A novel laminin-binding protein mediates microbial-endothelial cell interactions and facilitates dissemination of Lyme disease pathogens

Sandhya Bista[1*], Preeti Singh[1*], Quentin Bernard[1], Xiuli Yang[1], Thomas Hart[2,3], Yi-Pin Lin[2,4], Chrysoula Kitsou[1], Vipin Singh Rana[1], Fuming Zhang[5], Robert J. Linhardt [5,6,7], Kai Zhnag[8], Darrin R. Akins[9], Lucy Hritzo[10], Yuri Kim[10], Dennis J. Grab[10], J. Stephen Dumler [10], and Utpal Pal[1, 11 *]

[1] Department of Veterinary Medicine, University of Maryland, College Park, MD

[2]Department of Biological Science, State University of New York at Albany, Albany, NY

[3]Division of Infectious Diseases, Wadsworth Center New York State Department of Health, Albany, NY

[4]Department of Biomedical Science, State University of New York at Albany, Albany, NY

[5]Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY

[6]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY

[7]Departments of Biology and Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY

[8]Oral and Craniofacial Molecular Biology, School of Dentistry, Virginia Commonwealth University, Richmond, VA

[9] Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

[10] Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD

[11] Virginia-Maryland College of Veterinary Medicine, College Park, MD

* Equal contribution

Running Title: BB0406 supports spirochete dissemination through endothelial barriers

Corresponding author:
Utpal Pal, Department of Veterinary Medicine, University of Maryland, College Park, MD, USA
(upal@umd.edu)

NOTE

Acknowledgements

The authors are thankful to Kathryn Nassar for assistance with the preparation of this manuscript. The opinions expressed herein are those of the author(s) and are not necessarily representative of those of the Uniformed Services University of the Health Sciences (USUHS), the Department of Defense (DOD), or the United States Army, Navy, or Air Force.

Financial support

This research was supported by the National Institute of Allergy and Infectious Diseases (NIAID) Award Numbers R01AI080615, AI116620 and AI138949 and AI078958. C.K. is the recipient of the Deborah and Mark Blackman Postdoctoral Fellowship from Global Lyme Alliance.

Potential conflicts of interest

No reported conflicts.

Abstract

Borrelia burgdorferi conserved gene-products BB0406 and BB0405, members of a common *B. burgdorferi* paralogous gene family, share 59% similarity. While both gene-products can function as potential porins, only BB0405 is essential for infection. Here we show that, despite sequence homology and co-expression from the same operon, both proteins differ in their membrane localization attributes, antibody accessibility, and immunogenicity in mice. BB0406 is required for spirochete persistence in mammalian hosts, particularly for the disseminated infection in distant organs. We identified that BB0406 interacts with laminin, one of the major constituents of the vascular basement membrane, and facilitates spirochete transmigration across host endothelial cell barriers. A better understanding of how *B. burgdorferi* transmigrates through dermal and tissue vascular barriers and establishes disseminated infections will contribute to the development of novel therapeutics to combat early infection.

Keywords: *Borrelia burgdorferi,* BB0406, pathogen dissemination, laminin

Introduction

Lyme disease is a common vector-borne disease in North America and Europe that is inflicted by a group of atypical bacterial pathogens, *Borrelia burgdorferi* sensu lato [1-3]. The microbe is maintained in a complex enzootic infection cycle that involves *Ixodes* ticks and a vertebrate reservoir host. During blood meal engorgement, a tick infected with *B. burgdorferi* can transmit the pathogen to the host dermis. The spirochetes multiply at the tick-bite site and subsequently migrate to distant organs. After the initial infection of the dermal inoculation site, it remains unknown as to precisely how a fraction of spirochetes can invade the vasculature and then extravasate from the vessel in order to colonize distant organs. The colonization of the host organs, like the joints, heart, and central nervous system, can result in an array of serious clinical manifestations, such as Lyme arthritis, carditis, and neuroborreliosis [4]. Antibiotic treatment is usually effective when administered early during infection, although a subset of antibiotic-treated hosts can later experience a variable set of clinical symptoms, known as post-treatment Lyme disease syndrome (PTLDS) [5, 6]. The etiology, pathogenesis, and treatment of PTLDS remain unknown. Currently, a vaccine to prevent Lyme disease in humans is unavailable. Therefore, a better understanding of the infection process of *B. burgdorferi,* especially how the bacteria evade immune responses at the dermal inoculation site and disseminate through host vasculature, is fundamental to the development of new intervention strategies against the infection.

Hematogenous dissemination of a blood-borne pathogen is a critical initial process that remains poorly understood. The dissemination of spirochetes in the mammalian host requires temporal regulation of several virulence determinants, particularly surface proteins and adhesins,

which can interact with host ligands like extracellular matrix molecules (ECMs), aiding in *B. burgdorferi*-vascular system interactions, transmigration, and tissue colonization [7]. For example, *B. burgdorferi* BBK32 interacts with host fibronectin (Fn) and glycosaminoglycan (GAG) molecules, facilitating borrelial adhesion to vasculature via stabilization of the bacterial-endothelial interaction [8-11]. Similarly, an outer membrane (OM) surface protein, P66, has both porin and adhesin functions [12, 13], particularly as an integrin binding protein, and has demonstrated its ability to promote spirochete extravasation [14]. Further studies are required to identify *B. burgdorferi* OM proteins that promote vascular interactions and transmigration, which will assist in a better understanding of spirochete dissemination events, pathogenesis, and the development of preventive strategies.

The *B. burgdorferi* BB0406 gene-product is co-transcribed with two immediately upstream genes that encode BB0404 and BB0405 [15, 16]. The latter gene is recently characterized as an immune-invisible transmembrane protein that facilitates spirochete infection in mammals [15]. BB0406 and BB0405 share 59% similarity, are grouped into the same paralogous gene family, and are highly conserved in Lyme disease pathogens. Both BB0405 and BB0406 are capable of forming pores in large unilamellar vesicles, suggesting their potential functions as porins [16]. Here we report that BB0406 is a laminin-binding protein, which facilitates spirochete transmigration through the host endothelial barrier, thereby aiding the spirochete dissemination and survival in various distant organs in mammals.

Materials and Methods

Bacteria, mice, and ticks

B. burgdorferi isolates B31-A3, 297, and N40, and *B. garinii* isolate PBi, were grown in Barbour-Stoenner-Kelly-H (BSK-H) medium [15]. Four- to six-week-old C3H/HeN mice were purchased from the National Institutes of Health and Charles River Laboratories. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Biosafety Committee. The *Ixodes scapularis* ticks used in this study originated from a colony that is maintained in the laboratory.

Polymerase chain reaction

The oligonucleotide sequences for PCR primers are listed in Table S1. For gene expression analysis, the C3H mice (3 animals/group) were infected with a subcutaneous inoculum of *B. burgdorferi* (10^5 cells/mouse), and 14 days after infection, tissues from the skin, joints, heart, and bladder were collected. *B. burgdorferi*-infected mice were also parasitized by larval ticks (25 ticks/mouse) and collected after repletion. After larval molting, nymphs were allowed to engorge on naïve mice (25 ticks/animal) and were collected at 24, 48, and 96 hours after attachment. Total RNA was isolated from various tissues using TRIzol (Invitrogen), reverse transcribed to complementary DNA (cDNA), and treated with DNase (NEB). The relative abundances of the *bb0406* transcripts, normalized against *flaB* copies, were analyzed by quantitative PCR (qPCR) analysis using cDNA samples, as detailed [15]. The spirochete burdens in the ticks and murine tissues were assessed by measuring copies of the *flaB* transcripts,

using cDNA samples, through qPCR and normalizing against the corresponding tick or mouse *β*-actin levels, respectively, as detailed [15].

Generation of recombinant BB0406, antiserum, and immunoblotting

The *bb0406* gene was cloned into pET28a using specific primers (Table S1), and the recombinant protein without the N-terminal leader sequence was produced in *Escherichia coli*. Expression and purification were performed as described previously [15]. BB0406 antisera were generated in mice, and the titer and specificity were assessed using ELISA and Western blotting analysis, respectively, as described [15].

Generation of *bb0406* deletion mutants

The *bb0406* mutants were generated via homologous recombination by replacing BB0406 ORF with a kanamycin resistance (kanAn) cassette, as detailed [17]. The upstream and downstream DNA fragments of BB0406 were cloned into plasmid pXLF10601 and electroporated into *B. burgdorferi*. Transformants were selected with kanamycin (350 μg/mL) and analyzed for the desired integration of the kanAn cassette, loss of *bb0406* transcripts, and absence of polar effects on the transcription of the surrounding genes. Two *bb0406* deletion clones (designated as Mut1 and Mut2), retaining identical plasmids as wild-type isolates (except for non-essential plasmid lp 5), were used for further experiments. For *in vitro* growth analysis, spirochetes were diluted to a density of 10^5 cells/mL, grown until stationary phase (approximately 10^8 cells/mL), and counted by dark-field microscopy using a Petroff-Hausser cell counter.

Phenotypic analysis of genetically modified *bb0406* isolates

To examine the phenotypes of *bb0406* mutants, groups of mice (3 animals/group) were infected with either wild type or two *bb0406* mutant clones (Mut1 and Mut2) at a dose of 10^5 spirochetes per animal by intradermal needle inoculation. Blood, skin, joint, heart, and bladder samples from mice were collected at 7, 14, and 21 days after infection. Pathogen burdens in murine tissues were evaluated using qPCR. Portions of skin and spleen from the mice were cultured in BSK media to recover viable *B. burgdorferi*. For studies addressing the acquisition of spirochetes in ticks, mice were infected with borrelial isolates and, after 12 days, infested with naïve ticks (25 ticks/group). Ticks were collected after repletion, and pathogen burdens were assessed using qPCR. For transmission studies, naturally-infected nymphs or nymphs microinjected with *B. burgdorferi* isolates were allowed to feed on naïve mice (5 ticks/mouse, 3 mice/group). Engorged ticks were subjected to qPCR analyses of their spirochete levels. Mice were sacrificed 12 days after tick feeding, and tissues were isolated and assessed for spirochete burdens by qPCR and culture analysis.

For the intravenous injection study, groups of mice (3 animals/group) were intravenously infected with borrelial isolates (10^5 spirochetes/mouse). The pathogen burdens in the bloodstream and tissues were determined on day 14 of infection using qPCR. For the hyper-infection study, groups of mice (3 animals/group) were infected intradermally with wild type (10^5 spirochetes/mouse) or *bb0406* Mut1 (10^8 spirochetes/mouse). The spirochete burdens were assessed at the injection site and in murine tissues on day 14 of infection using qPCR.

Motility and bactericidal assays

Spirochete motility was evaluated by swimming plate assays, as previously described [18]. The diameters of the swimming rings reflected by mutants were recorded in millimeters and compared to the corresponding wild-type and a non-motile *flaB* mutant [19] as controls. BB0406 antibodies were tested for bactericidal activities against *B. burgdorferi* using a re-growth assay, as described [20].

THBMEC and ECIS assays

To test the effects of *B. burgdorferi* on the integrity of vascular barriers, we utilized an *in vitro* model of the human blood-brain barrier (BBB)[21-23] based on transformed human brain microvascular endothelial cell (THBMEC) monolayers and Electric Cell-substrate Impedance Sensing (ECIS) from Applied Biophysics Inc. (Troy, NY). About 10^5 THBMECs were grown in M199 media, as detailed [22], seeded in gold microelectrode arrays (8W10E+, Applied Biophysics), and exposed to 1-2 x 10^6 wild type *B. burgdorferi* or mutants at MOI of 1: 5-10. The resistance changes of THBMEC monolayers were continuously monitored for 24h and normalized to control M199-treated cells.

Transwell assay

The assay utilized an *in vitro* model of the human BBB represented by THBMECs grown on Transwell plates (Costar, Corning, NY) with permeable membrane inserts, as previously described [21]. After 24-36 hours of THBMEC growth, when the cellular resistance reached maximal steady-state values, 5-10 x 10^5 spirochete cells were added to the inserts at 5-10 MOI and incubated for 24-48 hours. Spirochetes that crossed the THBMEC monolayers were

collected from the bottom chambers, centrifuged, and used for qPCR to quantify transmigrated *B. burgdorferi* as a function of the total input.

Statistical Analysis

Data are presented as means and standard errors of the means (SEM) of at least two independent experiments. Significant differences between samples were determined using the Student's two-tailed *t*-test or one-way analysis of variance (ANOVA) test using GraphPad Prism 5.01, following logarithmic transformation of the data. A P-value of < 0.05 was considered significant.

Results

bb0406 encodes a weakly immunogenic antigen predominantly expressed during early murine infection

The *bb0406* mRNA is detectable in several borrelial isolates and strains in culture (Figure 1A, upper panel), although the protein remains nearly undetectable (Figure 1A, lower panel). Expression of *bb0406* is induced at 37°C in cultured cells (Figure 1B, left panel) and in ticks during spirochete transmission, but the transcripts are also detectable in various murine tissues (Figure 1B, right panel), with the most dramatic expression during early infection (Figure 1C). A low but noticeable antibody response against BB0406 is detectable in infected mice (Figure 1D), suggesting a poor abundance, or a weak immunogenicity and subsurface nature, of the antigen [24].

BB0406 supports *B. burgdorferi* dissemination and survival in mammalian hosts

To study the role of BB0406 in *B. burgdorferi* virulence, we generated *bb0406*-deficient *B. burgdorferi,* defined by a loss in expression of the target gene, but with no polar effects on the surrounding genes, including *bb0405* and *bb0407* (Figure 2A-C, Figure S1). Despite repeated attempts, we were unable to complement the gene; therefore, for all subsequent studies, we used two independent clones of isogenic *bb0406* mutants (Figure S2). The *bb0406* mutants did not reflect apparent growth defects (Figure S1, panel D).

Next, mice were infected with equal numbers (10^5 cells/animal) of wild type or *bb0406* mutant isolates (two independent clones, Mut1 or Mut2), via needle inoculation. Similar to wild-type cells, the *bb0406* mutants survived at the skin inoculation site until day 7, but their levels

decreased at day 14, both at the skin inoculation site (Figure 2A, upper panel) and at distant skin or other organs (Figure 2A, lower panel). We noted that the wild type and *bb0406* mutants induced comparable antibody responses, further supporting mutant survival during early infection (Figure S3). In agreement with a previous study [16], we found that anti-BB0406 antibodies have bactericidal activities (Figure S3). The detection of low levels of *bb0406* mutants in distant tissues could be due to their impaired ability to disseminate through blood (Figure 2B). Consequently, the survival defect of the *bb0406* mutants in mice also affected their entry or acquisition in ticks (Figure 2C). Similar to needle-borne infections (Figure 2A), *bb0406* mutants were also unable to establish tick-transmitted infections, although ticks that were artificially infected with equal numbers of either the wild type or *bb0406* mutant displayed similar spirochete levels (Figure S4, panel A). However, despite a robust antibody response comparable to mice infested with wild-type spirochetes (Figure S4, panel B), a significantly lower level of *bb0406* mutants was detected in mice after 14 days of tick engorgement (Figure 2D). Histological analyses of the tick-bite sites show no obvious differences in the migration of immune cells, suggesting that the function of BB0406 is unlikely to be related to evasion of the host's innate immunity (Figure S5). Consistent with low pathogen levels, none of the organs retrieved from mice infected with *bb0406* mutants were positive for *B. burgdorferi*, as measured by cultures using skin and spleen tissues (data not shown). Collectively, these results indicate that BB0406 is important for the establishment of spirochete infections in mice, including their hematogenous dissemination.

BB0406 assists spirochete dissemination through murine vasculature

As previous studies indicated that a greater mutant inoculum could rescue the phenotypic defects in tissue survival [25], we performed an infection experiment with a higher level of inoculum with *bb0406* mutants. Both mutant clones were severely impaired in their abilities to establish infection in disseminated tissues when mice were intradermally injected a 1000-times higher dose of the mutant clones (10^8 cells/animal), compared to the wild type (10^5 cells/animal) (Figure 3A and Figure 3B). We next assessed whether the tissue dissemination defect of *bb0406* mutants could be restored when they are directly introduced in the blood. Equal levels of mutants or wild-type isolates were introduced into murine blood vessels by intravenous injection. While similar spirochete levels were detected in the blood (Figure 3C), the burden of *bb0406* mutants in distant murine organs was significantly lower, as compared to the wild type, indicating that BB0406 might assist in pathogen dissemination from blood to tissues (Figure 3D). These results strongly suggest that BB0406 is not needed for spirochete survival in the blood, but that it may facilitate pathogen transmigration from the blood vessels. The deficiency in the migration of *bb0406* mutants is not due to spirochete motility or chemotaxis defects, as suggested by a swarm plate assay (Figure S6).

Deletion of *bb0406* impairs *B. burgdorferi* dissemination through human endothelial cell layers

We next used an *in vitro* model to directly test the hypothesis of whether BB0406 facilitates *B. burgdorferi* transmigration through endothelial barriers. We examined the spirochete transmigration through an endothelial barrier consisting of immortalized transformed human brain microvascular endothelial cells (THBMECs), which share characteristics of primary microvascular endothelial cells. The results indicate that both *bb0406* mutant clones induced a

markedly different THBMEC resistance, as compared to wild-type *B. burgdorferi* (Figure 4A), and are also impaired in their abilities to migrate through the endothelial barrier (Figure 4B), further suggesting that BB0406 facilitates pathogen dissemination through host vasculature.

BB0406 is a laminin-binding protein

We next examined the possibility that BB0406 may interact with extracellular matrix proteins (ECMs), particularly those that are associated with the host vasculature. To explore this, we tested recombinant BB0406 and another control membrane protein for their abilities to bind fibronectin, type I collagen, type IV collagen, elastin, laminin, heparan sulfate, chondroitin-4-sulfate, dermatan sulfate, and chondroitin-6-sulfate (and BSA, as a control), using a microtiter assay. The results indicate that BB0406 predominantly interacts with laminin, although a minor binding was also observed with heparan sulfate (Figure 5A). The binding affinity between BB0406 and laminin was evaluated (Figure 5B) and further confirmed by an independent surface plasmon resonance assay (Figure 5C). We next examined whether the BB0406 deficiency results in the impairment of the spirochete binding to laminin. To explore this, the wild type or *bb0406* mutant clones were separately incubated with laminin-coated microtiter wells, and bound cells were measured. Significantly decreased bindings of both clones (Mut1 and Mut2) to laminin were recorded (Figure 5D)

Discussion

The *B. burgdorferi* outer membrane (OM) protein BB0406 is co-transcribed with another OM protein, BB0405, with 59% similarity [15, 16]. While both proteins are capable of forming a membrane pore [16], only BB0405 was shown to support spirochete infection in mammals [15, 16]. In contrast to the prevailing notion that BB0406 has a redundant role in infection [16], we show that the protein facilitates spirochete survival in host tissues, possibly assisting in hematogenous dissemination of the pathogen via interaction with laminin, which is the major functional component of the basement membrane surrounding blood vasculature [26]. As previous studies [16] and our current data show that the anti-BB0406 antibodies also have bactericidal properties, interference with the function of BB0406 or the disruption of its interaction with laminin may lead to novel strategies to combat *B. burgdorferi* infection.

Our data suggesting that BB0406 is expressed at low levels *in vitro* is supported by previous mass spectrometry studies [27], which show that the protein remained undetectable in the OM of *B. burgdorferi*. Therefore, the bactericidal activity of anti-BB0406 antibodies against cultured spirochetes [16] is puzzling and may occur via the steric hindrance of antibodies which may bind to off-target yet homologous proteins, like BB0405. Notably, despite being a subsurface protein [24], BB0406 induces a detectable antibody response during murine infection (Figure 1D), while the highly antigenic and surface-exposed protein BB0405 failed to generate such responses. This differential development of host antibodies may be the result of differences in the posttranscriptional or posttranslational regulation of BB0405 and/or BB0406. In any case, either BB0405 [15, 16] or BB0406 is independently required for murine infectivity, especially at

the disseminated phase of infection. Although a redundant role of BB0406 in murine infection was proposed [16], a different mutagenesis strategy was used, which likely contributed to disparate phenotypic outcomes. While our study created a cleaner mutant via the deletion of a single targeted gene (BB0406), we were unable to complement the isolate; therefore, we used two independent mutant clones to analyze the phenotype. In contrast, the published study created a dual mutant by ablating the expression of two genes (both BB0405 and BB0406) and then genetically complementing either gene to generate two independent isolates that re-expressed either BB0405 or BB0406. Of note, native BB0406 displays a spectacular differential expression *in vivo* that varies both spatially (Figure 1B) and temporally (Figure 1C). As Shrestha et al. [16] used a constitutively active borrelial promoter (*flaB*) that overexpressed *bb0406*, it is possible that an abnormally regulated gene-product may impact the normal physiology of the organism, therefore affecting its virulence in mice. In either case, the outcomes of both studies highlighted that both BB0405 and BB0406 are expressed and maintained in *B. burgdorferi*, and thus are likely to have distinct or overlapping functions. Both genes, which share substantial similarity, have variable but detectable expression in ticks and mammalian hosts. Therefore, it is tempting to speculate that these gene-products may perform similar functions at different stages of the spirochete life cycle using conserved regions, and/or that they perform distinctly different functions in spirochete infectivity.

The vascular system is the main route of dissemination for tick-borne pathogens like *B. burgdorferi,* which colonizes several internal organs in mammals. Such hematogenous dissemination is a multi-step process including adhesion, tethering, dragging, stationary adhesion, and extravasation [28]; BB0406 is likely to be involved in one or multiple of these

processes. The BB0406-laminin interaction could be critical for the first step of bacterial adhesion to the basement membrane of the blood vessel. As one of the major structural and functional constituents of the vascular basement membrane, laminin's binding to BB0406 could compromise the integrity of the vessel wall, thereby facilitating pathogen passage through the endothelial barrier. Considering that laminin is involved in interactions with many other ECM proteins, including integrins and collagens [29, 30], its binding to spirochetes (via BB0406) could disrupt a plethora of its other functions that are relevant to cell attachment, differentiation, and movement. On the other hand, *B. burgdorferi* is known to produce additional laminin-binding proteins, such as the outer surface protein ErpX and several members of the basic membrane proteins [31, 32]. It remains unknown whether these spirochete proteins carry functional redundancy and bind similar laminin proteins in distinct mammalian hosts, or if each *B. burgdorferi* protein has a specific target laminin. The latter remains a high possibility, as there are at least 15 isoforms of laminin that exist in the basement membranes of the vessel walls of arterioles, capillaries, postcapillary venules, and venules, which display enormous variability in structure and expression [33].

In addition to the BB0406-laminin interaction, there are other OM proteins known to support spirochete dissemination through host vasculature. For example, BBK32, a spirochete adhesin that interacts with multiple host ECM molecules like fibronectin and glycosaminoglycans [8, 10, 11, 34], is involved in the early steps of *B. burgdorferi* endothelial adhesion and interactions with the host microvasculature [35]. In addition, the OM protein P66, which is an integrin-binding borrelial porin, was recently reported to assist in spirochete and vascular interactions, vascular transmigration, and extravasation, via P66-integrin interactions

[14]. There are many other bacterial porin and transporter proteins which are known to bind specific ECM proteins that promote microbial adherence [36]. In fact, the structures, surface locations, and abundances of bacterial porin and transporter proteins have likely contributed to their evolution as multifunctional proteins that ultimately support bacterial virulence. Future investigations into these intriguing mechanisms – in particular, how specific OM proteins, like BB0406, support the intravasation and extravasation of pathogens – will greatly enrich our knowledge of the biology of tick-borne infections and will inform the development of effective measures to control these diseases.

References

1. Mead PS. Epidemiology of Lyme disease. Infect Dis Clin North Am **2015**; 29:187-210.
2. Radolf JD, Caimano MJ, Stevenson B, Hu LT. Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. Nat Rev Microbiol **2012**; 10:87-99.
3. Steere AC, Coburn J, Glickstein L. The emergence of Lyme disease. J Clin Invest **2004**; 113:1093-101.
4. Steere AC, Strle F, Wormser GP, et al. Lyme borreliosis. Nat Rev Dis Primers **2016**; 2:16090.
5. Aucott JN, Crowder LA, Kortte KB. Development of a foundation for a case definition of post-treatment Lyme disease syndrome. Int J Infect Dis **2013**; 17:e443-9.
6. Marques A. Chronic Lyme disease: a review. Infect Dis Clin North Am **2008**; 22:341-60, vii-viii.
7. Caine JA, Coburn J. Multifunctional and Redundant Roles of Borrelia burgdorferi Outer Surface Proteins in Tissue Adhesion, Colonization, and Complement Evasion. Front Immunol **2016**; 7:442.
8. Fischer JR, LeBlanc KT, Leong JM. Fibronectin binding protein BBK32 of the Lyme disease spirochete promotes bacterial attachment to glycosaminoglycans. Infect Immun **2006**; 74:435-41.
9. Hyde JA, Weening EH, Chang M, et al. Bioluminescent imaging of *Borrelia burgdorferi* in vivo demonstrates that the fibronectin-binding protein BBK32 is required for optimal infectivity. Mol Microbiol **2011**; 82:99-113.
10. Lin YP, Chen Q, Ritchie JA, et al. Glycosaminoglycan binding by *Borrelia burgdorferi* adhesin BBK32 specifically and uniquely promotes joint colonization. Cell Microbiol **2014**.
11. Probert WS, Johnson BJ. Identification of a 47 kDa fibronectin-binding protein expressed by *Borrelia burgdorferi* isolate B31. Mol Microbiol **1998**; 30:1003-15.
12. Coburn J, Cugini C. Targeted mutation of the outer membrane protein P66 disrupts attachment of the Lyme disease agent, *Borrelia burgdorferi*, to integrin alpha-v-beta-3. Proc Natl Acad Sci U S A **2003**; 100:7301-6.
13. Skare JT, Mirzabekov TA, Shang ES, et al. The Oms66 (p66) protein is a *Borrelia burgdorferi* porin. Infect Immun **1997**; 65:3654-61.
14. Kumar D, Ristow LC, Shi M, et al. Intravital Imaging of Vascular Transmigration by the Lyme Spirochete: Requirement for the Integrin Binding Residues of the *B. burgdorferi* P66 Protein. PLoS Pathog **2015**; 11:e1005333.
15. Kung F, Kaur S, Smith AA, et al. A *Borrelia burgdorferi* Surface-Exposed Transmembrane Protein Lacking Detectable Immune Responses Supports Pathogen Persistence and Constitutes a Vaccine Target. J Infect Dis **2016**; 213:1786-95.
16. Shrestha B, Kenedy MR, Akins DR. Outer Membrane Proteins BB0405 and BB0406 Are Immunogenic, but Only BB0405 Is Required for *Borrelia burgdorferi* Infection. Infect Immun **2017**; 85.
17. Yang X, Coleman AS, Anguita J, Pal U. A chromosomally encoded virulence factor protects the Lyme disease pathogen against host-adaptive immunity. PLoS Pathog **2009**; 5:e1000326.
18. Li C, Wolgemuth CW, Marko M, Morgan DG, Charon NW. Genetic analysis of spirochete flagellin proteins and their involvement in motility, filament assembly, and flagellar morphology. J Bacteriol **2008**; 190:5607-15.
19. 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.

20. Yang X, Lenhart TR, Kariu T, Anguita J, Akins DR, Pal U. Characterization of unique regions of *Borrelia burgdorferi* surface-located membrane protein 1. Infect Immun **2010**; 78:4477-87.
21. Grab DJ, Nikolskaia O, Kim YV, et al. African trypanosome interactions with an in vitro model of the human blood-brain barrier. J Parasitol **2004**; 90:970-9.
22. Grab DJ, Nyarko E, Nikolskaia OV, Kim YV, Dumler JS. Human brain microvascular endothelial cell traversal by *Borrelia burgdorferi* requires calcium signaling. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases **2009**; 15:422-6.
23. Siddharthan V, Kim YV, Liu S, Kim KS. Human astrocytes/astrocyte-conditioned medium and shear stress enhance the barrier properties of human brain microvascular endothelial cells. Brain research **2007**; 1147:39-50.
24. Kenedy MR, Scott EJ, 2nd, Shrestha B, et al. Consensus computational network analysis for identifying candidate outer membrane proteins from *Borrelia* spirochetes. BMC Microbiol **2016**; 16:141.
25. Yang X, Qin J, Promnares K, Kariu T, Anderson JF, Pal U. Novel microbial virulence factor triggers murine lyme arthritis. J Infect Dis **2013**; 207:907-18.
26. Yousif LF, Di Russo J, Sorokin L. Laminin isoforms in endothelial and perivascular basement membranes. Cell Adh Migr **2013**; 7:101-10.
27. Yang X, Promnares K, Qin J, et al. Characterization of Multiprotein Complexes of the *Borrelia burgdorferi* Outer Membrane Vesicles. J Proteome Res **2011**; 10:4556-66.
28. Norman MU, Moriarty TJ, Dresser AR, Millen B, Kubes P, Chaconas G. Molecular mechanisms involved in vascular interactions of the Lyme disease pathogen in a living host. PLoS Pathog **2008**; 4:e1000169.
29. Nugroho RWN, Harjumaki R, Zhang X, et al. Quantifying the interactions between biomimetic biomaterials - collagen I, collagen IV, laminin 521 and cellulose nanofibrils - by colloidal probe microscopy. Colloids Surf B Biointerfaces **2019**; 173:571-80.
30. Venstrom K, Reichardt L. Beta 8 integrins mediate interactions of chick sensory neurons with laminin-1, collagen IV, and fibronectin. Mol Biol Cell **1995**; 6:419-31.
31. Brissette CA, Verma A, Bowman A, Cooley AE, Stevenson B. The *Borrelia burgdorferi* outer-surface protein ErpX binds mammalian laminin. Microbiology **2009**; 155:863-72.
32. Verma A, Brissette CA, Bowman A, Stevenson B. *Borrelia burgdorferi* BmpA is a laminin-binding protein. Infect Immun **2009**; 77:4940-6.
33. Nguyen NM, Senior RM. Laminin isoforms and lung development: all isoforms are not equal. Dev Biol **2006**; 294:271-9.
34. 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.
35. Ebady R, Niddam AF, Boczula AE, et al. Biomechanics of *Borrelia burgdorferi* Vascular Interactions. Cell Rep **2016**; 16:2593-604.
36. Paulsson M, Riesbeck K. How bacteria hack the matrix and dodge the bullets of immunity. Eur Respir Rev **2018**; 27.

Figure Legends

Figure 1. BB0406 is a weakly immunogenic and differentially-produced spirochete antigen. **A:** Production of BB0406 in various isolates and strains of *B. burgdorferi* sensu lato. The expression of *bb0406* was detected in B31, 297, N40, and *garinii* strains using qRT-PCR by measuring copies of target transcripts and normalizing against *flaB* copies (upper panel), while protein levels were detected by immunoblotting with specific antibodies against BB0406, BB0405, and FlaB (lower panel). **B:** BB0406 is differentially expressed both *in vitro* and *in vivo*. The expression of *bb0406* is measured in cultured cells at different temperatures (left panel) and during infection in mice and ticks (right panel) by qRT-PCR analysis. **C:** The temporal expression of *bb0406* at the spirochete inoculation site. Skin samples were collected from the injection sites at the indicated days and BB0406 expression was analyzed using qRT-PCR. **D:** Antibody responses against BB0406 during infection. The recombinant BB0406 protein was immunoblotted with antiserum collected from mice after 2 weeks of either needle-borne or tick-transmitted infection. Error bars represent the mean ± SEM of three independent experiments.

Figure 2. BB0406 supports *B. burgdorferi* dissemination and persistence in mammalian hosts.

A: Spirochete burden at various murine tissues. Mice (3 animals/group) were inoculated intradermally with equal numbers (10^5 spirochetes/mouse) of wild type (WT, white bar) or either of two independent clones of *bb0406* mutants (Mut1 or Mut2, black bars). The spirochete burdens at the dermal injection sites were assessed on day 7, which indicated a similar level in all groups (upper panel, $P > 0.05$), while on day 14, the pathogen burden in mutants was

significantly lower compared to the WT (right panel, *P<0.05). The lower panel shows decreased levels of mutant isolates in distant skin and other organs 14 days after infection (*P < 0.05). **B:** Spirochete burden in blood. The levels of wild-type or mutant isolates were analyzed in the blood on day 8 post-infection using qPCR (*P < 0.05). **C:** Spirochete acquisition in ticks. Mice (3 animals/group) were injected with wild-type or mutant isolates (10^5 spirochetes/mouse) for 12 days and then allowed to be parasitized by naïve ticks (25 ticks/group). The *B. burgdorferi* burdens in fed ticks were analyzed by measuring copies of *flaB* transcripts and normalizing to tick *β*-actin using qPCR. The levels of *bb0406* mutants were significantly decreased in ticks compared to the WT (*P<0.05). Error bars represent the mean ± SEM of three independent experiments. **D:** *bb0406* is required for transmission of spirochetes from ticks. Nymphal ticks were microinjected with either WT or *bb0406* mutants (Mut1 and Mut2) and then allowed to engorge on naïve mice (5 ticks/mouse and 3 animals/group). At 12 days after tick feeding, the spirochete burdens in murine tissues were assessed via qPCR by measuring copies of *flaB* transcripts and normalizing against mouse *β*-actin. The spirochete burdens in mice infected with Mut1 and Mut2 (black bars) were significantly lower than mice infected with WT (white bars) (*P < 0.05).

Figure 3. Hyperinfection or intravenous injection does not rescue infectivity of *bb0406* mutants. A: Spirochete burdens at the dermal inoculation sites after a high infection dose. Mice (3 animals/group) were infected either with WT (10^5 spirochetes/mouse) or a 1000-fold higher inoculum dose of the Mut1 mutant clone (10^8 spirochetes/mouse). After 14 days of infection, the levels of Mut1 isolates were measured by qPCR, which indicated a significantly lower level compared to the WT (*P<0.05). **B:** *bb0406* mutants fail to persist in mice even after a higher

level of inoculum. Mice were injected with the Mut1 isolate (10^8 spirochetes/animal), as detailed in panel A, and pathogen levels in distant murine tissues were determined by qPCR after 14 days of infection. Levels of the *bb0406* mutants were significantly lower in all mouse tissues, as compared to the WT (*P<0.05). **C:** Pathogen burden in blood. Mice (3 animals/group) were intravenously injected with either WT or *bb0406* mutants (Mut1 and Mut2) (10^5 spirochetes/mouse). On Day 14, spirochete burdens in the blood were determined by qPCR, which indicated similar pathogen levels in all groups (P>0.05). **D:** Intravenous injection did not rescue BB0406 mutant infectivity in mice. After 14 days of infection via intravenous injection, as detailed in panel C, the levels of *B. burgdorferi* in murine tissues were analyzed using qPCR. Levels of the *bb0406* mutants were significantly lower in all mouse tissues, as compared to the WT (*P<0.05). Error bars represent the mean ± SEM of three independent experiments.

Figure 4. Deletion of *bb0406* impairs *B. burgdorferi* dissemination through human endothelial cell layers.

A: Altered resistance of transformed human brain microvascular endothelial cells (THBMECs) during transmigration of *bb0406* mutants. Wild-type or mutant spirochetes were allowed to transmigrate through THBMECs grown in transwell membranes, and the changes in transendothelial electrical resistance were monitored in real-time by electric cell-substrate impedance sensing. **B:** *bb0406* mutants are impaired in their ability to cross THBMEC layers. The proportions of spirochetes transmigrating between the transwell chambers were measured by qPCR.

Figure 5. BB0406 is a laminin-binding protein. A: Binding of recombinant BB0406 to various ECM proteins was examined by a microtiter assay. The histidine-tagged BB0406, or OspD (as a negative control), at a 2-μM concentration, were added to quadruplicate wells coated with fibronectin (Fn), type I collagen (Collagen I), type IV collagen (Collagen IV), elastin, laminin (Ln), heparan sulfate (Hep-SO4), chondroitin-4-sulfate (Chon-4-SO4), dermatan sulfate (Derm-SO4), chondroitin-6-sulfate (Chon-6-SO4), or BSA as control. Bound proteins were measured by ELISA. Shown is the average OD_{405} of six independent experiments ± SEM. Asterisks indicate that BB0406 binds to laminin or heparan sulfate in statistically higher levels ($P < 0.05$) relative to OspD. **B:** Saturation kinetics of BB0406-laminin interaction. The indicated concentrations of recombinant histidine-tagged BB0406 or OspD were added to quadruplicate wells coated with laminin (Ln), heparan sulfate ($HepSO_4$), or BSA (data not shown), and the protein binding was quantified by microtiter assays. The experiments were performed on three independent occasions; the samples were run in duplicate each time. All experiments were performed with a single preparation of recombinant proteins. Shown is one representative experiment from the average OD_{405} ± SEM of two replicates. The K_D value of BB0406 binding to $HepSO_4$ cannot be evaluated, as the binding was not saturated due to extremely weak binding activity. The K_D value shown in the inset, which represents the laminin-binding affinity of BB0406, was determined from the average of three experiments. **C:** BB0406-laminin interaction studied by surface plasmon resonance assay. About 1.6 to 1000 nM of histidine-tagged BB0406 was flowed over a surface coated with 10μg of laminin (Ln). Shown is a representative of four experiments performed on two separate occasions. The k_{on} ($1.66 \pm 0.75 \times 10^6$ $s^{-1}M^{-1}$) and k_{off} values (0.31 ± 0.021 s−1) were obtained from the average of these four experiments. The K_D for the relevant interaction is shown in the inset. **D:** The lack of BB0406 results in the

reduced levels of spirochete binding to laminin. About 10^7 cells of the wild-type *B. burgdorferi* strain (WT) or the *bb0406* mutant clones (Mut 1 and Mut2) were incubated with laminin (top panel) or BSA as negative control (bottom panel) on microtiter plate wells. After washing, the bound cells were measured by a microtiter assay using an antibody that recognizes the *B. burgdorferi* strain B31. Each bar represents the mean of 12 independent determinations ± SEM. Significant ($P< 0.05$) differences in binding relative to the wild-type *B. burgdorferi* strain B31-A3 are indicated ("*").

Legends for supplementary figures

Figure S1: Generation of *bb0406* mutant *B. burgdorferi.* **A:** Schematic drawings of wild-type (WT) and *bb0406* mutant (*bb0406*$^-$) isolates at the *bb0406* locus. Genes from *bb0404* to *bb0408* (white arrows) and the kanamycin-resistance cassette driven by the *B. burgdorferi flaB* promoter (*flaB-kan*) (black arrow) are indicated. Primers P1–P4 (arrowheads) were used to amplify the 5′ and 3′ arms for homologous recombination, and regions flanking the *bb0406* locus were ligated on either side of the *flaB-kan* cassette to obtain the mutagenic construct. **B:** Integration of the mutagenic construct, *flaB-kan*, in the intended genomic locus. Primers 5-10 (arrowheads) were employed for polymerase chain reactions (PCR) using DNA isolated from the WT or *bb0406* mutant *B. burgdorferi,* and subjected to agarose gel electrophoresis. **C:** RT-PCR analysis of the *bb0406* transcript and the polar effects of mutagenesis. Total RNA was isolated from the WT or two independent clones of the *bb0406* mutant (designated as Mut1 and Mut2), converted to cDNA, used to amplify regions within *bb0406, flaB*, and genes surrounding the *bb0406* locus

(*bb0405* and *bb0407*), and visualized on a gel. **D:** Growth kinetics of spirochetes. *B. burgdorferi* were diluted to a density of 10^5 cells/mL and grown at 34°C in BSK-H medium. Samples were counted under a dark field microscope every 24 hours using a Petroff-Hausser cell counter. The growth patterns of *bb0406* mutants and WT are comparable (*P >0.05).

Figure S2: Plasmid profile of WT and *bb0406* mutants. DNA was isolated from the WT and *bb0406* mutant isolates. The presence of all plasmids in spirochetes was detected using PCR. Both *bb0406* mutants (Mut1 and Mut2) have the same set of endogenous plasmids as the isogenic WT spirochete.

Figure S3: *B. burgdorferi bb0406* mutants induce antibody responses, while BB0406 antibodies are borreliacidal. A: Antibody responses in mice after spirochete infection. Mice (3 animals/group) were inoculated intradermally with equal numbers of WT, Mut1 or Mut2 (10^5 spirochetes per mouse). After 14 days of infection, serum was collected and used to immunoblot *B. burgdorferi* proteins. Despite near clearance (Figure 1), the BB0406 mutants still induced detectable antibody responses. **B:** Bactericidal property of BB0406 antibodies. Spirochetes were incubated with normal mouse serum (NMS), OspA antibodies (positive control), and BB0406 antibodies in the absence of active complement. After 48hrs incubation, a re-growth assay was performed to recover the viable *B. burgdorferi*. The growth of the spirochetes after incubation with BB0406 antisera was significantly decreased compared to that with NMS (*P<0.5)

Figure S4: *bb0406* deletion did not impair *B. burgdorferi* persistence in ticks. A: Persistence of spirochetes in fed ticks. Nymphal ticks were microinjected with equal levels of wild-type

(WT) or *bb0406* mutants (Mut1 and Mut2). After overnight incubation, the ticks were allowed to feed on naïve mice (5 ticks/mouse; 3 animals/group). Ticks were collected when replete, and their spirochete burdens were assessed via qPCR. **B:** Antiserum responses during tick-transmitted infection. The *B. burgdorferi* proteins were immunoblotted with serum collected from mice 14 days after infection with either WT or *bb0406* mutants (Mut1 and Mut2). Similar to syringe-borne infections (Figure S3A), *bb0406* mutants also induced antibody responses during tick-transmitted infections.

Figure S5: Histology of *B. burgdorferi* infected-murine skin at tick-bite sites. Ticks were microinjected with wild type or *bb0406* mutants and allowed to parasitize mice (10 ticks/mouse). 4 days after the placement of ticks, cellular infiltrates (arrows) at the tick-bite site were analyzed by hematoxylin-eosin staining.

Figure S6: Deletion of *bb0406* has no impact on *B. burgdorferi* motility. **A:** A representative of swimming plate assays. The *ΔflaB* mutant, a previously constructed non-motile mutant, is included to determine the size of the inoculum. **B:** Averaged sizes of swimming rings of WT, Mut1, Mut2, and Δ*flaB* isolates from 4 agar plates: WT (12.75 ± 0.5 mm); Mut1 (12 ± 0.82 mm); Mut2 (12.25 ± 0.5 mm); and Δ*flaB* (7.13 ± 0.25 mm).