 12474386]
10. Revel AT, Talaat AM, Norgard MV. DNA microarray analysis of differential gene expression in *Borrelia burgdorferi*, the Lyme disease spirochete. Proc Natl Acad Sci U S A 2002;99:1562–7. [PubMed: 11830671]
11. Tokarz R, Anderton JM, Katona LI, Benach JL. Combined effects of blood and temperature shift on *Borrelia burgdorferi* gene expression as determined by whole genome DNA array. Infect Immun 2004;72:5419–32. [PubMed: 15322040]
12. Liang FT, Nelson FK, Fikrig E. Molecular adaptation of *Borrelia burgdorferi* in the murine host. J Exp Med 2002;196:275–80. [PubMed: 12119353]
13. Rosa PA, Tilly K, Stewart PE. The burgeoning molecular genetics of the Lyme disease spirochaete. Nat Rev Microbiol 2005;3:129–43. [PubMed: 15685224]
14. Purser JE, Lawrenz MB, Caimano MJ, Howell JK, Radolf JD, Norris SJ. A plasmid-encoded nicotinamidase (PncA) is essential for infectivity of *Borrelia burgdorferi* in a mammalian host. Mol Microbiol 2003;48:753–64. [PubMed: 12694619]
15. Jewett MW, Lawrence K, Bestor AC, et al. The critical role of the linear plasmid lp36 in the infectious cycle of *Borrelia burgdorferi*. Mol Microbiol 2007;64:1358–74. [PubMed: 17542926]
16. Pal U, Wang P, Bao F, et al. *Borrelia burgdorferi* basic membrane proteins A and B participate in the genesis of Lyme arthritis. J Exp Med 2008;205:133–41. [PubMed: 18166585]
17. Yang XF, Pal U, Alani SM, Fikrig E, Norgard MV. Essential role for OspA/B in the life cycle of the Lyme disease spirochete. J Exp Med 2004;199:641–8. [PubMed: 14981112]
18. Grimm D, Tilly K, Byram R, et al. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc Natl Acad Sci U S A 2004;101:3142–7. [PubMed: 14970347]
19. Pal U, Yang X, Chen M, et al. OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. J Clin Invest 2004;113:220–30. [PubMed: 14722614]
20. Shi Y, Xu Q, McShan K, Liang FT. Both decorin-binding proteins A and B are critical for the overall virulence of *Borrelia burgdorferi*. Infect Immun 2008;76:1239–46. [PubMed: 18195034]
21. Blevins JS, Hagman KE, Norgard MV. Assessment of decorin-binding protein A to the infectivity of *Borrelia burgdorferi* in the murine models of needle and tick infection. BMC Microbiol 2008;8:82. [PubMed: 18507835]
22. Seshu J, Esteve-Gassent MD, Labandeira-Rey M, et al. Inactivation of the fibronectin-binding adhesin gene *bbk32* significantly attenuates the infectivity potential of *Borrelia burgdorferi*. Mol Microbiol 2006;59:1591–601. [PubMed: 16468997]
23. Li X, Liu X, Beck DS, Kantor FS, Fikrig E. *Borrelia burgdorferi* lacking BBK32, a fibronectin-binding protein, retains full pathogenicity. Infect Immun 2006;74:3305–13. [PubMed: 16714558]
24. Revel AT, Blevins JS, Almazan C, et al. bptA (*bbe16*) is essential for the persistence of the Lyme disease spirochete, *Borrelia burgdorferi*, in its natural tick vector. Proc Natl Acad Sci U S A 2005;102:6972–7. [PubMed: 15860579]
25. Pal U, Dai J, Li X, et al. A Differential Role for BB0365 in the Persistence of *Borrelia burgdorferi* in Mice and Ticks. J Infect Dis 2008;197:148–155. [PubMed: 18171298]
26. Li X, Pal U, Ramamoorthi N, et al. The Lyme disease agent *Borrelia burgdorferi* requires BB0690, a Dps homologue, to persist within ticks. Mol Microbiol 2007;63(3):694–710. [PubMed: 17181780]
27. Coleman AS, Yang X, Kumar M, et al. *Borrelia burgdorferi* complement regulator-acquiring surface protein 2 does not contribute to complement resistance or host infectivity. PLoS ONE 2008;3:3010e.
28. Hubner A, Revel AT, Nolen DM, Hagman KE, Norgard MV. Expression of a *luxS* gene is not required for *Borrelia burgdorferi* infection of mice via needle inoculation. Infect Immun 2003;71:2892–6. [PubMed: 12704164]
29. Li X, Neelakanta G, Liu X, et al. Role of outer surface protein D in the *Borrelia burgdorferi* life cycle. Infect Immun 2007;75:4237–44. [PubMed: 17620358]
30. Stewart PE, Bestor A, Cullen JN, Rosa PA. A tightly regulated surface protein of *Borrelia burgdorferi* is not essential to the mouse-tick infectious cycle. Infect Immun 2008;76:1970–8. [PubMed: 18332210]

31. Tilly K, Grimm D, Bueschel DM, Krum JG, Rosa P. Infectious cycle analysis of a *Borrelia burgdorferi* mutant defective in transport of chitobiose, a tick cuticle component. Vector Borne Zoonotic Dis 2004;4:159–68. [PubMed: 15228817]
32. Nowalk AJ, Gilmore RD Jr, Carroll JA. Serologic proteome analysis of *Borrelia burgdorferi* membrane-associated proteins. Infect Immun 2006;74:3864–73. [PubMed: 16790758]
33. Nowalk AJ, Nolder C, Clifton DR, Carroll JA. Comparative proteome analysis of subcellular fractions from *Borrelia burgdorferi* by NEPHGE and IPG. Proteomics 2006;6:2121–34. [PubMed: 16485259]
34. Stewart PE, Hoff J, Fischer E, Krum JG, Rosa PA. Genome-wide transposon mutagenesis of *Borrelia burgdorferi* for identification of phenotypic mutants. Appl Environ Microbiol 2004;70:5973–9. [PubMed: 15466540]
35. Elias AF, Stewart PE, Grimm D, et al. Clonal polymorphism of *Borrelia burgdorferi* strain B31 MI: implications for mutagenesis in an infectious strain background. Infect Immun 2002;70:2139–50. [PubMed: 11895980]
36. Pal U, Li X, Wang T, et al. TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. Cell 2004;119:457–68. [PubMed: 15537536]
37. Pal U, Montgomery RR, Lusitani D, et al. Inhibition of *Borrelia burgdorferi*-tick Interactions *in vivo* by outer surface protein A antibody. J Immunol 2001;166:7398–403. [PubMed: 11390491]
38. Skare JT, Shang ES, Foley DM, et al. Virulent strain associated outer membrane proteins of *Borrelia burgdorferi*. J Clin Invest 1995;96:2380–92. [PubMed: 7593626]
39. Barthold SW, Hodzic E, Tunev S, Feng S. Antibody-mediated disease remission in the mouse model of Lyme borreliosis. Infect Immun 2006;74:4817–25. [PubMed: 16861670]
40. Wang X, Ma Y, Weis JH, Zachary JF, Kirschning CJ, Weis JJ. Relative contributions of innate and acquired host responses to bacterial control and arthritis development in Lyme disease. Infect Immun 2005;73:657–60. [PubMed: 15618212]
41. Schwan TG. Temporal regulation of outer surface proteins of the Lyme-disease spirochaete *Borrelia burgdorferi*. Biochem Soc Trans 2003;31:108–12. [PubMed: 12546665]
42. Ostberg Y, Carroll JA, Pinne M, Krum JG, Rosa P, Bergstrom S. Pleiotropic effects of inactivating a carboxyl-terminal protease, CtpA, in *Borrelia burgdorferi*. J Bacteriol 2004;186:2074–84. [PubMed: 15028692]
43. Buist G, Steen A, Kok J, Kuipers OP. LysM, a widely distributed protein motif for binding to (peptido) glycans. Mol Microbiol 2008;68:838–47. [PubMed: 18430080]
44. Bolton MD, van Esse HP, Vossen JH, et al. The novel *Cladosporium fulvum* lysin motif effector Ecp6 is a virulence factor with orthologues in other fungal species. Mol Microbiol 2008;69:119–36. [PubMed: 18452583]
45. Motaleb MA, Corum L, Bono JL, et al. *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. Proc Natl Acad Sci U S A 2000;97:10899–904. [PubMed: 10995478]
46. Heilmann C, Hartleib J, Hussain MS, Peters G. The multifunctional *Staphylococcus aureus* autolysin Aaa mediates adherence to immobilized fibrinogen and fibronectin. Infect Immun 2005;73:4793–802. [PubMed: 16040992]

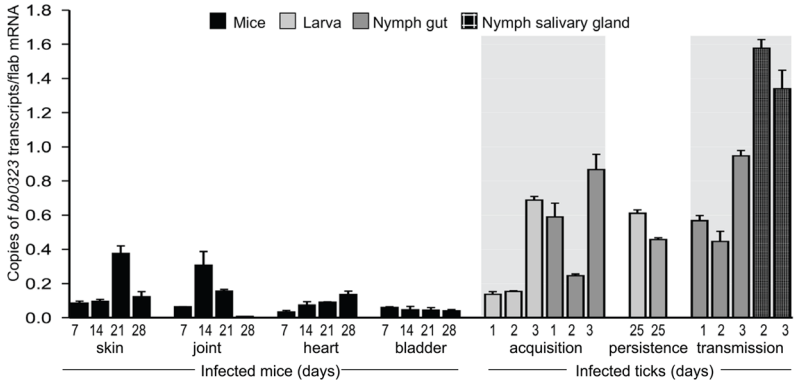

Figure 1. Consistent expression of *bb0323* throughout the enzootic life cycle of *B. burgdorferi*

Total RNA was isolated from multiple tissues covering the major stages of mouse-tick infection cycle and *bb0323* transcripts were measured using quantitative RT-PCR and presented as copies of *bb0323* transcript per copy of *flaB* transcript. Shaded area denotes duration of tick feeding. Error bars represent the mean ± SEM from four quantitative RT-PCR analyses of two independent murine-tick infection experiments.

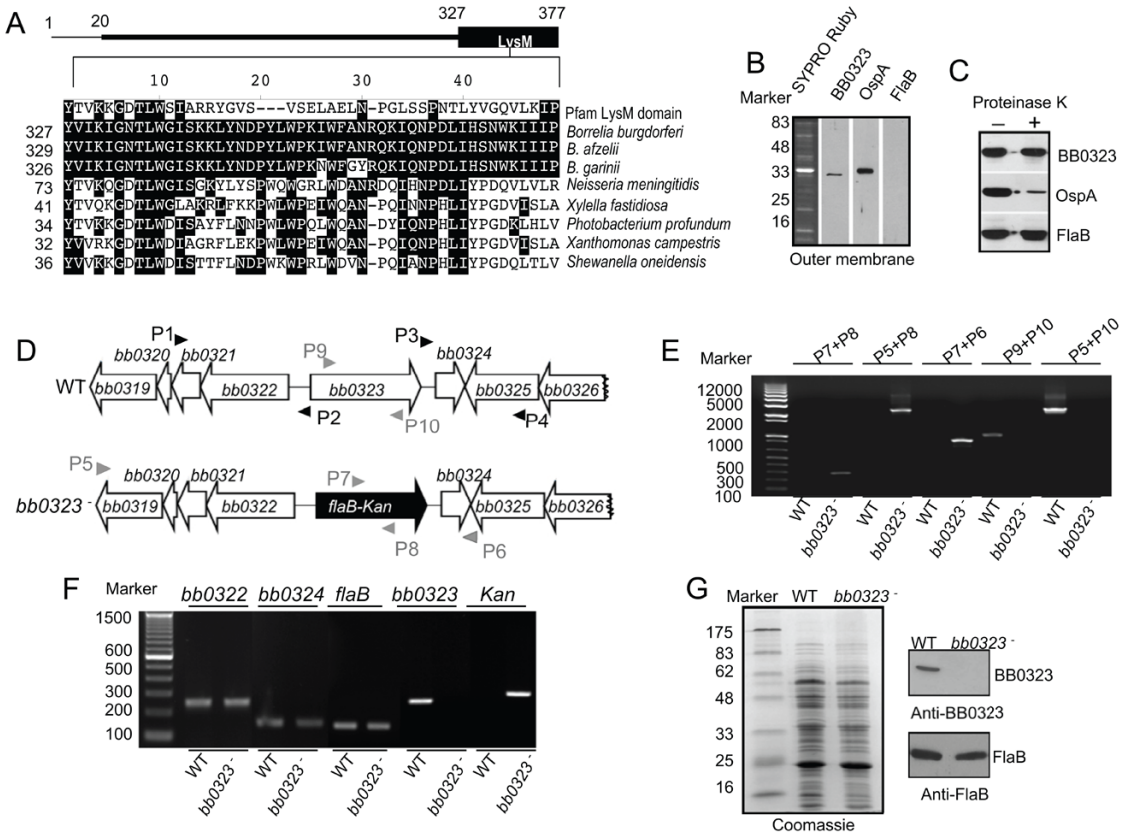

Figure 2. Construction and analysis of the *bb0323* mutant *B. burgdorferi*

A, Position and amino acid sequence alignment of putative LysM-like domain of *B. burgdorferi* BB0323. The upper panel represents BB0323 protein indicating an amino terminal signal peptide (encompassing the first 20 amino acids) and a carboxyl terminal LysM domain. Lower panel shows amino acid sequence alignment of LysM-like domain of BB0323 to a designated LysM domain (Pfam accession number PF01476) or to orthologs in major *B. burgdorferi sensu lato* isolates and representative non-spirochete proteins with closest homologies. Dashes indicate gaps introduced to optimize alignments and identical amino acids are shaded. The numbers on the left are the positions of the amino acid residues in the proteins, for which corresponding species and annotations are indicated in the supplementary text. *B*, BB0323 is detectable in the outer membrane. Outer membrane vesicles were separated by sucrose density gradient centrifugation, separated by SDS-PAGE, and stained with SYPRO Ruby or immunoblotted with BB0323, OspA and FlaB antibodies. *C*, BB0323 is partially sensitive to proteinase K-mediated degradation of *B. burgdorferi* surface proteins. Viable *B. burgdorferi* were incubated with (+) or without (−) proteinase K for the degradation of protease-sensitive surface proteins and processed for immunoblot analysis using BB0323 antibodies. *B. burgdorferi* OspA and FlaB antibodies were utilized as controls for surface-exposed and sub-surface proteins, respectively. *D*, Schematic drawings of the wild type (WT) and the *bb0323* mutant (*bb0323*$^{-}$) isolates at the *bb0323* locus. Genes *bb0319* - *bb0326* (white box arrows) and the kanamycin-resistance cassette driven by the *B. burgdorferi flaB* promoter (*flaB-Kan*, black box arrow) are indicated. Primers P1–P4 (black arrow-heads) were used to amplify 5′ and 3′ arms for homologous recombination, and regions flanking up- and down-stream of the *bb0323* locus were ligated on either side of the *flaB-Kan* cassette as detailed in the text. *E*, Integration of the mutagenic construct, *flab-Kan*, in the intended genomic locus. Primers 5–10 (gray arrow-heads, positions indicated in figure 2*D*) were used for PCR analysis

using isolated DNA from wild type (WT) or mutant *B. burgdorferi* (*bb0323*$^{-}$) 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. *F*, RT-PCR assessment of *bb0323* ablation and the polar effects of mutagenesis. Total RNA was isolated from wild type *B. burgdorferi* (WT) or *bb0323* mutant (*bb0323*$^{-}$) converted to cDNA and used to amplify regions within *bb0323*, *flaB*, kanamycin and genes surrounding *bb0323* locus (*bb0322* and *bb0324*) and visualized on a gel. *G*, Protein analysis of wild type and mutant isolates. Equal amounts of protein were by SDS-PAGE gel, and either stained with Coomassie blue (left panel) or immunoblotted with BB0323 and FlaB antibodies (right panel). Migration of protein standards is shown to the left in kDa.

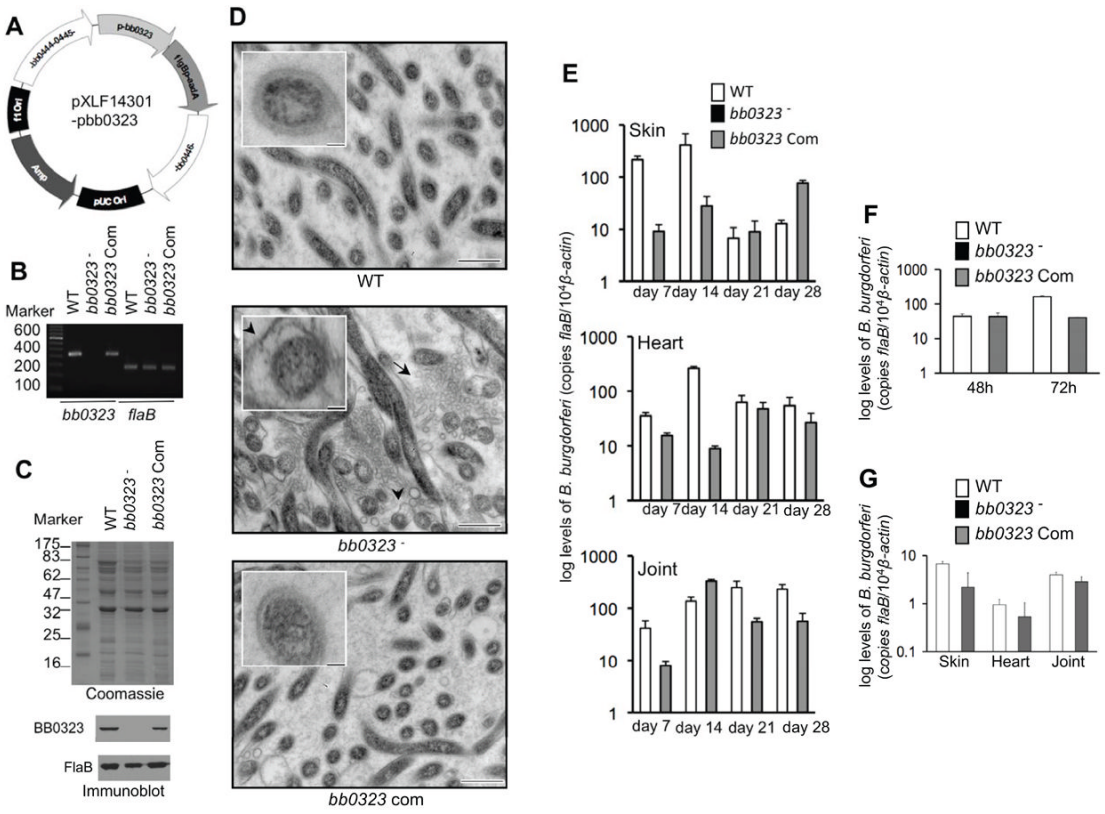

Figure 3. Complementation of *bb0323* mutant *B. burgdorferi* with *bb0323* restores spirochete infectivity throughout an experimental enzootic cycle

A, Construction of DNA construct (pXLF14301-pbb0323) for chromosomal integration of the *bb0323*. The open reading frame of *bb0323* and its native promoter together with streptomycin resistance gene (*aadA*) was cloned into pXLF14301, which contains *B. burgdorferi* inserts for integration of the complemented gene in spirochete chromosome. *B*, RT-PCR analysis of the *bb0323* transcripts. Total RNA was isolated from the wild type (WT), *bb0323* mutant (*bb0323*$^{-}$) or *bb0323*-complemented *B. burgdorferi* (*bb0323* Com), converted to cDNA and subjected to PCR analysis. *C*, Reproduction of BB0323 protein by the complemented *B. burgdorferi*. Lysates of *B. burgdorferi* were separated on a SDS-PAGE gel, which was either stained with Coomassie blue (upper panel) or immunoblotted with specific antibodies (lower panels). *D*, Complementation of *bb0323* mutant with *bb0323* gene restores ultrastructural defects of mutant cells. *B. burgdorferi* were grown at a density of 5×10^{7}/ml in BSK medium and processed for transmission electron microscopy. Arrows indicate enhanced shedding of vesicular structures or blebs, possibly derived from the outer membrane, in the mutants. Inset shows higher magnification images of a representative single cell indicating altered organization of outer membrane in the *bb0323* mutant. Bar = 500 nm (inset Bar = 100 nm). *E*, Complementation restores spirochete infectivity in mice. Spirochete burden in groups of infected mice (5 animals/group) was analyzed by quantitative RT-PCR at days 7, 14, 21 or 28 by measuring copies of the *flaB* gene and normalized against mouse *β-actin* levels. Bars represent the mean ± SEM of relative tissue levels of pathogen from three independent animal infection experiments. *F*, *bb0323*-complemented *B. burgdorferi* are acquired by ticks. Mice were inoculated with spirochetes (5 mice/group) and following two weeks of infection, naïve *I. scapularis* nymphs (25 ticks/mice) were allowed to feed on mice. Spirochete burdens were assessed in ticks by qRT-PCR, by measuring copies of the *B. burgdorferi flaB* gene and normalized against tick *β-actin* levels. Bars represent the mean ± SEM from three independent

experiments. *G*, *bb0323*-complemented *B. burgdorferi* persisted through larval-nymphal molt and transmit from feeding ticks to mice. *B. burgdorferi*-infected nymphs were generated by feeding larva on infected mice, which were allowed to feed to repletion on naïve mice (3 tick/mouse, 5 animal/group). Murine tissues were isolated at day 14 after repletion and the spirochete burdens were assessed by qRT-PCR. Bars represent the mean ± SEM from two independent experiments.

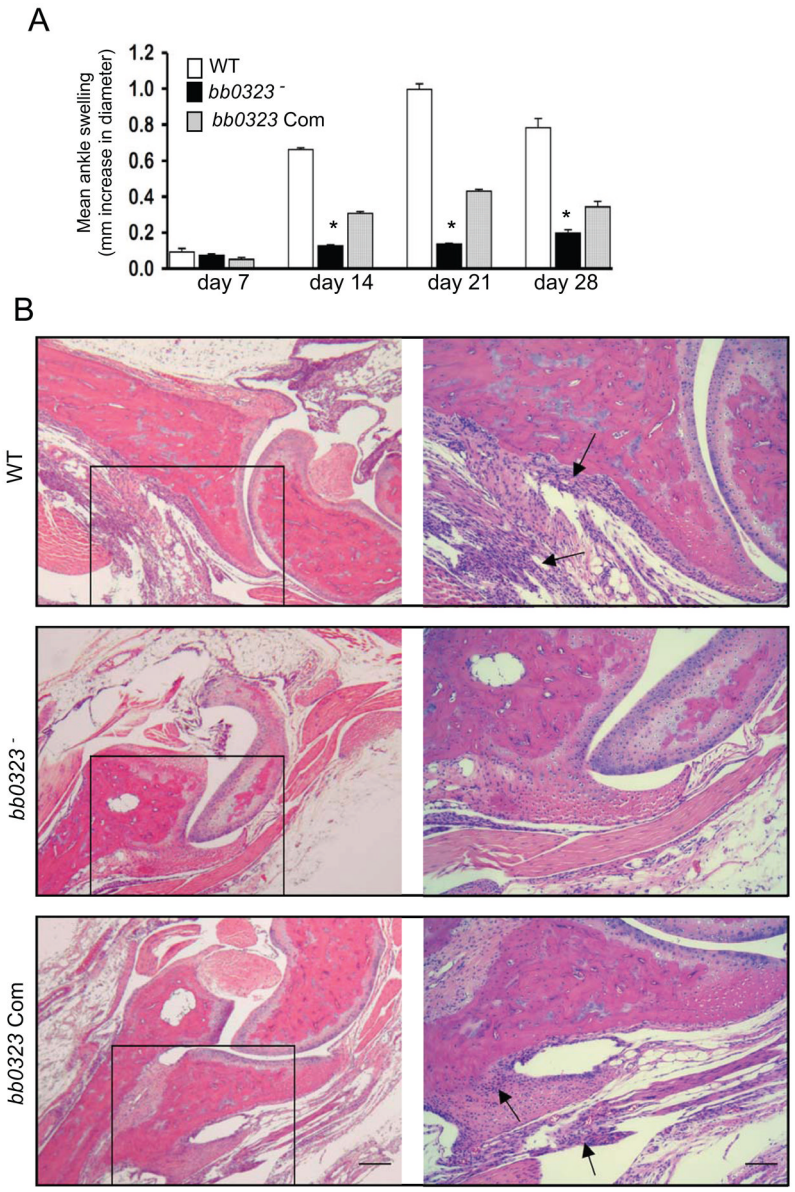

Figure 4. Deficiency of *B. burgdorferi bb0323* affects the severity of arthritis in mice

A, Severity of joint swelling in *B. burgdorferi*-infected mice. Groups of mice (5 animals/group) were infected with the wild type (WT), *bb0323* mutant (*bb0323*$^{-}$) or *bb0323*-complemented *B. burgdorferi* (*bb0323* Com), and assessed for joint swelling. Bars represent the mean ± SEM from three independent infection experiments. Differences in the joint swelling between groups of mice infected with *bb0323* mutant and those with the *bb0323*-complemented isolates were highly significant (*$P < 0.01$) at all time points, except for day 7. *B*, Representative demonstration of joint histology in mice infected with wild type or genetically-manipulated *B. burgdorferi* isolates. Twenty-one days following spirochete infection, tibiotarsal joints were analyzed for histopathology. The left panel indicates low-resolution (40x, bar = 400 μm) joint images. Higher-resolution (200x, bar = 80 μm) images of selected areas from corresponding sections (marked by box) are shown in the right panels.

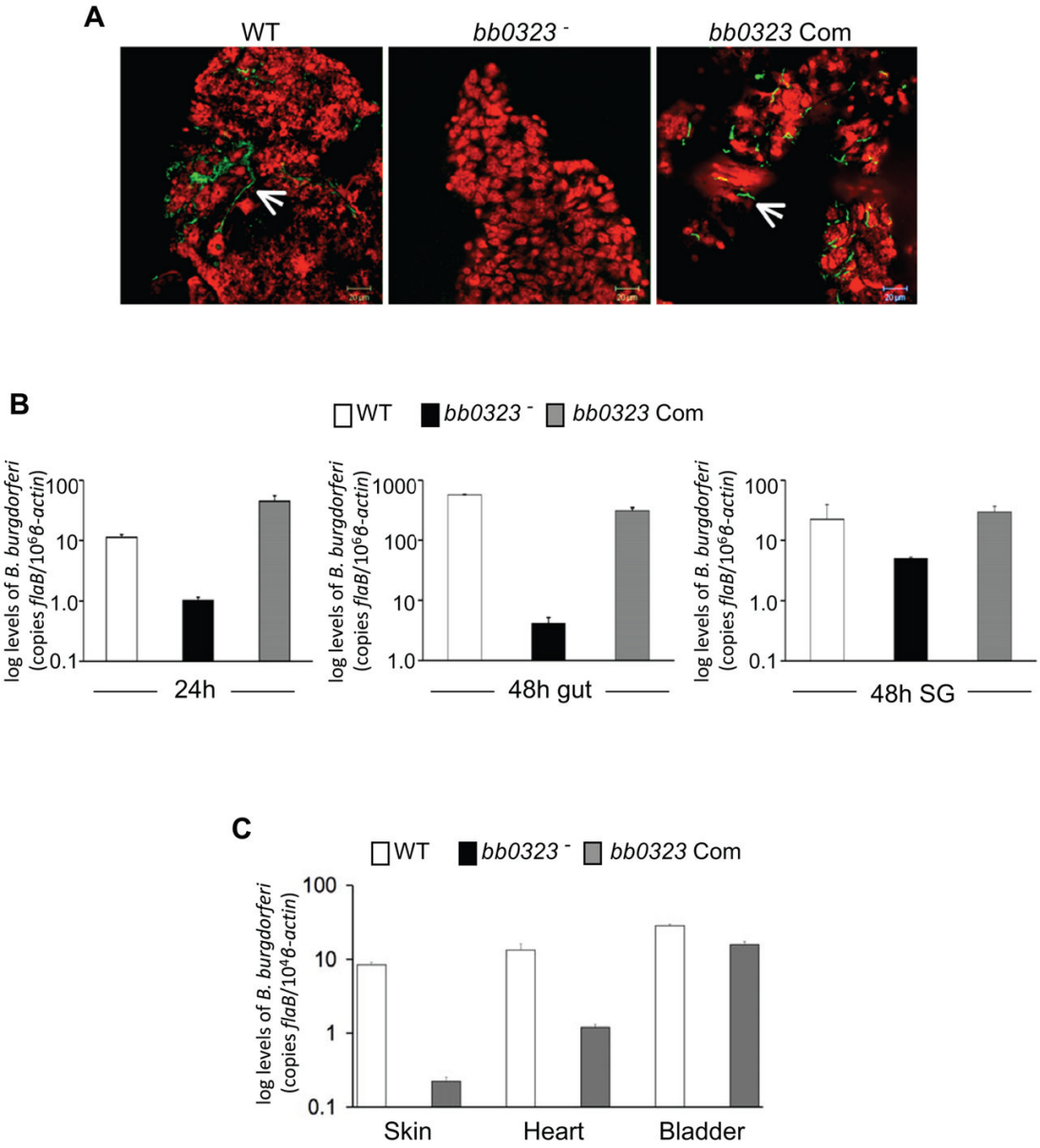

Figure 5. *bb0323* is required for *B. burgdorferi* to persist in ticks and for transmission to naïve host

A, *bb0323* mutants were unable to persist in unfed *Ixodes* nymphs. Nymphal ticks were microinjected with *B. burgdorferi* (WT), *bb0323* mutant (*bb0323*$^{-}$) or *bb0323*-complemented *B. burgdorferi* (*bb0323* Com), and spirochete burdens in the gut of unfed ticks were analyzed at 14 days after injection. The spirochetes (arrow) were labeled with FITC-labeled goat anti-*B. burgdorferi* antibody (shown in green), and the nuclei of the gut cells were stained with propidium iodide (shown in red). Images were obtained using a confocal immunofluorescence microscope at 400x magnification, and presented as merge image for clarity. *B*, Decreased burdens of microinjected *bb0323* mutants in feeding ticks. Nymphal ticks were microinjected with *B. burgdorferi*, as described in figure 5*A*, and placed on naïve mice 48 hours after injection. Spirochete burden was analyzed by quantitative PCR analysis of whole ticks at 24 hours and dissected gut or salivary glands at 48 hours of feeding by measuring copies of the *B. burgdorferi flaB* gene, normalized against tick *β-actin* levels. Bars represent the mean ± SEM of relative

tissue levels of *B. burgdorferi* from three independent animal infection experiments. Differences between *bb0323* mutant burdens and those with complemented or wild type isolates were significant at all time points and tissues ($P < 0.02$ or lower). *C*, *bb0323* mutants were unable to transmit to mice. Nymphal ticks were microinjected as described in figure 5*B*, and placed on naïve mice following 48 hours after injection. Ticks were allowed to engorge and transmission of *B. burgdorferi* was assessed by measuring copies of the *flaB* gene, normalized against mouse *β-actin* levels, in indicated murine tissues after 14 days of tick feeding. Bars represent the mean ± SEM of relative tissue levels of *B. burgdorferi* from three independent animal infection experiments.