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A Novel Laminin-Binding Protein Mediates Microbial-Endothelial Cell Interactions and Facilitates Dissemination of Lyme Disease Pathogens

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Borrelia burgdorferi conserved gene products BB0406 and BB0405, members of a common *B. burgdorferi* paralogous gene family, share 59% similarity. Although both gene products can function as potential porins, only BB0405 is essential for infection. Here we show that, despite sequence homology and coexpression from the same operon, both proteins differ in their membrane localization attributes, antibody accessibility, and immunogenicity in mice. BB0406 is required for spirochete survival 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.

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 precisely how a fraction of spirochetes can invade the vasculature and then extravasate from the vessel to colonize distant organs.

The colonization of the host organs in humans, 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, although a subset of antibiotic-treated hosts can later experience a variable set of clinical symptoms, known as posttreatment Lyme disease syndrome [5, 6]. When infested via *B. burgdorferi*–infected ticks or inoculated via syringe, many laboratory animals [7], including C3H mice [8, 9] are susceptible to *B. burgdorferi* infection, which may mimic many aspects of clinical Lyme disease [2]. 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 (ECM) molecules, aiding in *B. burgdorferi*–vascular system interactions, transmigration, and tissue colonization [10]. For example, *B. burgdorferi* BBK32 interacts with host fibronectin and glycosaminoglycan molecules, facilitating borrelial adhesion to vasculature via stabilization of the bacterial-endothelial interaction [11–14]. Similarly, an outer membrane (OM) surface protein, P66, has

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both porin and adhesin functions [15, 16], particularly as an integrin-binding protein, and has demonstrated its ability to promote spirochete extravasation [17]. Further studies are required to identify *B. burgdorferi* OM proteins that promote vascular interactions and transmigration, which will help in our understanding of spirochete dissemination events, pathogenesis, and the development of preventive strategies.

The *B. burgdorferi* bb0406 gene product is cotranscribed with 2 immediately upstream genes that encode bb0404 and bb0405 [18, 19]. The latter gene has recently been characterized as a transmembrane protein that facilitates spirochete infection in mammals [18]. 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 [19]. In the current article, 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

The *B. burgdorferi* isolates B31-A3, 297, and N40, and the *Borrelia garinii* isolate PBi, were grown in Barbour-Stoenner-Kelly-H medium [18]. C3H/HeN mice were purchased from the National Institutes of Health and Charles River Laboratories. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Biosafety Committee. The ticks used were originated from a colony that is maintained in the laboratory.

Polymerase Chain Reaction

The primers sequences are listed in Supplementary Table 1. For gene expression analysis, the C3H mice (3 animals per group) were infected with a intradermal inoculum of *B. burgdorferi* (10^5 cells per mouse), and 14 days after infection, tissues were collected. Infected mice were parasitized by larval ticks (25 ticks per mouse) that were collected after repletion. After molting, nymphs were allowed to engorge on mice (25 ticks per animal) and were collected at 24, 48, and 96 hours after attachment. Total RNA was isolated from tissues using TRIzol reagent (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 means of quantitative polymerase chain reaction (qPCR) using cDNA samples, as detailed elsewhere [18]. The spirochete burdens in the tissues were assessed by measuring copies of the *flaB* transcripts through qPCR, using cDNA samples, and by normalizing against the corresponding β-actin levels [18].

Generation of Recombinant BB0406, Antiserum, and Immunoblotting

The *bb0406* gene was cloned into pET28a using specific primers (Supplementary Table 1), and the recombinant protein was produced in *Escherichia coli,* as described elsewhere [18]. BB0406 antisera were generated in mice, and the titer and specificity were assessed using enzyme-linked immunosorbent assay (ELISA) and Western blotting analysis, respectively, as described elsewhere [18].

Generation of *bb0406* Deletion Mutants

The *bb0406* mutants were generated via homologous recombination by replacing *bb0406* open reading frame with a kanamycin resistance cassette, as detailed elsewhere [20]. 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 kanamycin resistance 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 nonessential 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 to the stationary phase (approximately 10^8 cells/mL), and counted by means of 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 3 mice were infected with either wild-type isolates or either of the mutant clones (10^5 spirochetes per animal) by intradermal inoculation. Tissue samples were collected at 7, 14, and 21 days after infection. Pathogen burdens in murine tissues were evaluated using qPCR. Portions of skin and spleen were cultured in Barbour-Stoenner-Kelly medium to recover viable spirochetes. For studies addressing the acquisition of pathogens in ticks, mice were infected with borrelial isolates and, after 12 days, infested with naive nymphs (25 ticks per 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* were fed on naive mice (5 ticks per mouse, 3 mice per group). Engorged ticks were subjected to qPCR analyses of their spirochete levels. Mice were euthanized 14 days after tick feeding, and tissues were assessed for spirochete burdens by qPCR and culture analysis.

For the intravenous injection study, groups of mice (3 animals per group) were intravenously infected with borrelial isolates (10^5 spirochetes per mouse). The pathogen burdens in the bloodstream and tissues were determined on day 14 of infection, using qPCR. For the hyperinfection study, separate groups of mice (3 animals per group) were infected intradermally with wild-type *B. burgdorferi* (10^5 spirochetes per mouse) or Mut1 or Mut2 clones (10^8 spirochetes per mouse). The spirochete burdens were assessed by means of qPCR at the injection site and in murine tissues on day 14 of infection.

Motility and Bactericidal Assays

Spirochete motility was evaluated by swimming plate assays, as described elsewhere [21], using a nonmotile *flaB* mutant [22] as controls. BB0406 antibodies were tested for bactericidal activities against *B. burgdorferi* using a regrowth assay, as described elsewhere [23].

Transformed Human Brain Microvascular Endothelial Cell and Electric Cell-Substrate Impedance Sensing Assays

To test the effects of *B. burgdorferi* on the integrity of vascular barriers, we used an in vitro model of the human blood-brain barrier [24–26] based on transformed human brain microvascular endothelial cell (THBMEC) monolayers and electric cell-substrate impedance sensing (ECIS; Applied Biophysics). About 10^5 THBMECs were grown in M199 medium, as detailed elsewhere [25], seeded in gold microelectrode arrays (8W10E+; Applied Biophysics), and exposed to 1–2 × 10^6 wild-type *B. burgdorferi* or mutants at a multiplicity of infection of 1:5–10. The resistance changes of THBMEC monolayers were monitored for 24 hours and normalized to control M199 medium–treated cells.

Transwell Assay

The assay used an in vitro model of the human blood-brain barrier represented by THBMECs grown on Transwell plates (Costar) with permeable membrane inserts, as described elsewhere [24]. After 24–36 hours of THBMEC growth, when the cellular resistance reached maximal steady-state values, 5–10 × 10^5 spirochete cells were added to the inserts at a multiplicity of infection of 5–10 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.

Assays for *B. burgdorferi*–Extracellular Matrix Interaction

The binding of *B. burgdorferi* proteins to various ECM molecules was analyzed using assays detailed assays elsewhere, such as ELISAs [27] and surface plasmon resonance [28]. Binding of *B. burgdorferi* cells to laminin was determined using methods reported elsewhere [27, 29].

Statistical Analysis

Data are presented as means and standard errors of the means of ≥2 independent experiments. Significant differences between samples were determined using Student 2-tailed *t* tests or 1-way analysis of variance, using GraphPad Prism 5.01 software, following logarithmic transformation of the data. Differences were considered significant at $P < .05$.

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 1*A, upper panel*), although the protein remains nearly undetectable (Figure 1*A*, *lower panel*). Expression of *bb0406* is induced at 37°C in cultured cells (Figure 1*B, left panel*) and in ticks during spirochete transmission, but the transcripts are also detectable in various murine tissues (Figure 1*B, right panel*), with the most dramatic expression during early infection (Figure 1*C*). A low but noticeable antibody response against BB0406 is detectable in infected mice (Figure 1*D*), suggesting a poor abundance, or a weak immunogenicity and subsurface nature, of the antigen [30].

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* (Figures 2*A–C* and Supplementary Figure 1). Despite repeated attempts, we were unable to complement the gene; therefore, for all subsequent studies, we used 2 independent clones of isogenic *bb0406* mutants (Supplementary Figure 2). The *bb0406* mutants did not reflect apparent growth defects (Supplementary Figure 1*D*).

Next, mice were infected with equal numbers (10^5 cells per animal) of wild-type or *bb0406* mutant isolates (2 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 2*A, upper panel*) and at distant skin or other organs (Figure 2*A, lower panel*). We noted that the wild-type and *bb0406* mutants induced comparable antibody responses, further supporting mutant survival during early infection (Supplementary Figure 3*A*). In agreement with a previous study [19], we found that anti-BB0406 antibodies have bactericidal activities (Supplementary Figure 3*B*). The detection of low levels of *bb0406* mutants in distant tissues could be due to their impaired ability to disseminate through blood (Figure 2*B*). Consequently, the survival defect of the *bb0406* mutants in mice also affected their entry or acquisition in ticks (Figure 2*C*).

Similar to needle-borne infections (Figure 2*A*), *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 (Supplementary Figure 4*A*). However, despite a robust antibody response comparable to that in mice infested with wild-type spirochetes (Supplementary Figure 4*B*), a significantly lower level of *bb0406* mutants was detected in mice after 14 days of tick engorgement (Figure 2*D*). 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 (Supplementary Figure 5). 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

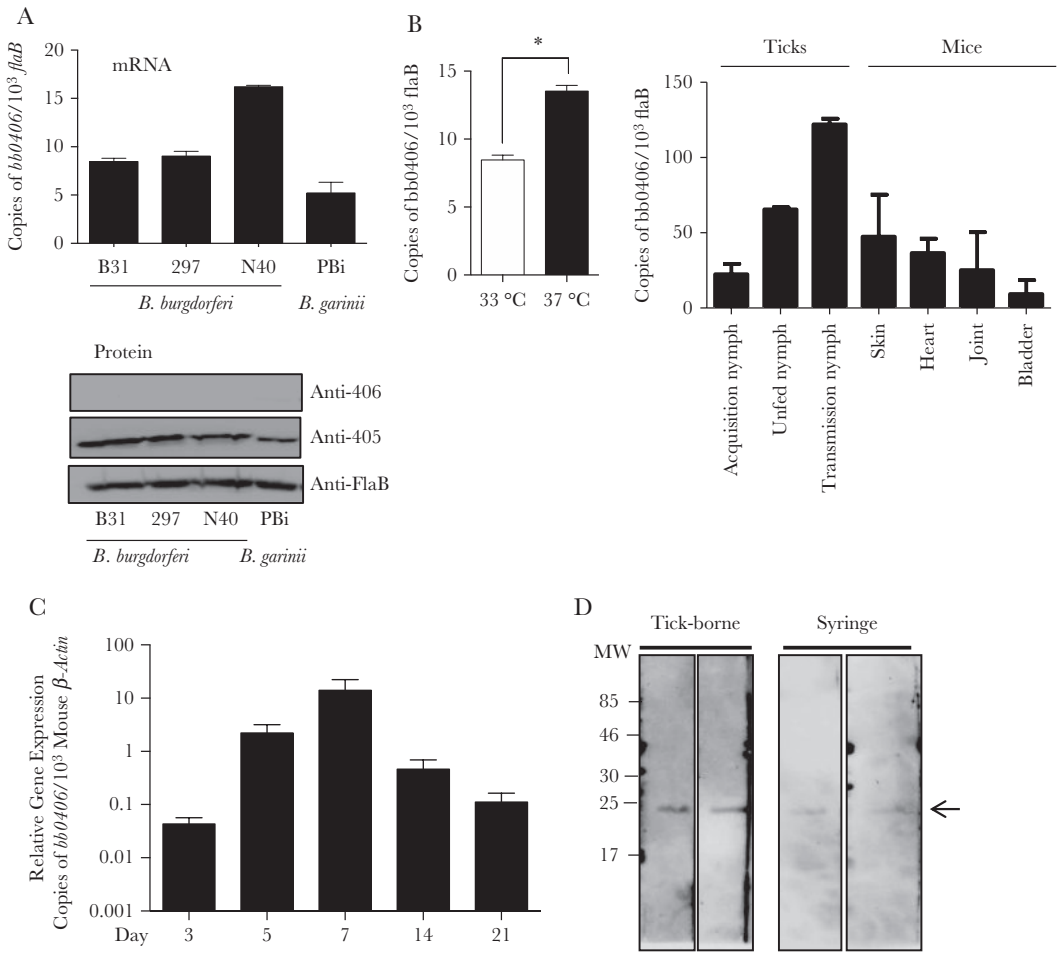

Figure 1. BB0406 is a weakly immunogenic and differentially produced spirochete antigen. *A,* Production of BB0406 in various isolates and strains of *Borrelia burgdorferi* sensu lato. The expression of *bb0406* was detected in B31, 297, N40, and *Borrelia garinii* strains using quantitative reverse-transcription polymerase chain reaction (qRT-PCR), by measuring copies of target transcripts and normalizing against *flaB* copies (*upper panel*), while detecting protein levels 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 *B. burgdorferi* B31 isolates 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. Mice were infected with *B. burgdorferi* B31 cells and 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 (2 mice per group) collected from after 2 weeks of either needle-borne (*right panel*) or tick-transmitted (*left panel*) infection. Error bars represent means and standard errors of the mean for 3 independent experiments. $*P < .05$. Abbreviations: mRNA, messenger RNA; MW, molecular weight.

the establishment of spirochete infections in mice, including their hematogenous dissemination.

BB0406 Assists Spirochete Dissemination Through Murine Vasculature

Because previous studies indicated that a greater mutant inoculum could rescue the phenotypic defects in tissue survival [31], 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 per animal), compared with the wild type (10^5 cells per animal) (Figure 3*A* and *B*). 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. Although similar spirochete levels were detected in the blood (Figure 3*C*), the burden of *bb0406* mutants in distant murine organs was significantly lower, compared with the wild type, indicating that BB0406 might assist in pathogen dissemination from blood to tissues (Figure 3*D*). 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 results of a swim plate assay (Supplementary Figure 6).

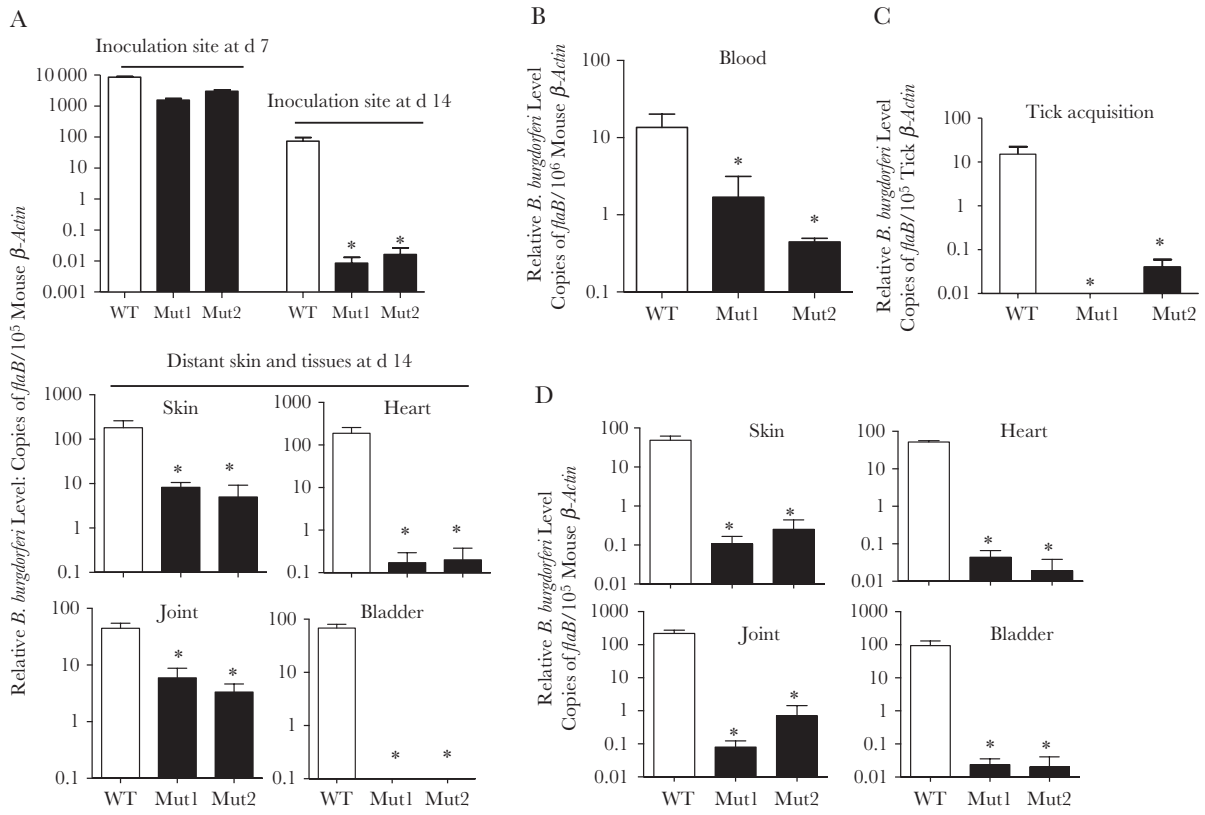

Figure 2. BB0406 supports *Borrelia burgdorferi* dissemination and persistence in mammalian hosts. *A,* Spirochete burden at various murine tissues. Mice (3 animals per group) were inoculated intradermally with equal numbers (10^5 spirochetes per mouse) of *B. burgdorferi* B31 wild-type (WT) cells (*white bar*) or either of 2 isogenic independent clones of *bb0406* mutants (Mut1 or Mut2) (*black bars*). *Upper panel,* spirochete burdens at the dermal injection sites were assessed on day 7 (*left*), which indicated a similar level in all groups ($P > .05$), while on day 14, the pathogen burden in mutants was significantly lower compared with the WT (*right*). *Lower panel,* Decreased levels of mutant isolates in distant skin and other organs 14 days after infection. $^*P < .05$. *B,* Spirochete burden in blood. The levels of WT or mutant isolates were analyzed in the blood on day 8 after infection using quantitative polymerase chain reaction (qPCR). $^*P < .05$. *C,* Spirochete acquisition in ticks. Mice (3 animals per group) were injected with WT or mutant isolates (10^5 spirochetes per mouse) for 12 days and then allowed to be parasitized by naive ticks (25 ticks per 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 with the WT. $^*P < .05$. Error bars represent the means and standard errors of the mean for 3 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 naive mice (5 ticks per mouse and 3 animals per group). At 14 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 in mice infected with WT (*white bars*), $^*P < .05$.

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 THBMECs, which share characteristics of primary microvascular endothelial cells. The results indicate that both *bb0406* mutant clones induced markedly different THBMEC resistance, compared with wild-type *B. burgdorferi* (Figure 4*A*), and are also impaired in their abilities to migrate through the endothelial barrier (Figure 4*B*), 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 ECM proteins, 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 bovine serum albumin, as a control), using qualitative ELISA. The results indicate that BB0406 predominantly interacts with laminin and heparan sulfate (Figure 5*A*), although an unsaturated binding with the latter ligand was recorded (Figure 5*B*). The binding affinity between BB0406 and laminin was evaluated using quantitative ELISA (Figure 5*B*) and further confirmed with an independent

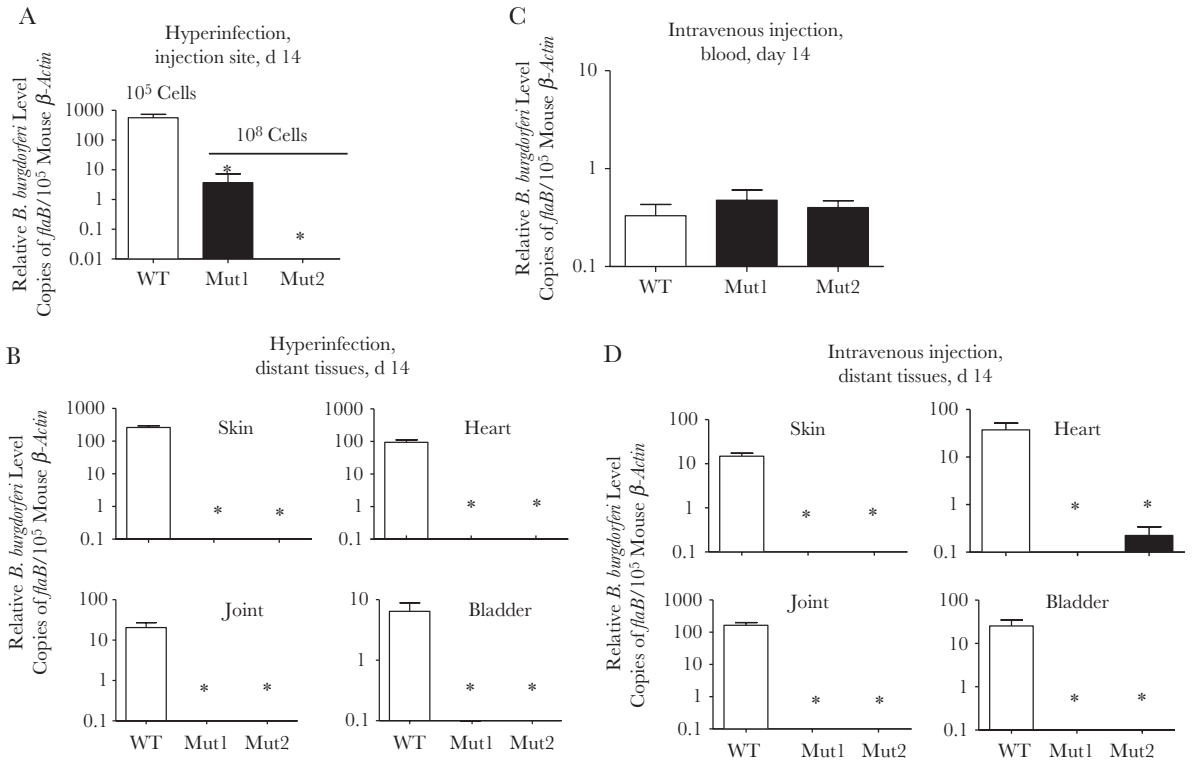

Figure 3. Hyperinfection or intravenous injection does not rescue infectivity of *bb0406* mutants. *A,* Spirochete burdens at the dermal inoculation sites after a high infection dose. Mice (3 animals per group) were infected either with wild-type (WT) (10^5 spirochetes per mouse) or a 1000-fold higher inoculum dose of the mutant clones (10^8 spirochetes per mouse). After 14 days of infection, the levels of mutant isolates were measured by quantitative polymerase chain reaction (qPCR), which indicated a significantly lower level compared with the WT. **P* < .05. *B, bb0406* mutants fail to persist in mice even after a higher level of inoculum. Mice were injected with the Mut1 or Mut2 isolate (10^8 spirochetes per animal), as detailed in *A,* and pathogen levels in distant murine tissues were determined with qPCR after 14 days of infection. Levels of the *bb0406* mutants were significantly lower in all mouse tissues, as compared with the WT. **P* < .05. *C,* Pathogen burden in blood. Mice (3 animals per group) were intravenously injected with either WT or *bb0406* mutants (Mut1 and Mut2) (10^5 spirochetes per mouse). On day 14, spirochete burdens in the blood were determined by qPCR, which indicated similar pathogen levels in all groups (*P* > .05). *D,* Intravenous injection did not rescue BB0406 mutant infectivity in mice. After 14 days of infection via intravenous injection, as detailed in *C,* the levels of *Borrelia burgdorferi* in murine tissues were analyzed using qPCR. Levels of the *bb0406* mutants were significantly lower in all mouse tissues, compared with the WT. **P* < .05. Error bars represent means and standard errors of the mean for 3 independent experiments.

surface plasmon resonance assay (Figure 5*C*). 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 (or bovine serum albumin, as a control), and bound cells were measured. Significantly decreased bindings of both clones (Mut1 and Mut2) to laminin were recorded (Figure 5*D*).

DISCUSSION

The *B. burgdorferi* OM protein BB0406 is cotranscribed with another OM protein, BB0405, with 59% similarity [18, 19]. Although both proteins are capable of forming a membrane pore [19], only BB0405 was shown to support spirochete infection in mammals [18, 19]. In contrast to the prevailing notion that BB0406 has a redundant role in infection [19], 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 [32]. Because previous studies [19] 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 findings of previous mass spectrometry studies [33], which show that the protein remained undetectable in the OM of *B. burgdorferi*. Therefore, the bactericidal activity of anti-BB0406 antibodies against cultured spirochetes [19] is puzzling and may occur via the steric hindrance of antibodies, which may bind to off-target yet homologous proteins, such as BB0405. Notably, despite being a subsurface protein [30], BB0406 induces a detectable antibody response during murine infection (Figure 1*D*), whereas the highly antigenic and surface-exposed protein BB0405 failed to generate such responses. This

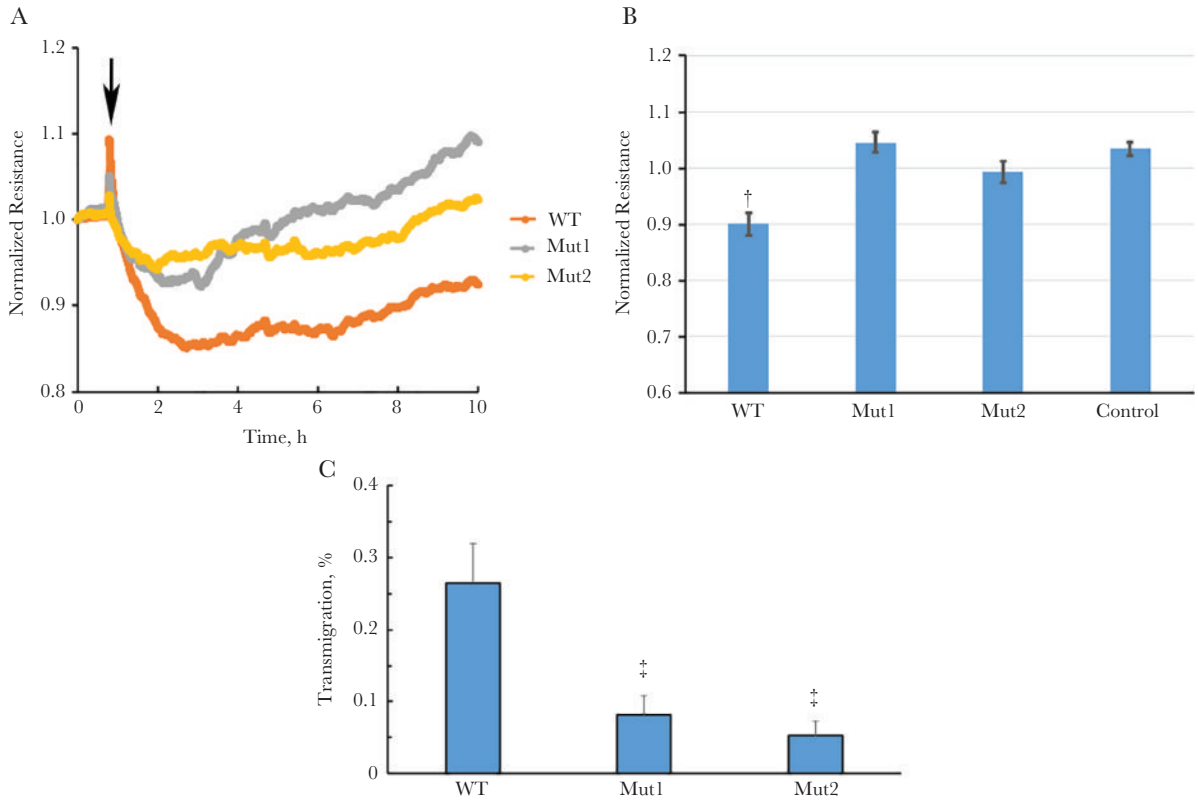

Figure 4. Deletion of *bb0406* impairs *Borrelia burgdorferi* dissemination through human endothelial cell layers. *A,* Representative time-resolved electric cell-substrate impedance sensing (ECIS; Applied Biophysics) traces of resistance changes across the transformed human brain microvascular endothelial cell (THBMEC) monolayers on addition (*arrow*) of *B. burgdorferi* wild-type (WT), mutant 1 (Mut1) and mutant 2 (Mut2). *B,* Resistance changes across THBMEC monolayers after 6 hours of incubation with WT and mutant *B. burgdorferi*. Results for the control show resistance changes on addition of M199 medium only. Data are means and standard errors of the mean (n = 3). †$P < .02$. *C,* Deletion of BB0406 dramatically decreased ability of *B. burgdorferi* to cross the blood-brain barrier. A total of 10^6 WT or mutant *B. burgdorferi* were added into the medium above the 3.0 µmol/L pore membrane inserts with confluent THBMEC monolayers after maximum transendothelial electrical resistance. After 6 hours, spirochetes that crossed the monolayer were collected from the lower chamber and quantified using quantitative polymerase chain reaction. Data are presented as the percentage of the input spirochetes. ‡$P < .005$ for 2 independent experiments in triplicate.

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 [18, 19] 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 [19], a different mutagenesis strategy was used, which likely contributed to disparate phenotypic outcomes. Whereas 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 2 independent mutant clones to analyze the phenotype. In contrast, the published study created a dual mutant by ablating the expression of 2 genes (both bb0405 and bb0406) and then genetically complementing either gene to generate 2 independent isolates that reexpressed either BB0405 or BB0406. Of note, native BB0406 displays a spectacular differential expression in vivo that varies both spatially (Figure 1*B*) and temporally (Figure 1*C*). Shrestha et al [19] used a constitutively active borrelial promoter (*flaB*) that overexpressed *bb0406*, and it is possible that an abnormally regulated gene product may affect 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 are substantially similar, 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 multistep process including adhesion, tethering, dragging, stationary adhesion, and extravasation [34]; BB0406 is likely to be

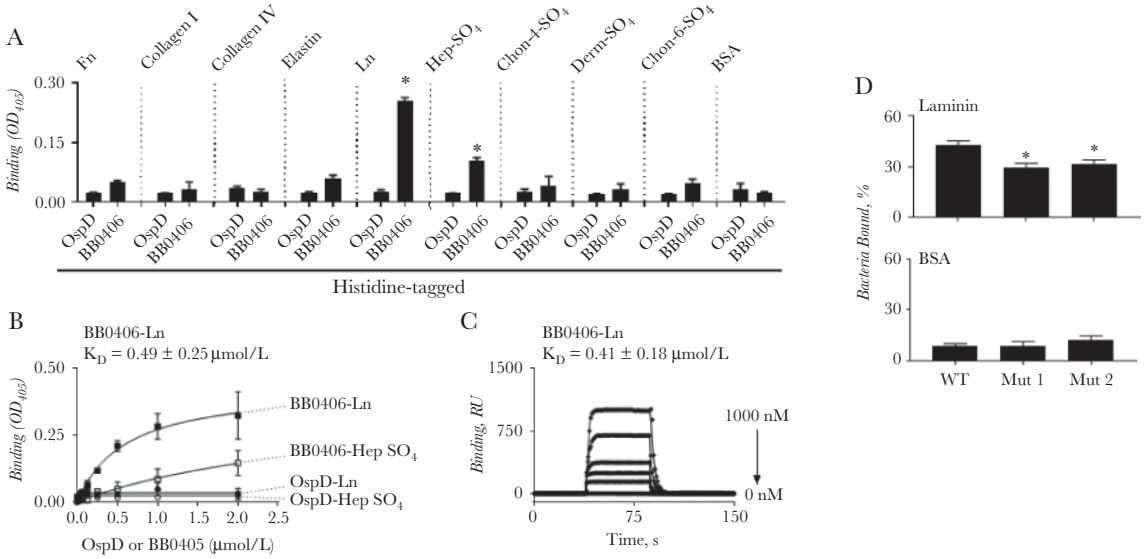

Figure 5. BB0406 is a laminin-binding protein. *A*, Binding of recombinant BB0406 to various extracellular matrix (ECM) proteins was examined by a microtiter assay. The histidine-tagged BB0406, or OspD (as a negative control), at a 2-µmol/L concentration, were added to quadruplicate wells coated with 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 bovine serum albumin (BSA) as a control. Bound proteins were measured by enzyme-linked immunosorbent assay. Shown is the average optical density at 405 nm (OD_{405}) of 6 independent experiments standard errors of the mean. * $P < .05$ for comparison of BB0406 binding to laminin or heparan sulfate relative to OspD. *B,* Saturation 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 3 independent occasions; the samples were run in duplicate each time. All experiments were performed with a single preparation of recombinant proteins. Shown is 1 representative experiment from the mean OD_{405} and standard errors of the mean for 2 replicates. The K_D value of BB0406 binding to $HepSO_4$ cannot be evaluated, because the binding was not saturated owing 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 3 experiments. *C*, BB0406-laminin interaction studied by surface plasmon resonance assay. About 1.6 to 1000 nmol/L of histidine-tagged BB0406 was flowed over a surface coated with 10 µg of laminin (Ln). Shown is a representative of 4 experiments performed on 2 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 4 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 (WT) *B. burgdorferi* strain or the *bb0406* mutant clones (Mut1 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 and standard errors of the mean. * $P < .05$ for differences in binding relative to the WT *B. burgdorferi* strain B31-A3. Abbreviations: kon, rate constant of association; koff is the rate constant of dissociation; M, molar; nM, nanomolar; 0nM, 0 nM; RU, relative unit.

involved in one or more 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. Because one of the major structural and functional constituents of the vascular basement membrane, the binding of laminin 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 [35, 36], 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 [37, 38], which may interact with specific target laminin and contribute to pathogen survival and pathogenesis. This remains a possibility, because there are ≥15 isoforms of laminin in the basement membranes of the vessel walls of arterioles, capillaries, postcapillary venules, and venules, which display enormous variability in structure and expression [39].

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 [11, 13, 14, 40], is involved in the early steps of *B. burgdorferi* endothelial adhesion and interactions with the host microvasculature [41]. In addition, the OM protein P66, an integrin-binding borrelial porin, was recently reported to assist in spirochete and vascular interactions, vascular transmigration, and extravasation, via P66-integrin interactions [17]. Many other bacterial porin and transporter proteins are known to bind specific ECM proteins that promote microbial adherence [42]. 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, in immunocompetent or in immunodeficient hosts (like SCID mice)—will greatly enrich our knowledge of the biology of tick-borne infections and host immune responses and will inform the development of effective measures to control these diseases.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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