Research article

Assessment of decorin-binding protein A to the infectivity of Borrelia burgdorferi in the murine models of needle and tick infection

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Abstract

Background: Decorin-binding proteins (Dbps) A and B of Borrelia burgdorferi, the agent of Lyme disease, are surface-exposed lipoproteins that presumably bind to the extracellular matrix proteoglycan, decorin. B. burgdorferi infects various tissues including the bladder, heart, joints, skin and the central nervous system, and the ability of B. burgdorferi to bind decorin has been hypothesized to be important for this disseminatory pathogenic strategy.

Results: To determine the role of DbpBA in the infectious lifecycle of B. burgdorferi, we created a DbpBA-deficient mutant of B. burgdorferi strain 297 and compared the infectious phenotype of the mutant to the wild-type strain in the experimental murine model of Lyme borreliosis. The mutant strain exhibited a 4-log decrease in infectivity, relative to the wild-type strain, when needle inoculated into mice. Upon complementation of the DbpBA-mutant strain with DbpA, the wild-type level of infectivity was restored. In addition, we demonstrated that the DbpBA-deficient mutant was able to colonize Ixodes scapularis larval ticks after feeding on infected mice and persist within the ticks during the molt to the nymphal state. Moreover, surprisingly, the DbpBA-mutant strain was capable of being transmitted to naïve mice via tick bite, giving rise to infected mice.

Conclusion: These results suggest that DbpBA is not required for the natural tick-transmission process to mammals, despite inferences from needle-inoculation experiments implying a requirement for DbpBA during mammalian infection. The combined findings also send a cautionary note regarding how results from needle-inoculation experiments with mice should be interpreted.

Background

The causative agent of Lyme disease, Borrelia burgdorferi, is introduced into a mammalian host via tick bite, whereupon the organisms enter the skin, disseminate hematogenously, and persist in the presence of a strong host immune response [1]. The dissemination and persistence of B. burgdorferi within a mammalian host is thought to be predicated, at least in part, on the organism’s ability to bind molecules of the extracellular matrix (ECM) [2-4], inasmuch as these interactions have been shown to be important for other bacterial pathogens [5]. Among various ECM components, B. burgdorferi binds to type I collagen [4], fibronectin [6,7], integrins [8,9], the proteoglycan decorin [10], and glycosaminoglycans (GAGs) [11,12]. The B. burgdorferi proteins described as ECM-binding proteins include BBK32 [6,13], Bgp (borrelia-GAG binding protein) [12], P66 (Oms66) [14], decorin-binding protein (Dbp) A, and DbpB [10,15]. These proteins, and perhaps...
other as yet unidentified molecules, may play a significant role in the infectivity and pathogenesis phenotypes of *B. burgdorferi*.

Our laboratory has been interested in DbpA as a vaccine candidate for the prevention of Lyme disease and the contribution of DbpA and DbpB to *B. burgdorferi* pathogenesis and infectivity [16,17]. Since their *in vitro* characterization as decorin-binding proteins [10,15], the DbpA and DbpB lipoproteins have been implicated as potential contributors to adhesion and colonization of *B. burgdorferi* within mammalian hosts [15,18]. The genes that encode DbpA and DbpB reside in an operon, *dbpBA*, on linear plasmid 54 (lp54) and are found within many *B. burgdorferi sensu lato* isolates [19]. Neither protein is expressed by *B. burgdorferi* within the tick vector [17], however, expression of *dbpA* (and presumably *dbpB*) is upregulated in the mammalian host after ticks deposit spirochetes into the skin [20,21]. DbpA and DbpB expression likely remains high for the duration of mammalian infection, as inferred by the presence of antibodies against both antigens in the serum of mice as late as one year after infection (Hagman, unpublished data). The presence of antibodies against DbpA in the serum of patients with late-stage, disseminated Lyme disease also is well-documented [22], providing added support for DbpA expression by *B. burgdorferi* during chronic infection.

Although the combined data to date suggest an important role for the decorin-binding proteins of *B. burgdorferi* during mammalian infectivity, virtually all data inferring the importance of DbpA and DbpB thus far have been indirect. The first direct investigation into the role of the *dbpBA* operon in the infectious lifecycle of *B. burgdorferi* was carried out by Shi et al [23]. In this study, mutational analysis of *dbpBA* in *B. burgdorferi* strain B31 indicated that neither DbpA, nor DbpB was essential in the murine needle-challenge infection model of borreliosis. However, there was evidence suggesting that these mutants exhibited a modest level of attenuation in immunocompetent mice. Unfortunately, a comprehensive 50% infectious dose (ID$_{50}$) was not included in this report to further investigate this possible defect, nor was genetic complementation of the mutation performed. This latter point is of particular importance given the genetic plasticity of *B. burgdorferi* and the possibility that spontaneous loss of an endogenous borrelial plasmid might account for the apparent defect in this mutant. To more directly examine the role of both DbpA and DbpB in the murine/tick model of Lyme disease, a *dbpBA*-deficient mutant and a *dbpA* genetic complement of the mutant were generated in the infectious strain 297 of *B. burgdorferi*. Phenotypic assessment of mouse needle infectivity by ID$_{50}$ analysis and *Ixodes scapularis* tick colonization and tick-transmis-

**Results**

**Construction and characterization of a *dbpBA*-deficient mutant**

To assess the roles that DbpA or DbpB play in the infectivity and pathogenesis of *B. burgdorferi*, we created a *dbpBA*-deficient mutant by allelic exchange of a PlfgB-kan cassette for the majority of the *dbpBA* operon (Fig. 1A). Infectious Bb297 was chosen for construction of the *dbpBA* mutant because it is a human isolate [24] and prior infectious mutants of this strain have been readily created [25-28].

Bb297 was electroporated with the suicide vector, pKHD-bdpAko, containing a PlfgB-kan cassette flanked by 1.5 kb of DNA on the left side of the *dbpBA* operon and 890 bp of DNA on the right side of the *dbpBA* operon (Fig. 1A). This allelic exchange strategy relies on a double crossover, homologous recombination event to replace the *dbpBA* operon with the PlfgB-kan cassette. Several kanamycin-resistant transformants were obtained, and the presence (Fig. 1B) and orientation (Fig. 1C) of the PlfgB-kan cassette within the *dbpBA* operon was confirmed by PCR analysis.

During genetic manipulation and *in vitro* cultivation, *B. burgdorferi* may spontaneously lose endogenous plasmids that are not required for growth *in vitro*, but are essential for mammalian infection. At least two plasmid-encoded genes, *vlsE* [29] and *pncA* (BBE22) [30], fit this criteria. One kanamycin-resistant transformant, BbKH500, retained both *vlsE* and *pncA* (Fig. 2) and was analyzed by immunoblotting to confirm the loss of DbpA and DbpB. As expected, DbpA and DbpB were absent from BbKH500 (Figs. 3A and 3B). BbKH500 exhibited identical doubling times when compared to wild-type Bb297 (data not shown) and PCR-based plasmid profiling revealed that the endogenous plasmid profile of BbKH500 matched that of the parent Bb297 (Fig. 2).

**Loss of *DbpBA* significantly reduces the infectivity of *B. burgdorferi* in mice when introduced via needle inoculation**

To determine the contribution of DbpA and DbpB to the infectivity of *B. burgdorferi* in mice, C3H/HeJ mice were challenged intradermally via needle inoculation with increasing numbers of BbKH500 (10$^4$, 10$^5$, 10$^6$ and 10$^7$ spirochetes). As a positive control, an additional five mice were inoculated with wild-type Bb297 at a dose of 10$^5$ spirochetes (ID$_{50}$ of approximately 50 spirochetes; [27]). The results are shown in Table 1. Whereas the mice infected with 10$^5$ of Bb297 showed culture positive ear punch biopsies at two weeks post-infection, needle challenge of naïve mice with 10$^4$ BbKH500 did not produce an infec-
Construction of dbpBA-deletion mutant BbKH500 and Prom-dbPA complementation vector and PCR confirmation

**Figure 1**

**Construction of dbpBA-deletion mutant BbKH500 and Prom-dbPA complementation vector and PCR confirmation.** (A) Strategy for the replacement of the dbpBA operon with the FlgB-kan and complementation with pKH2000. pKH-dbPAko was the pGEM-T easy-based suicide plasmid used to transform Bb297 for the homologous recombination of the kanamycin-resistance gene into the dbpBA operon. "A" denotes Ascl sites. FlgB-kan denotes the kanamycin-resistance marker expressed from the flgB promoter. The borrelial shuttle vector containing the dbpBA Prom fused to the dbpA ORF (pKH2000) was transformed into BbKH500 to restore DbpA expression. Oligonucleotide primers used for PCR are indicated with short arrows. (B) PCR using primers ko5 and ko4 (shown in panel A). The first two lanes are undigested PCR products from Bb297 and BbKH500, whereas the second two lanes are the corresponding PCR products digested with Ascl. (C) Lanes 1, 3 and 5 are PCR products derived from BbKH500 template DNA and lanes 2, 4 and 6 are PCR products derived from Bb297 template DNA. Primer pairs used in PCR are indicated above the lanes. FlaB5' and FlaB3' primers amplify flaB of B. burgdorferi. DNA size standards (M) are shown in base pairs on the left.
tion in any mouse (n = 15), even when the infection was allowed to progress for 14 weeks. Seventeen mice challenged with 10^5 and ten mice challenged with 10^6 BbKH500 also did not show signs of infection (negative ear-punch biopsy culture) at four weeks post-challenge (Table 1). However, after 10 weeks, three mice from the group infected with 10^5 BbKH500 and two mice from the group challenged with 10^6 BbKH500 were shown to be infected by positive ear-punch biopsy cultures. Additionally, mice were challenged with either 10^5 or 10^6 BbKH500 and ear-punch biopsies were harvested 14 weeks after infection (Table 1). From the group that received 10^5 spirochetes, two mice were infected (n = 10), and from the group that received 10^6 spirochetes, four mice were infected (n = 10). All of the mice challenged with 10^7 of BbKH500 had positive ear-punch cultures as early as five-weeks post-challenge, and as late as 14 weeks post-infection (n = 5). These spirochetes from ear punch-positive cultures were analyzed by diagnostic PCR as described above, and confirmed to be Bb297 or BbKH500 (data not shown). Aliquots of these cultures also were passed to media containing kanamycin to confirm resistance and sensitivity of BbKH500 and Bb297, respectively.

Seroconversion analyses performed on a subset of mice also revealed that none of the mice infected with BbKH500 showed antibody reactivity against DbpA or DbpB (data not shown). Furthermore, only mice that exhibited culture-positive ear punch cultures showed significant serum antibody reactivity with P39 (data not shown). Analysis of all infection results yielded an ID_{50} for BbKH500 of 1.2 × 10^6 (p < 0.001) compared with ID_{50} of approximately 50 bacteria for wild-type Bb297 [27].

**Complementation of the dbpBA-deletion mutant with DbpA restores infectivity in needle-challenged mice**

The results above suggested that the dbpBA operon is required for full infectivity of *B. burgdorferi* when mice are infected via needle inoculation. However, during genetic manipulation of *B. burgdorferi*, it is not uncommon to lose one or more plasmid(s) which potentially contribute to infection, therefore genetic complementation is necessary to definitely ascribe the attenuated phenotype in BbKH500 to the dbpBA lesion. Although BbKH500 carries a mutation in both DbpA and DbpB, we chose to complement with only DbpA because i) DbpA is better characterized than DbpB [31,32] and ii) experimentation suggests that DbpA is the prominent Dbp in *B. burgdorferi* [15,33]. To restore DbpA expression in BbKH500, a shuttle plas-
mid carrying a copy of *dbpA* driven by its native promoter was constructed (Fig. 1A). Since *dbpA* is the second gene in the *dbpBA* operon, we chose to clone the promoter for this operon upstream of the *dbpA* gene and then insert the fusion product in the multiple-cloning site of pJD51. BbKH500 cells were transformed with this shuttle plasmid (pKH2000), yielding clones resistance to both kanamycin and streptomycin. These transformants were screened by PCR to verify the presence of the complementing plasmid (Fig. 3C). Clones shown to contain the complementing plasmid were checked for *vlsE* and *pncA* (Fig 2.), as described above. PCR-based plasmid profiling, performed on one of the transformants that contained both *pncA* and *vlsE*, revealed a profile identical to the parent strain, BbKH500 (Fig. 2). This clone, designated BbKH501, was selected for further analysis and DbpA expression was confirmed by both SDS-PAGE (Fig. 3A) and immunoblot analysis (Fig. 3B). Proteinase K digestion of BbKH501 and Bb297, under conditions that left FlaB intact, demonstrated surface exposure of DbpA in BbKH501 and Bb297, under conditions that left FlaB intact, demonstrated surface exposure of DbpA in both strains (Fig. 3D). Three groups of five C3H/HeJ mice were needle inoculated (i.d.) with BbKH501 at 10², 10³ or 10⁴ borreliae per mouse. Ear punch biopsies were harvested at either two weeks (10³ and 10⁴ doses) or three weeks (10² dose); all 15 mice became infected, as confirmed by positive ear-punch biopsy cultures. Although a precise determination of the ID₅₀ of BbKH501 could not be calculated, the fact 100% of the mice were infected with 10² bacteria suggests that the ID₅₀ is less than 10² spirochetes (4-logs lower than BbKH500). Aliquots of these cultures were passed to media containing streptomycin and kanamycin selection to confirm resistance. Diagnostic PCR also confirmed that the spirochetes growing out of the ear punch biopsy were BbKH501 (data not shown). Seroconversion analyses carried out on a subset of mice also revealed that the mice infected with BbKH501 showed antibody reactivity against DbpA, but not DbpB (data not shown). These data confirm that DbpA is necessary for wild-type levels of *B. burgdorferi* infectivity in mice when introduced via needle inoculation, and that DbpB is dispensable for this aspect of the infectious process.

Neither DbpA nor DbpB is required for acquisition of *B. burgdorferi* by ticks or infection of mice via tick bite

To determine whether *I. scapularis* ticks could acquire borreliae lacking *dbpA* and *dbpB*, naïve larvae were allowed to feed to repletion on mice infected with BbKH500 or on mice infected with Bb297. Larvae were collected after feeding, allowed to molt, and the unfed nymphs were examined for the presence of *B. burgdorferi* by IFA; approximately 11 weeks had elapsed since these ticks had fed to repletion as larvae. Microscopic examination of multiple fields revealed approximately one spirochete per field in the BbKH500-infected ticks (23 ticks examined), as opposed to 20–30 spirochetes per field in the Bb297-infected ticks (10 ticks examined). All Bb297-infected ticks examined were positive for *B. burgdorferi*. Of the ticks that had fed on BbKH500-infected mice, 80% were positive for spirochetes.
Table 2: Assessing infectivity of BbKH500 via tick inoculation of C3H/Hej mice

| Strain       | Ticks/mouse | Mouse infectivity |
|--------------|-------------|-------------------|
| Bb297        | 5           | 2/2               |
| BbKH500      | 5           | 1/2               |
|              | 10          | 4/6               |

*a Varying number of ticks infected with BbKH500 were allowed to feed to repletion on naive mice.

*b Infection rates were assessed at three weeks post-infection by culturing BbKH500 from ear-punch biopsies.

Because the ticks maintained strain BbKH500 through their molt, it was important to assess whether the loss of DbpA and DbpB affected B. burgdorferi transmission to mice via tick bite. To test this, five BbKH500-infected nymphs were placed on each of two naive mice and ten BbKH500-infected nymphs were placed on each of six naive mice. An additional two naive mice received five Bb297-infected ticks each. The ticks were allowed to feed to repletion, and at three weeks post-infection, ear-punch biopsies were harvested from all mice. The ear-punch biopsies were cultured in BSK medium and the cultures examined by dark-field microscopy for the presence of B. burgdorferi. These results are shown in Table 2. One of the two mice infected with five ticks harboring BbKH500 was infected as demonstrated by a positive ear-punch culture, and four of the six mice infected with ten ticks harboring BbKH500 were infected. Both of the mice on which the Bb297-infected ticks fed had positive ear-punch biopsy cultures. These spirochetes were analyzed by diagnostic PCR as described above, and confirmed to be Bb297 or BbKH500 (data not shown). Seroconversion analyses performed on a subset of the BbKH500-infected mice also revealed that none of the mice infected with BbKH500 showed antibody reactivity against DbpA or DbpB (data not shown). Furthermore, only mice that exhibited culture-positive ear punch cultures showed significant antibody reactivity against P39 (data not shown). Taken together, these data indicate that DbpA and DbpB are not essential for the transmission of B. burgdorferi from ticks to mice.

Discussion
The importance of ECM-binding proteins to the pathogenic strategy of B. burgdorferi is not currently known. It is presumed that borreliae bind mammalian host cells during infection because binding to various ECM molecules and to tissue culture cells has been demonstrated in vitro [4,34,35]. Of the known ECM-binding proteins of B. burgdorferi, Bgp [36] and BBK32 [37] have been deleted from infectious strains N40 and B31, respectively, and shown to be dispensable for infectivity in the mouse model of Lyme disease. A deletion of integrin-binding protein, P66, in a noninfectious strain of B. burgdorferi, HB19 [38], caused the spirochetes to lose their ability to bind b3- chain integrins [14] but the phenotype of a P66 mutant in an infectious strain of B. burgdorferi is currently unknown. The remaining two known ECM-binding proteins, DbpA and DbpB, were the focus of this work due to multiple lines of evidence suggesting a role for one or both proteins during infection of mammalian hosts [15,18,31]. To directly address the contribution of both DbpA and DbpB to B. burgdorferi infectivity, we created BbKH500, a dbpBA-deletion mutant of the human isolate Bb297, and used this strain to challenge mice with increasing numbers of spirochetes. We observed a 4-log reduction in infectivity of the mutant strain when the spirochetes were needle inoculated into mice. Complementation of the dbpBA-mutant with DbpA alone in the dbpBA-mutant restored infectivity of B. burgdorferi to wild-type levels.

Prior to this work, Fischer et al. [18] had restored DbpA and DbpB expression (via shuttle plasmid) to B. burgdorferi strain B314, a non-adherent, noninfectious derivative of strain B31 that has lost several plasmids, including lp54 [39]. Although expression of the Dbp molecules restored binding of the spirochetes to purified decorin, dermatan sulphate, and human epithelial cells [18], experiments to test the infectivity of B314 expressing DbpA and DbpB in the murine model of Lyme disease were implausible due to the loss of plasmids that are required for mammalian infection [39]. A recent study by Shi et al. [23] assessed the infectivity of a DbpA,B mutant of B. burgdorferi strain 5A18NP1, a B31 derivative that lacks the BBE02 gene [40]. Based on needle-challenge results obtained from a single inoculation dose of 105 bacteria (80% infection), Shi et al. [23] ascertained that the dbpBA locus was not required for infectivity. However, in the present study, we observed a 4-log increase in the ID50 of BbKH500 (>106 bacteria) and a 19% infection rate with a dose of 105 BbHK500 when mice were infected by needle inoculation. The reason for the difference in infectivity levels of the dbpBA-deficient mutants reported by Shi et al. and BbKH500 created in our laboratory is unknown at this time. One possible explanation for this disparity could be due to our use of Bb297 as the parental strain for the present mutational analysis, whereas Shi et al. [23] utilized a clonal derivative of B31 that is a BBE02-mutant. This variability also could be explained by spirochete enumeration differences prior to needle challenge. As mentioned in the Methods, significant attention was given to the enumeration of BbKH500 prior to needle inoculation of mice.

It should be noted that during the course of the study described herein, a subsequent report on the role of DbpBA was published by Shi et al. [41], which, unlike their previous study, included genetic complementation experiments. Our current findings agree with those of this most recent report in that Shi et al. also observed a 4-log
increase in ID₅₀ values with their dbpBA mutant [41]. In addition, Shi et al. observed a defect in the ability of the dbpBA mutant to colonize heart, joint and skin tissues, suggesting an overall deficiency in dissemination [41]. The observation that mice infected with BbKH500 showed a delay in infectivity (10- to 14-weeks post-infection) also suggests that BbKH500 might be attenuated with respect to its capacity to disseminate through the host. However, whereas we were able to compensate fully for the loss infectivity in the dbpBA mutant by complementing with dbpA alone (as assessed by ear punch biopsy culture), Shi et al. reported that both dbpA and dbpB were required to restore the infectivity of their dbpBA mutant [41]. Although the precise reason for this disparity is unknown at this time, there are several differences between the experimental approaches of these studies that might account for these differing results. First, it is possible that some intrinsic differences(s) in the bacterial strains utilized might account for the variation observed between these two studies. Specifically, as noted above, strain 297 was the parental strain used in the current study, and Shi et al. utilized a highly-transformable clone (5A13) of strain B31 that lacks both lp56 and the virulence-associated plasmid lp25 as the background for their mutagenesis experiments [30,41]. Second, the disparity in our results might be due to the use of different strains of mice in these two studies; C3H/HeJ mice were used in our study, whereas Shi et al used BALB/c [41]. This may be relevant because numerous studies have reported that experimental infection of C3H strains of mice with B. burgdorferi results in significantly different disease pathologies [42,43], higher spirochetal loads in multiple tissues [44], and different cytokine responses [45,46] by comparison to similarly infected BALB/c mice. Although, it is difficult to precisely predict how the reported differences in pathogenesis observed in these two distinct murine backgrounds might be impacted by DbpA and/or DbpB during infection, it is reasonable to suspect that these differences might account for some of the disparity in the results of complementation experiments obtained in the aforementioned studies.

Even though DbpA was required for full infectivity by Bb297 in mice when introduced by needle inoculation (intradermally), neither DbpA, nor DbpB, was required for infection of larval I. scapularis ticks or for transmission of BbKH500 from infected ticks to naïve mice. That DbpA was not required for uptake of the spirochetes by larval ticks was not surprising due to our earlier work demonstrating that DbpA is not expressed by B. burgdorferi harbored within the tick vector [17]. However, based on the data we obtained from needle inoculation of mice with BbKH500, it was surprising that as few as five nymphal ticks could transmit BbKH500. This is especially surprising because semi-quantitative analysis of the total number of spirochetes observed in the midguts of unfed ticks infected with BbKH500 showed a lower spirochete density (approx. 1/field) by comparison to number of bacteria present in the dissected midguts of the Bb297-infected ticks (20–30/field). While these data might suggest a possible defect in either the efficiency of acquisition of the dbpBA mutant or the capacity of this mutant to persist in the tick midgut, there were still sufficient numbers of the mutant spirochetes in these ticks to infect naïve mice. Since it is not known precisely how many spirochetes are transmitted by ticks during the feeding process [47], it was impossible to directly compare the number of B. burgdorferi transmitted by tick bite to our needle-challenge experiments. Spirochete numbers within salivary glands during tick feeding have been estimated between 20 [47] and 61 spirochetes [48] per salivary gland pair. These data suggest that an individual tick deposits far fewer than 10⁴ spirochetes during the feeding process, and our results have shown that by the needle-inoculation route, 10⁴ BbKH500 are not infectious.

In addition to the data presented by Fischer et al. [18], additional reports have attempted to address the role of DbpA, DbpB, and the ECM proteoglycan, decorin, with respect to the infectivity and pathogenesis of B. burgdorferi. Brown et al. described B. burgdorferi infection of decorin-deficient mice (Dcn⁻/⁻) [49]. Brown et al. [49] found that, by comparison to Dcn⁺/⁺ mice, Dcn⁻/⁻ mice challenged with a higher dose of B. burgdorferi (10⁴) had i) fewer infected joints, ii) a reduction in the severity of arthritis, but iii) no significant defect in colonization of the other tissues. Whereas 10⁴ wild-type B. burgdorferi were infectious for Dcn⁻/⁻ mice in the studies by Brown et al. [49], the Bb297 dbpBA-mutant was unable to infect mice at this dose. When considered together, these data support the hypothesis that DbpA, in addition to binding decorin, may have an additional ligand(s) or has another function critical for infectivity. At the present time, the only other known ligand recognized by DbpA is the GAG dermatan sulfate [18], but the contribution of this interaction to the pathogenesis of B. burgdorferi remains to be elucidated.

Conclusion

Despite the disparities between the results of the complementation experiments described in the current study and those obtained by Shi et al. [41], the overall results of our needle-inoculation experiments are in agreement with the most recent conclusion of Shi et al. that mutation of dbpBA results in significant attenuation of B. burgdorferi in the murine model of Lyme borreliosis. However, the observation that the dbpBA mutant showed a significant reduction in infectivity when the mice were needle inoculated is overshadowed by the finding that this same mutant was capable of infecting mice via tick challenge.
The fact that DbpA and DbpB are dispensable for infection via the tick-mediated route of infection suggests that *B. burgdorferi* transmitted via tick bite are in some way phenotypically different than their *in vitro*-cultivated counterparts, and/or that tick-derived salivary components, such as Salp15, may assist *B. burgdorferi* during the early infection process [50]. Taken together, these results emphasize the importance of characterizing the impact of a given gene in the infectious lifecycle of *B. burgdorferi* using the natural tick vector, as opposed to using only the artificial needle-challenge model.

**Methods**

**Bacterial strains and growth conditions**

Infectious, low-passage *B. burgdorferi* strain 297 (Bb297) [51] was used for these studies. Bacteria were cultivated *in vitro* in either Barbour-Stoenner-Kelley (BSK)-II [52] or BSK-H Incomplete medium (Sigma-Aldrich, St. Louis, MO) supplemented with 6% normal rabbit serum (Pel-Freeze Biologicals, Rogers, AR) at 35°C with 5% CO₂. When necessary, BSK media was supplemented with borrelia antibiotic mix (BAM; Sigma-Aldrich), 600 μg/ml kanamycin, or 700 μg/ml streptomycin.

**Generation of DbpBA deletion mutant in Bb297**

The *dbpBA*-deficient Bb297 strain was created by allelic exchange of the *dbpBA* operon with a kanamycin-resistance cassette, PflgB-kan [53], derived from pBSV2 [54]. The mutagenesis construct, pKHdbpBAko, was created by generating two PCR products that constituted the left and right flanking regions of the *dbpBA* operon which then were joined via an Ascl restriction site, thus deleting a significant portion of the *dbpBA* operon. Takara EX Taq polymerase (Takara Bio Inc., Shiga, Japan) and oligonucleotide primers ko1 and ko2 (Table 3) were used to amplify the left arm and ko3 and ko4 (Table 3) were used to amplify the right arm; primers ko2 and ko3 were modified such that Ascl restriction sites would be introduced into the "middle termini" of the two PCR fragments. The resulting PCR products then were digested with Ascl and ligated together. The linear ligated product was used as the template in a second PCR amplification containing the primers ko1 and ko4. The resulting PCR product, representing the joined flanking regions, was cloned into pGEM-T Easy vector (Promega Corp., Madison, WI) to generate pGEMT-dbpBA-AscI. The PflgB-kan cassette was from pJD55, a derivative of pJD44 in which the original aph [3′]-IIIa was replaced with the PflgB-kan cassette of pBSV2 [27,54]. In pJD55, the PflgB-kan cassette is flanked by Ascl sites which facilitated the cloning of the marker into the unique Ascl site within pGEMT-dbpBA-AscI to create pKHdbpBAko. Primers kan5' and kan3' were used with primers ko1 and ko4 to determine the orientation of the PflgB-kan cassette with respect to the *dbpBA* operon.

Table 3: Oligonucleotide primers used for cloning and PCR confirmation.

| Designation | Sequence |
|-------------|----------|
| ko1         | GGATCTTAAGAAATTTCAATTTTTT |
| ko2         | TATAGCGGCGCCCAATACGCGACCAAAT |
| ko3         | TATAGCGGCGCCCTGAGAGAATCTCCTCAACT |
| ko4         | TTTAGATCTAAGGTGATATAAAAATTGGCAGG |
| ko5         | AAACAAAGCTTAAAATCTCAACAGC |
| kan5'       | AGCCATATTCACAGGGAAGGCG |
| kan3'       | TTATCATACAGGATTATATAACC |
| vlsE-5'     | GATGCAGAGAAAGCAGCCTGGCAGGTTATG |
| vlsE-3'     | TATAAGCTTCTACGAAGAGTCTTATTAAACAGCAGTCTCAAC |
| BBE22-5'    | AAATTAATTTCTTTGTCAACCAAC |
| BBE22-3'    | TATATTAAGCTTCTTTGTGTCG |
| FlaB5'      | ATGATTATCAATCATATTACACAGCATATTA |
| FlaB3'      | TTATCTAAGCATAAACAAATATTGGG |
| comp1       | GGCTTCTTCTTTTATTTTAAGAGG |
| comp2       | CATATGGTCTTCTCTCTATTAAATTTAGTTAATTTAAAACTTACAAC |
| comp3       | AGATCTCATATTAAATGTAAATAAAACCTTT |
| comp4       | GCATGCGTTTTGGGATTTGCTTAAAC |
| comp5       | GTAGCTCCTCTTTTGCTTC |

* Ascl site underlined.
* Ndel site underlined.
* Sphi site underlined.
medium containing the appropriate concentration of antibiotic(s) and aliquoted in 96-well tissue culture plates (Corning, Lowell, MA). Transformants were recovered 7–21 days after plating from wells in which a red to orange color change of the medium was observed. The presence of viable spirochetes was confirmed visually by dark-field microscopy and clones expanded into BSK medium supplemented with kanamycin.

Transformants were verified as dbpBA-deficient mutants by diagnostic PCR using primers ko5 and ko4 (Table 3) followed by analytical restriction enzyme digestion of the PCR product with Ascl. DNA for PCR analysis was extracted from borreliae harvested from the expansion cultures. The PCR product generated from amplification of Bb297 DNA (wild-type) is of similar size to that generated by amplification of DNA from a dbpBA::PflgB-kan mutant, but the former lacks Ascl sites. Therefore, to verify that the dbpBA operon was replaced by the PflgB-kan cassette, PCR products from both Bb297 and mutant-derived were digested with Ascl prior to agarose-gel electrophoresis. The presence of the vlsE and pncA (BBE22) genes in kanamycin-resistant transformants was confirmed by PCR using vlsE-5’ and vlsE-3’ for vlsE amplification or BBE22-5’ and BBE22-3’ for BBE22 amplification; refer to Table 3 for sequence information. FlaB5’ and FlaB3’ primers, which amplified the flaB gene of B. burgdorferi, were used as a control for DNA integrity. A single dbpBA-deficient clone, BbKH500, that retained vlsE and pncA, was chosen for additional PCR-based analyses to compare the endogenous plasmid content of this clone to Bb297. The sequences of the primers utilized for plasmid profiling are provided in Table 4. Nine of the primer pairs have been previously described by Eggers et al. [55]. The remaining primers utilized are unique from those cited by Eggers et al. and were designed primarily based on sequence data from strain 297.

**Construction of shuttle plasmids for genetic complementation of BbKH500 with DbpA**

Complementation of DbpA in BbKH500 was achieved by transforming electrocompetent BbKH500 with the B. burgdorferi-shuttle plasmid, pKH2000. Because dbpB precedes dbpA in the native operon, it was first necessary to clone the promoter for the dbpBA operon directly in front of the dbpA gene from Bb297, thereby removing the dbpB gene. The dbpBA promoter region (Prom) was amplified by PCR using primers comp1 and comp2 and the dbpB open reading frame (ORF) was amplified using primer comp3 and comp4. To facilitate fusion of the Prom and dbpA open reading frame fragment, an NdeI restriction site was introduced into the 3’ end of oligonucleotide comp2 and the 5’ end of primer comp3. Following PCR amplification, the resulting PCR fragments were digested with NdeI and ligated together. A second PCR amplification was performed using the ligation product as the template and the oligonucleotides comp1 and comp4 for primers. The resultant PCR product was digested with BglII (a unique BglII site is located in the PCR product, 11 bp downstream of the 3’ end of the comp1 primer) and SphI (5’ end of comp4 primer) then cloned into the BglII and SphI sites of pJD51, a derivative of pJD44 [27], that contains the aadA gene encoding streptomycin resistance in B. burgdorferi [56], to create pKH2000. Electroporation of BbKH500 was performed as described above and transformants were selected in the presence of kanamycin and streptomycin. Antibiotic-resistant clones first were checked for the presence of the Prom-dbpa construct by PCR using primers ko5 and comp5 (the latter anneals near the middle of the dbpA coding strand); Bb297 = 1.2 kb product; BbKH500 = no product; Prom-dbpa = 535 bp product. Next, DbpA expression in clones identified by PCR confirmation was assessed by immunoblot analysis as described below. Clones that expressed DbpA from the Prom-dbpa construct were assessed for the presence of the vlsE and BBE22 genes by PCR amplification using the primers described above. One clone, BbKH501, was chosen for further characterization. PCR-based plasmid profiling was performed on BbKH501, as described above, to compare the plasmid content of this clone to BbKH500 and Bb297.

**Proteinase K digestion of B. burgdorferi**

Intact, motile borreliae were exposed to 200 μg of proteinase K (40 mg/ml; Fisher Scientific, Pittsburgh, PA) or were sham treated for 40 min at room temperature. To stop the reaction, 10 μl of phenylmethylsulfonyl fluoride (50 mg/ml in isopropanol; Sigma) was added to each sample and the bacteria prepared for SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis as described below.

**Immunoblot analysis**

*B. burgdorferi* whole-cell lysates were generated by washing the spirochetes with wash buffer (10 mM HEPES, 150 mM NaCl, pH 7.5) three times, incubating the cells in BugBuster plus Benzonase solution (Novagen, Madison, WI) overnight then adding an equal volume of 2× SDS-PAGE running buffer (Bio-Rad Laboratories, Hercules, CA) for a final concentration of 10⁷ bacteria/ml. Whole-cell lysates were separated via electrophoresis through 12.5% SDS-polyacrylamide gels (approximately 10⁷ spirochetes per lane) and transferred to nitrocellulose (0.45 μm; Bio-Rad Laboratories) for immunoblot analysis. Nitrocellulose membranes were probed with either 6B3-DbpA, a mouse monoclonal antibody that specifically recognizes DbpA, chicken anti-*B. burgdorferi* FlaB IgY, or rat anti-DpbB anti-sera. The monoclonal antibody, 6B3-DbpA, was produced in collaboration with the Antibody Production Core facility at UT Southwestern and the pol-
yclonal rat anti-DbpB antisera was described previously [16]. The chicken anti-FlaB antibody was produced in collaboration with Lampire Biological Laboratories (Pipersville, PA). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG), donkey anti-chicken IgY, or goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:10,000–1:30,000 for chemiluminescent detection. Immunoblots were developed using Immobilon Chemiluminescent Western HRP Substrate (Millipore, Billerica, MA) and exposed to X-ray film (Phenix Research Products, Hayward, CA).

**Infection of mice by needle inoculation**

Prior to use of the cultures in needle-inoculation experiments, the bacterial cell density in each culture was accurately determined by counting spirochetes in no fewer than 60 microscopic fields (400× magnification) using dark-field microscopy. Cultures exhibiting cell aggregation were not used for infections as the presence of clumps

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**Table 4: Oligonucleotide primers used for plasmid profiling.**

| Primer   | Sequence                  | Reference |
|----------|---------------------------|-----------|
| lp54 5'  | ATGAGCAAAAAAGTAATTTAATAT | [55]      |
| lp54 3'  | CACTAATTCTTGTATTTAATCTAT | [55]      |
| cp26 5'  | ATGCCTCAAAAAGTGAGATAAAAA | [55]      |
| cp26 3'  | TAGCTTAAATTTAAATTGTTGAT  | [55]      |
| cp9 5'   | ATGCAAATATATACATGCAAAAT  | [55]      |
| cp9 3'   | ATCTTCTCAGATATTTTATATA   | [55]      |
| lp17 5'  | GTGATACACCCAAAAGGTCATTTA | [55]      |
| lp17 3'  | CAAATAATGTGATATTTTTAAAGA | [55]      |
| lp25 5'  | AAATTAATTTTGTGCAAACCAAC |          |
| lp25 3'  | TATATTAAGCTCTTTTGGCTTGCG |          |
| lp28-1 5' | GATGCAGAAGGCTGTCGTCAGTTA | This study|
| lp28-1 3' | TATAAGGCTTCTATCAGAGCTCTTTATTTAAACGAGCAGTCTCAAC | This study|
| lp28-2 5' | ATGCGCTATTACATATTGCG   | [55]      |
| lp28-2 3' | AATCTTGGAAGACCTCTGACCTTTTA | [55]    |
| lp28-3 5' | CTGAAAATGAGAGAAGCGGGTGG  | [55]      |
| lp28-3 3' | TAGGCTAAATACCAATTGCTAACAAT | [55]    |
| lp28-4 5' | ATGAAATGATCATATAATTGCAAATCTA | [55]    |
| lp28-4 3' | AATCCGACAGATCTGTTGTCACAG | [55]      |
| lp38 5'  | ATGATTATTTCCAACAAACGCCC  | This study|
| lp38 3'  | ATGTGTTTCTTCTAATTGCTGCAATCCCATC | This study|
| lp36 5'  | TCCTTTCTCTCTGTTTGTTTGTTGGGTTTT | This study|
| lp36 3'  | ATGAAATGGAAATAATTGACATACAC | [55]      |
| lp5 3'   | AATATTAGGTGAAGATTATAATTATAATT | [55]    |
| lp21 5'  | TGGTTGTTCTTAAACCGACGCTT |          |
| lp21 3'  | TTGTGTTTCTAATGCTGCTGAATTTGCAAC | This study|
| Chrom 5' | GATTATCTAATATAGTATAATTGCT | [55]      |
| Chrom 3' | ATATATATATATATATATATATATAT | [55]      |
| cpi18-1 5' | AGGGGAAATGATTTAATGTAATACTTCA | This study|
| cpi18-1 3' | AGTTTTTCCAAAAATTTTGGCGAT | This study|
| cpi18-2 5' | TCAGAAAAGCATACCTACATGCAACATC | This study|
| cpi18-2 3' | AATAATCTACCTTTCTACGGGCTGATA | This study|
| cpi21-1 5' | GTTCTTCTACATACCAAGCAAGG | This study|
| cpi21-1 3' | GCCTCTCTTCTAATCCCTTATCA | This study|
| cpi22-2 5' | CAAGCGAGTTTATTCCCCTCTAA | This study|
| cpi22-2 3' | ATATATGATTTGCTCATTATTTGGAAT | This study|
| cpi23-3 5' | ACTTGCTAAGGAGCAGCTTCAATTAGAAT | This study|
| cpi23-3 3' | CTTAATACATTATCTGTTTTCGACTTATCA | This study|
| cpi24-5 5' | GTATAAATGCTTTTTGTTTAAACGACAC | This study|
| cpi24-5 3' | GAAACTCCTTCTCATTACCTTACATAC | This study|
| cpi25-3 5' | GCCTTATAAGGCAATAGTTTTAAGG | This study|
| cpi25-3 3' | AGATTTTCAAGCCGCTTCTACCAAAA | This study|
| cpi26-3 5' | GGTGCTTTAGACACAGAGATGTG | This study|
| cpi26-3 3' | GAACAAATTTCAAGTCTATTACCCCTTTTA | This study|
| cpi27-3 5' | GTCAAATTTAAGCTGTTTTAGGTAT | This study|
| cpi27-3 3' | TATTTACTAAATCTTATTTTCAAATTTTCA | This study|
Colonization of *Ixodes scapularis* larvae with *B. burgdorferi*

Female C3H/HeJ mice were needle inoculated as described above with either Bb297 (10^4 spirochetes) or BbKH500 (100 μl of a post-exponential growth phase culture; 10^6-10^7 spirochetes). Infection of mice was confirmed by ear-punch biopsy culture; 4- and 8-weeks post-inoculation for Bb297 and BbKH500, respectively. Naïve, pathogen-free *I. scapularis* larvae, obtained from the Department of Entomology and Plant Pathology at Oklahoma State University (Stillwater, OK), were allowed to feed to repletion on the infected mouse individually housed in cages with raised wire-bottoms above water to facilitate recovery of the ticks. Fed larvae were collected and washed sequentially with 70% ethanol, deionized water (dH2O), 1× Fungizone (Gemini Bio-Products, West Sacramento, CA), and dH2O before placing them in 100% cotton fabric-lined Petri dish molting chambers for storage until they molted to the nymphal stage. The molting chambers containing the ticks were housed in a humidified chamber (97–98% humidity) containing saturated potassium sulfate solution at 20°C with a 16 h light, 8 h dark cycle. Unfed nymphs were collected and stored in autoclaved glass vials containing approximately 1 cm of sand. The vials were closed with vented lids and the ticks housed as described above.

Direct immunofluorescence assay (IFA) on B. burgdorferi-infected ticks

Prior to placement of unfed (flat) nymphs on naïve mice, five nymphs from each of two individual mice were dissected on silylated slides (CEL Associates, Inc., Pearland, TX) in 50 μl phosphate-buffered saline containing 10 mM MgCl₂. At the time testing was performed, approximately 11 weeks had elapsed since these ticks had fed to repletion as larvae. Midgut tissues were extracted from the ticks, allowed to dry on the slides, blocked with Tris-buffered saline with 0.1% Tween-20, then probed with a FITC-conjugated rabbit anti-*B. burgdorferi* antibody (Fitzgerald Industries International, Inc., Concord, MA) as described previously.

Transmission of *B. burgdorferi* from infected nymphal ticks to naïve mice

To assess the transmissibility of strain Bb297 and BbKH500 from flat nymphs to naïve mice, either five or ten ticks were allowed to feed to repletion on three- to five-week-old female C3H/HeJ mice. At three-weeks post-infestation, ear-punch biopsies were harvested from each mouse and cultured in BSK-H medium without antibiotics to determine infection status. Borreliae from the ear-punch biopsy cultures were transferred to BSK-H medium supplemented with kanamycin and streptomycin to confirm their antibiotic-resistance phenotypes.

Statistical analysis

Statistical analysis was performed with assistance from the UT Southwestern Clinical Sciences Department. Both chi-square and Fisher’s exact test were applied to the mouse infection data in pair-wise comparisons between experimental groups.

Authors’ contributions

JSB and KEH performed experiments and analyzed results. JSB, KEH, and MVN participated in experimental designs and co-wrote the manuscript. All authors read and approved the manuscript.

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