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The BBA33 lipoprotein binds collagen and impacts *Borrelia burgdorferi* pathogenesis

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Summary

Borrelia burgdorferi , the etiologic agent of Lyme disease, adapts to the mammalian hosts by differentially expressing several genes in the BosR and Rrp2-RpoN-RpoS dependent pathways, resulting in a distinct protein profile relative to that seen for survival in the *Ixodes* spp. tick. Previous studies indicate that a putative lipoprotein, BBA33, is produced in an RpoS-dependent manner under conditions that mimic the mammalian component of the borrelial lifecycle. However, the significance and function for BBA33 is not known. Given its linkage to the BosR/Rrp2-RpoN-RpoS regulatory cascade, we hypothesized that BBA33 facilitates *B. burgdorferi* infection in the mammalian host. The deletion of *bba33* eliminated *B. burgdorferi* infectivity in C3H mice, which was rescued by genetic complementation with intact *bba33*. With regard to function, a combinatorial peptide approach, coupled with subsequent *in vitro* binding assays, indicated that BBA33 binds to collagen type VI and, to a lesser extent, collagen type IV. Whole cell binding assays demonstrated BBA33-dependent binding to human collagen type VI. Taken together, these results suggest that BBA33 interacts with collagenous structures and may function as an adhesin in a process that is required to prevent bacterial clearance.

Introduction

The spirochetal bacterium *Borrelia burgdorferi*, the etiologic agent of Lyme disease, infects mammalian hosts via the bite of *Ixodes* spp. ticks (Samuels, 2011; Radolf *et al*., 2012). When humans become infected, they present with nondescript flu-like symptoms with most developing a characteristic skin lesion referred to as a erythema migrans (recently reviewed in Shapiro, 2014). If untreated, the infection can progress to a multistage disorder as the

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Supporting information

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spirochete disseminates to distant organ sites, resulting in carditis, neurological disorders and arthritis. Recently, the CDC reported that more than 300,000 cases of Lyme disease are diagnosed annually in the United States, indicating that Lyme borreliosis is a significant, re-emerging infectious disease (Hinckley *et al.*, 2014). Furthermore, the lack of an effective vaccine underscores the need to better understand factors that contribute to the pathogenic potential of *B. burgdorferi*.

Borrelia burgdorferi establishes infection by interaction with connective tissue of the skin and disseminates to deeper tissues following colonization (Cabello *et al.*, 2007). Early in the infectious process, *B. burgdorferi* is known to align with host collagen (Barthold *et al.*, 1991) and interact with other structures found within the extracellular matrix (ECM) in a process that involves the function of borrelial adhesins. Known surface-exposed adhesins that recognize host structures found in the ECM include the following: the lipoproteins DbpA and DbpB that bind decorin and glycosoaminoglycans (GAGs) (Guo *et al.*, 1995; Fischer *et al.*, 2003; Blevins *et al.*, 2008; Weening *et al.*, 2008); BBK32 that interacts with fibronectin and GAGs (Probert and Johnson, 1998; Kim *et al.*, 2004; Fischer *et al.*, 2006; Seshu *et al.*, 2006; Norman *et al.*, 2008); BmpA and ErpX that engage laminin (Brissette *et al.*, 2009; Verma *et al.*, 2009); P66 and BBB07 that adhere to integrin proteins (Behera *et al.*, 2008; Ristow *et al.*, 2012); and CspA and CspZ that bind to complement proteins, fibronectin, laminin and collagens (Rogers and Marconi, 2007; Kenedy *et al.*, 2009; Hallström *et al.*, 2010; Hallström *et al.*, 2013). Several of these proteins are categorized as MSCRAMMs (for microbial surface component that recognize adhesive matrix molecules) (Patti *et al.*, 1994), and some (Dbp's, BBK32, and P66) are linked to borrelial pathogenesis (Seshu *et al.*, 2006; Weening *et al.*, 2008; Lin *et al.*, 2012; Ristow *et al.*, 2012).

It is well established that *B. burgdorferi* modulates its gene expression as it cycles between the tick vector and the mammalian hosts they infect (Akins *et al.*, 1998; Ohnishi *et al.*, 2001; Liang *et al.*, 2002; Revel *et al.*, 2002; Brooks *et al.*, 2003; Narasimhan *et al.*, 2003; Ojaimi *et al.*, 2003; Samuels, 2011), including genes that encode infection-associated lipoproteins (Zhang *et al.*, 1997; Grimm *et al.*, 2004; Pal *et al.*, 2004; Seshu *et al.*, 2006; Weening *et al.*, 2008; Radolf *et al.*, 2012). For mammalian infection, the Rrp2-RpoN-RpoS regulatory pathway plays an important role in this process (Hubner *et al.*, 2001; Boardman *et al.*, 2008; Samuels, 2011; Radolf *et al.*, 2012) as this regulatory cascade is required for the expression of *ospC*, *dbpBA* and *bbk32*, all of which are required for optimal mammalian infection (Grimm *et al.*, 2004; Pal *et al.*, 2004; Seshu *et al.*, 2006; Weening *et al.*, 2008; Samuels, 2011; Radolf *et al.*, 2012). In addition to these pathogenesis-associated genes, several additional genes are regulated in a similar manner; however, the importance and function of the proteins they encode are not known. One such gene that is RpoS-dependent is *bba33*, which is found on the 54 kb linear plasmid of *B. burgdorferi* strain B31 (Fraser *et al.*, 1997). Previous studies showed that *bba33* was up-regulated 29-fold in implanted dialysis membrane chambers and 8.2-fold *in vitro* in an RpoS-dependent manner (Caimano *et al.*, 2007), and required Rrp2 for maximal expression (Boardman *et al.*, 2008), suggesting that BBA33 is produced under mammalian-like conditions. In support of this contention, Kumar *et al.* (2010) found that *bba33* is induced during a blood meal in ticks and is expressed in infected mouse skin. Furthermore, microarray and RT-PCR experiments revealed that *bba33* transcripts were significantly up-regulated in heart tissue but poorly expressed in the central

nervous system in *B. burgdorferi* infected nonhuman primates (Narasimhan *et al.*, 2003), suggesting that *bba33* expression is spatially regulated and thus may contribute to the dissemination and secondary colonization of *B. burgdorferi* to targeted organs. Taken together, these data suggested that BBA33 was linked to *B. burgdorferi* virulence; however, its function was unknown.

By analogy to other infection-associated lipoprotein-encoding genes that are regulated in a similar manner [e.g. *dbpA* or *bbk32* (Seshu *et al.*, 2006; Blevins *et al.*, 2008; Weening *et al.*, 2008)], we hypothesized that the protein encoded by *bba33* could contribute to pathogenesis-associated functions. We now report that the loss of *bba33* renders *B. burgdorferi* essentially noninfectious. A combinatorial peptide approach suggested that BBA33 recognizes a sequence that maps to human collagen type VI. Given its abundance in the skin and other sites colonized by *B. burgdorferi*, as well as the known association of *B. burgdorferi* with collagen (Barthold *et al.*, 1991), we evaluated the ability of BBA33 to bind collagen and mediate bacterial attachment to collagen substrates. The results presented herein demonstrate that BBA33 plays an important role in early stages of borrelial infection due presumably to its ability to interact with collagenous substrates.

Results

Molecular analysis of bba33

Given the significance of several RpoS-regulated *B. burgdorferi* genes in mammalian infection (Hubner *et al.*, 2001; Pal *et al.*, 2004; Blevins *et al.*, 2008; Weening *et al.*, 2008; Radolf *et al.*, 2012), including several that map to the linear 54 kb plasmid (lp54) of *B. burgdorferi* strain B31 (Blevins *et al.*, 2008; Weening *et al.*, 2008; Gilmore *et al.*, 2010; Kumar *et al.*, 2010), we sought to determine whether RpoS-dependent BBA33 contributed to borrelial pathogenesis. As a first step, we genetically deleted *B. burgdorferi bba33* as depicted in Fig. 1A. Briefly, a construct that replaced the entire *bba33* locus with a streptomycin-resistant (Str^R) cassette was made and designated pHZ001. This construct was transformed into strain ML23, and transformants were screened by PCR for allelic exchange of the native *bba33* gene with the Str^R marker (Fig. 1B). ML23 is a strain that lacks the 25 kb linear plasmid that encodes a restriction/modification gene (*bbe02*); as such, this strain is more readily transformable via allelic exchange (Seshu *et al.*, 2006; Weening *et al.*, 2008) but is also noninfectious since the *bbe22*/*pncA* locus is essential for survival following experimental infection (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001; Purser *et al.*, 2003). Infectivity can be restored via transcomplementation of *bbe22*/*pncA* on the shuttle vector pBBE22 (Purser *et al.*, 2003).

To assess the status of the Str^R transformants relative the parent strain, PCR was employed. Consistent with the deletion of *bba33*, a 1296 bp PCR product was amplified from the putative Δ*bba33* strain using primers 1 and 4, relative to the expected 841 bp fragment from the parent strain (Fig. 1B). As predicted, primers 4 and 5 failed to amplify any fragment from the parent strain ML23 pBBE22*luc*, whereas a Str^R-cassette containing 995 bp fragment was amplified from the putative Δ*bba33* mutant (Fig. 1B). Furthermore, primers 2 and 3 amplified a 672 bp fragment from the parent, but not from the putative Δ*bba33* mutant (Fig. 1B). The resulting Δ*bba33* mutant strain was designated HZ001 and then transformed

with pBBE22*luc* to both complement mammalian infectivity with *bbe22*, which is necessary in strains lacking lp25 (Purser *et al.*, 2003), and provide a constitutively expressed borrelial codon-optimized luciferase (*luc*) gene (Blevins *et al.*, 2007).

To genetically complement the *bba33* deletion *in trans*, intact *bba33* with both upstream (210 bp) and downstream (129 bp) sequences included was cloned into pBBE22*luc* (Hyde *et al.*, 2011a). The resulting construct, designated pHZ300, was transformed into the *bba33* deletion strain HZ001. PCR analysis of the transformed deletion strain (Fig. 2B) showed a 672 bp fragment similar to that seen for the initial parent strain (Fig. 1B). *B. burgdorferi* plasmid profiles of all strains were evaluated to ensure no further loss of plasmid DNA (Labandeira-Rey and Skare, 2001) (data not shown). All three strains made PncA/BBE22 protein and produced light, consistent with the presence of the *pncA* locus and firefly *luc* on pBBE22*luc* respectively (data not shown). Furthermore, all isolated strains grew at the same rate indicating that the loss of *bba33* did not alter *B. burgdorferi* replication *in vitro* (Fig. S1).

Conditions that mimic the mammalian host environment induce bba33

To determine whether BBA33 was produced in the deletion strain HZ001 pBBE22*luc* relative to its parent (ML23 pBBE22*luc*) and genetic complement (HZ001 pHZ300), we subjected protein lysates to Western immunoblot analysis. Prior studies indicated that temperature, pH and CO_2 content affected gene expression in *B. burgdorferi* (Stevenson *et al.*, 1995; Carroll *et al.*, 1999; Hyde *et al.*, 2007; Samuels, 2011). Furthermore, *bba33* was induced under conditions that result in the production of several virulence-associated proteins, including OspC, BBK32 and DbpA (Grimm *et al.*, 2004; Pal *et al.*, 2004; Seshu *et al.*, 2006; Weening *et al.*, 2008), which require Rrp2/RpoS for their induction (Hubner *et al.*, 2001; Yang *et al.*, 2003; Caimano *et al.*, 2007; Boardman *et al.*, 2008). To assess whether the aforementioned variables contributed to *bba33* induction, we subjected the aforementioned three strains to growth at either 32°C, 1% CO_2, pH 7.6 (our conventional growth conditions) or 37°C, 5% CO_2, pH 6.8 to mimic the mammalian host conditions *in vitro*. When equivalent amounts of *B. burgdorferi* proteins from these conditions were resolved by SDS-PAGE, lp54-encoded BBA33 was detected in the parent strain ML23 pBBE22*luc* grown at 37°C, 5% CO_2, pH 6.8 (Fig. 3, bottom panel, right), consistent with the induction of mammalian-induced proteins, including OspC (Fig. 3, middle panel). In contrast, lp54-encoded BBA33 was not detectable in ML23 pBBE22*luc* when grown at 32°C, 1% CO_2, pH 7.6 (Fig. 3, bottom panel, left). In fact, BBA33 levels were only observed at 37°C, 5% CO_2, pH 6.8; no other combination of temperature, CO_2 content or pH resulted in detectable levels of BBA33 (not shown). As expected, BBA33 was not detected in the Δ*bba33* mutant HZ001 pBBE22*luc*, regardless of growth condition (Fig. 3, lower panel). In contrast, when *bba33* is expressed from a cp9-derived shuttle vector in the genetic complement strain HZ001 pHZ300 (Fig. 3, lower panel), the levels of BBA33 produced were high independent of the conditions imposed, presumably due to the increased copy number of the episomal DNA relative to its native location on lp54, which may titrate out regulatory components that affect *bba33* expression. In support of this premise, previous studies estimated the copy number of cp9 at 5–10 per cell relative to linear replicons (Tilly *et al.*, 2006; Beaurepaire and Chaconas, 2007). In addition, a similar effect was seen when *dbpBA* was complemented *in*

trans; that is, shuttle-vector encoded DbpA was produced at levels that were approximately eightfold greater than the lp54-encoded version (Weening *et al.*, 2008). Given that *dbpBA* and *bba33* are similarly regulated (Blevins *et al.*, 2008; Weening *et al.*, 2008), it is not surprising that *in trans* encoded BBA33 is produced at a similarly high level.

BBA33 is a surface-exposed lipoprotein

The primary sequence of *B. burgdorferi* BBA33 predicts a lipoprotein with a leader peptidase II signal peptide at its amino terminus. To provide supporting evidence that BBA33 was surface exposed, we subjected intact *B. burgdorferi* strain ML23 pBBE22*luc* to proteinase K treatment. These cells were grown at 37°C, 5% CO_2, pH 6.8, to induce detectable levels of BBA33 (Fig. 3). The data showed that BBA33 was degraded in intact *B. burgdorferi* cells under conditions where P66 was accessible to protease [producing a known 50 kDa stable product; (Bunikis *et al.*, 1998)], but subsurface endoflagella (FlaB) was not (Fig. 4). The addition of detergent Triton X-100 together with proteinase K resulted in the loss of all antigens tested providing further support that the cells treated with PBS or proteinase K alone were intact (Fig. 4). Additional studies confirmed that BBA33 partitioned with Triton X-114, consistent with its predicted status as a lipoprotein (not shown). Taken together, these results demonstrate that BBA33 is a surface-exposed outer membrane lipoprotein of *B. burgdorferi*.

The loss of bba33 results in the rapid clearance of *B. burgdorferi*

Next, we asked if *B. burgdorferi* cells lacking *bba33* were attenuated using the murine infection model. To track the active infection of cells lacking BBA33, we infected C3H/HeN mice with the parent strain (ML23 pBBE22*luc*), the Δ*bba33* strain (HZ001 pBBE22*luc*) and the *bba33* complement strain (HZ001 pHZ300), all containing borrelial codon-optimized firefly luciferase at doses of 10^3 and 10^5 cells and measured light emission from these cells over time (Figs 5 and S2). At the lower inoculum dose, a signal is not detected above background levels for any of the strains prior to day 4. At day 4, a clear signal is observed in mice infected with the parent and complement strain (Fig. 5). The signal expanded with a peak at day 7 and then began to wane commensurate with the development of the adaptive immune response (Figs 5 and S2). At the low dose, the Δ*bba33* mutant did not emit light above background for any of the time points tested (Fig. 5). For the high dose, an equivalent light signal was detected at 24 hours following infection for all strains tested (Figs 5 and S2). However, at day 4 and beyond, no signal above background was detected in the Δ*bba33* strain HZ001 pBBE22*luc* (Fig. 5). Conversely, the parent and *bba33* complement strains exhibited similar light emission and dissemination profiles (Figs 5 and S2).

After 21 days, the mice infected with the parent, Δ*bba33*, and *bba33* complement, at both inoculum doses, were sacrificed and organ harvested for cultivation and quantitative PCR (qPCR) analysis. Consistent with the imaging data, the absence of *bba33* resulted in the inability of *B. burgorferi* to survive or colonize mice from any organ site tested at any inoculum dose tested including up to an infection dose of 10^7 Δ*bba33 B. burgdorferi* (Table 1). This is in stark contrast to the parent and complement strains, which were recovered from all organs independent of dose (Table 1).

To quantify the bacterial burden, qPCR was performed on total DNA extracted from the skin, lymph node, tibiotarsal joint and heart from mice infected with the parent, the Δ*bba33* mutant, and *bba33* complemented strains harvested at the same time as the *in vitro* cultivated samples. Consistent with the cultivation data shown in Table 1, few genomic equivalents were detected in tissues infected with the Δ*bba33* mutant HZ001 pBBE22*luc* at a 10^3 or 10^5 inoculum (Fig. 6A and B respectively). Also in line with the prior infection data (Fig. 5 and Table 1), the parent and complemented strains exhibited similar colonization phenotypes indicating that the defect observed in the mutant was due to the loss of BBA33 and not a secondary mutation (Fig. 6A and B). Interestingly, the overproduction of BBA33 in complemented strains resulted in lower colonization in joint tissue relative to the parent strain independent of infectious dose, yet exhibited similar bacterial burden in heart tissue (Fig. 6A and B). Taken together, these data indicate that BBA33 is required for infectivity in this genetic background. Furthermore, the absence of BBA33 from the surface of *B. burgdorferi* leads to rapid clearance, suggesting that BBA33 is required for the establishment of a localized infection and resistance to the host innate immune response.

Recombinant BBA33 mediates binding to collagens

Given that BBA33 was an infectivity-associated surface-exposed lipoprotein, we were next interested in identifying a function for this protein. To this end, we screened random cyclic peptide phage display libraries to identify peptide sequences that would selectively recognize recombinant BBA33 (rBBA33; Mullen *et al.*, 2006; Sergeeva *et al.*, 2006; Barbu *et al.*, 2010). We found that the number of phage binding to immobilized rBBA33 increased relative to background after several rounds of panning (based on BSA binding), suggesting a specific enrichment for BBA33. After the aforementioned panning, the DNA corresponding to the peptide insert from 100 randomly selected plaques was sequenced. An alignment of the deduced amino acids revealed enrichment for the sequence PGEPGLN, which is found in the human collagen type VI alpha 3 chain. However, in collagen, this sequence is present in the triple helix collagenous domains, and it is unlikely that all residues identified in the cyclic peptide would be available for interactions in the structurally restricted triple helix. To explore the possibility that BBA33 can bind collagens, we used an ELISA-type binding assay where increasing concentrations of purified rBBA33 protein were incubated in wells coated with different collagen types and other ECM proteins. A concentration-dependent binding of BBA33 to type VI collagen was noted with saturation at 2 μM and half maximum binding (e.g. apparent K_D) at approximately 350 nM (Fig. 7A). In contrast, purified OspC did not bind to collagen type VI at any concentration tested (Fig. 7A) indicating that the BBA33::collagen type VI interaction is specific. BBA33 showed dose-dependent binding to collagen type IV but not to human collagen type I, fibronectin and murine laminin, indicating specificity for a subset of targets (Fig. 7B). When BBA33 was subjected to heat inactivation, the binding to collagen observed was eliminated, suggesting that the native conformation was required for dose-dependent binding (not shown).

Native BBA33 binds to type VI collagen

We next sought to determine whether native BBA33, presented on the surface of *B. burgdorferi* cells, could promote bacterial attachment to the identified BBA33 targets. A significant difference in borrelial cell attachment was observed to collagen type VI when the

bba33 complement (HZ001 pHZ300; designated C) was compared with the parent strain ML23 pBBE22*luc* (designated P) and Δ*bba33* strain HZ001 pBBE22*luc* (designated M) when the cells are grown at 32°C, 1% CO_2, pH 7.6 (Fig. 8, left); in contrast, no difference was seen in the attachment to laminin to any of the strains tested (Fig. 8). Under these conditions, the levels of BBA33 are low in the parent strain (Fig. 3); therefore, the low level of binding observed for the parent strain ML23 pBBE22*luc* is consistent with the lack of BBA33 produced when the parent is grown under noninducing conditions and thus, not surprisingly, comparable with the Δ*bba33* mutant (Fig. 8, left). However, when the cells were grown under conditions that promote the production of BBA33 (Fig. 3; e.g., 37°C, 5% CO_2, pH 6.8), a significant difference in collagen type VI binding was observed between the parent ML23 pBBE22*luc* and the Δ*bba33* mutant HZ001 pBBE22*luc*; no significant differences to laminin binding were noted (Fig. 8, right). As expected, the *bba33* complement HZ001 pHZ300 strain binds collagen type VI under inducing conditions (Fig. 8, right) as shuttle vector encoded BBA33 is synthesized at high levels independent of growth parameters (Fig. 3). These data are consistent with the induction of *bba33* under the 'mammalian-like' induction conditions and provide further support that surface-exposed native BBA33 specifically recognizes collagen type VI.

Discussion

Interaction with the host ECM is an essential part of the pathogenic process of *B. burgdorferi*. *B. burgdorferi* is transmitted from ticks to mammalian host during a blood meal and, as a result, is deposited within the dermal layer of the skin where collagen and other components of the ECM reside (Cabello *et al.*, 2007; Radolf *et al.*, 2012). Some of the first micrographs of *B. burgdorferi* from infected tissue showed spirochetal bacteria aligned with collagen fibrils (Barthold *et al.*, 1991; Cabello *et al.*, 2007). Subsequent infectivity studies demonstrated that the association of *B. burgdorferi* with ECM tissue is required for persistence (Cabello *et al.*, 2007; Brissette and Gaultney, 2014), although the basis for all *B. burgdorferi*–ECM interactions was not completely defined.

In this study, we describe a collagen binding lipoprotein from *B. burgdorferi*, designated BBA33, which is required for experimental Lyme borreliosis. To date, the only data associated with *bba33* is its dependence on the BosR/Rrp2-RpoN-RpoS regulatory pathways for expression (Caimano *et al.*, 2007). Despite the observation that *bba33* was subject to the same degree of regulation observed for other borrelial virulence determinants (Hubner *et al.*, 2001; Yang *et al.*, 2003; Caimano *et al.*, 2007; He *et al.*, 2007; Boardman *et al.*, 2008), notably *ospC*, *dbpBA* and *bbk32* (Hubner *et al.*, 2001; He *et al.*, 2007), no function was known for BBA33. Using phage display, we identified a sequence found in type VI collagen as a target for BBA33. To biochemically validate the predicted BBA33–collagen interaction, we used an ELISA-based assay and found that BBA33 binds to collagen types IV and VI in a dose-dependent manner but does not bind to collagen I, laminin or fibronectin (Fig. 7B). To address the ability of surface-exposed native BBA33 to recognize ECM proteins, we incubated whole cells producing BBA33 with human collagen type VI and laminin. The results in Fig. 8 show that the genetic complement strain HZ001 pHZ300, which produces large amounts of BBA33, significantly enhances the ability of the spirochete to adhere to collagen type VI relative to the parent strain (ML23 pBBE22*luc*) and Δ*bba33* strain (HZ001

pBBE22*luc*) that are grown under conditions where BBA33 is not produced (Fig. 3). These data appear to contradict earlier findings that saw no binding of *B. burgdorferi* to collagens (Guo *et al.*, 1995). However, upon reflection, this observation may be consistent with our findings as lp54-encoded *bba33* is only expressed in the parent strain under conditions that mimic mammalian host adapted conditions (Fig. 3). The prior work was done using conventionally grown *B. burgdorferi* (Guo *et al.*, 1995), which represents experimental conditions that do not yield detectable levels of BBA33 (Fig. 3). In addition, when the parent strain ML23 pBBE22*luc* is grown under inducing conditions, the level of collagen type VI binding increases significantly consistent with the production of BBA33 within this same environment (Fig. 3).

The ability of bacteria to adhere to collagen type VI is known for a few pathogens, including *Legionella* and some *Staphylococcus* spp. (Liu *et al.*, 2004; Wagner *et al.*, 2007; Bober *et al.*, 2010), but is still poorly characterized relative to other pathogen–collagen interactions (Zong *et al.*, 2005; Liu *et al.*, 2007). It is important to note that, in the case of collagen type VI binding by the *Staphylococcus capitis* protein SdrX, the recombinant protein bound to several types of collagens in ELISA-based binding assays but only type VI collagen when *S. capitis* cells were used to query collagen binding (Liu *et al.*, 2004). As such, the ability of *B. burgdorferi* cells to bind to collagen type VI provides similar support for BBA33-type VI collagen interactions.

The recognition of collagen type VI by BBA33 is of further interest given the recent reports demonstrating the ability of activated macrophages to secrete this particular collagen in a nonfibrillar form as is normally seen in fibroblasts (Schnoor *et al.*, 2008). Two molecules that stimulate the production and secretion of type VI collagen include IL-10 and TGF-β1, both of which are induced early in *B. burgdorferi* infection (Codolo *et al.*, 2008; Lazarus *et al.*, 2008). In addition, both of these cytokines negate the inflammatory response of cells, including activated macrophages (Bogdan and Nathan, 1993; Oosting *et al.*, 2014) and, by virtue of their ability to secrete collagens, notably type VI collagen (Schnoor *et al.*, 2008), might create an additional platform for *B. burgdorferi* to bind. Based on the concurrent deactivation of previously activated macrophages, this microenvironment may promote localized colonization of *B. burgdorferi* and assist in the dissemination of spirochetes to distal sites. Based on our current data, it is unclear if this is operative *in vivo*; however, the dramatic attenuation of cells lacking BBA33 suggests that this lipoprotein plays an important role early in *B. burgdorferi* infection.

Type VI collagen also interacts with both biglycan and decorin, two proteoglycans that *B. burgdorferi* recognizes via the surface exposed lipoproteins, DbpA and DbpB (Brown *et al.*, 1999; Fischer *et al.*, 2003). It is tempting to speculate that *B. burgdorferi* infection stimulates the production of type VI collagen locally within the skin via innate immune cells and fibroblasts to generate a scaffold that borrelial cells can actively engage via a number of adhesins that bind to ECM targets. Further experimentation is required to test this hypothesis.

Perhaps the more striking result presented herein is the absolute loss of infectivity seen for the Δ*bba33* mutant strain HZ001 pBBE22*luc*. The isolation and characterization of

additional isogenic mutants in other *B. burgdorferi* genetic backgrounds will be important to further validate the findings reported herein. In this regard, a prior transposon mutant library screen demonstrated that *bba33* transposon mutants are infectious in only 16% of the tissues evaluated (Lin *et al.*, 2012). This degree of attenuation for *bba33* mutants is comparable with transposon insertion mutants in the *dbpBA* locus (Lin *et al.*, 2012), which encode the virulence-associated decorin binding protein adhesins (Guo *et al.*, 1995; Cabello *et al.*, 2007). Mutants in *dbpBA* exhibit a significant infectivity defect with an approximate three log increase in ID_{50} (Blevins *et al.*, 2008; Weening *et al.*, 2008) and are cleared early in the host as assessed by *in vivo* imaging in a manner that is indistinguishable from the Δ*bba33* strain (Fig. 5 and Hyde *et al.*, 2011a). Given the similarities of the *dbpBA* and *bba33* mutant phenotypes, the transposon mutant screen provides additional genetic evidence that BBA33 is involved in *B. burgdorferi* pathogenesis.

From our imaging data of bioluminescent spirochetes (Fig. 5), it is clear that the Δ*bba33* mutant is cleared early in the infectious process at time points where the ML23 pBBE22*luc* parent and the resulting genetic complement are replicating *in vivo*, suggesting that BBA33 may assist in the resistance to innate immune clearance. It is curious that the complement protein, C1q, contains a collagen-like motif (Kishore and Reid, 2000). Although C1q is involved in the classical pathway of complement, a serum-sensitive *B. burgdorferi sensu lato* isolate*, B. garinii*, is resistant to serum when C1q is selectively depleted (van Dam *et al.*, 1997), suggesting that the interaction of C1q and *Borrelia* isolates is key for innate survival. If and how BBA33 and C1q interact are currently being evaluated.

A diverse set of pathogens are known to bind to mammalian collagen-rich tissues to promote colonization (Umemoto *et al.*, 1997; Bober *et al.*, 2010; Foster *et al.*, 2014). The structure of several of these interactions has been resolved yielding important insight into the binding process (Zong *et al.*, 2005). The best characterized is the Cna collagen binding protein from *Staphylococcus aureus*, which uses a collagen-hug mechanism to bind host collagen (Zong *et al.*, 2005). This interaction is important for pathogenesis as Cna is required for several pathogenic features associated with experimental *S. aureus* infections, including ocular keratitis, osteomyelitis and arthritis (Foster *et al.*, 2014). The portion of Cna that mediates collagen binding is composed of two subdomains, each adopting an IgG-like fold (Zong *et al.*, 2005). This same beta-pleated sheet rich structure is seen or presumed for the homologous collagen binding protein from *Streptococcus* and *Enterococcus* spp (Lannergard *et al.*, 2003; Liu *et al.*, 2007). Several algorithms predict that BBA33 is devoid of beta-pleated sheet structure and instead is largely alpha helical in nature. If this is true, then the mechanisms that BBA33 employs to bind collagen are likely to be novel relative to that seen for Cna and related collagen binding proteins.

In summary, herein, we demonstrate that *B. burgdorferi* BBA33 is a surface-exposed lipoprotein that is expressed preferentially under conditions that mimic the mammalian host environment. In addition, we show that BBA33 mediates specific interaction of *B. burgdorferi* with collagen substrates (i.e. human collagen type VI and murine type IV collagen). Despite a long history of *B. burgdorferi*–collagen interactions, to our knowledge, BBA33 represents the first *B. burgdorferi* protein that recognizes human collagens in a dose-dependent manner. Imaging of bioluminescent Δ*bba33 B. burgdorferi* showed that the loss

of BBA33 results in rapid clearance, which suggests a role in resistance to innate immune mechanisms. Further studies are warranted to investigate the significance of this interaction *in vivo*.

Experimental procedures

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are described in Table 2. *Escherichia coli* strains were grown with aeration in Luria broth media at 37°C. Concentrations of antibiotics used in *E. coli* for selective pressure are as follows: kanamycin, 50 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}. *B. burgdorferi* B31 ML23 (Labandeira-Rey and Skare, 2001) and derivative strains were grown in BSK-II media supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR, USA) under either conventional microaerobic conditions at 32°C, at pH 6.8 or pH 7.6, under a 1% or 5% CO_2 atmosphere, or at 37°C, pH 6.8 or pH 7.6, under a 1% or 5% CO_2 atmosphere. For most experiments, *B. burgdorferi* cultures were grown at either 32°C, 1% CO_2, pH 7.6 (noninducing or conventional conditions) or at 37°C, 5% CO_2, pH 6.8 (inducing conditions). Growth was scored daily by dark field microscopy. Antibiotics used for the selective pressure in *B. burgdorferi* are as follows: kanamycin at 300 μg ml^{-1} and/or streptomycin at 50 μg ml^{-1}, depending on genetic composition. The use of infectious *B. burgdorferi* in this study was reviewed and approved by the Institutional Biosafety Committee at Texas A&M University.

Genetic modification of *B. burgdorferi*

Borrelia burgdorferi deleted for *bba33* was generated via homologous recombination by replacing all of *bba33* with a P_{flgB}-*aadA* (Str^R) antibiotic cassette (Frank *et al.*, 2003). To accomplish this, a 1526 bp fragment upstream of *bba33* was amplified using the primer pairs BamHI-A33us-recF and flgB-A33us-recR (Table 3) that created a 5′ overhang to pCR2.1 with the *Bam*HI site and a 3′ overhang homologous to P_{flgB} sequences from pKFSS1 (Table 3). An additional 1527 bp PCR product, which was directed to sequences downstream from *bba33*, was engineered with primers aadA-A33ds-recF and A33ds-XhoI-recR (Table 3) with overlapping sequences to sequences downstream to the stop codon of the *aadA* locus (Str^R) at the 5′ end, and sequences homologous to pCR2.1, including the *Xho*I site. Finally, a PCR product containing the P_{flgB}-Str^R cassette was amplified from plasmid pKFSS1 using primer set FlgB-F and aadA-R (Table 3). All three PCR products, each containing homologous sequences, along with pCR2.1 digested with *Bam*HI and *Xho*I, were combined and subjected to Gibson assembly as outlined by the manufacturer (New England Biolabs). The resulting construct was designated pHZ001 (Table 3). Transformation of strain ML23 with linearized pHZ001 was done as previously described (Samuels, 1995; Weening *et al.*, 2008; Hyde *et al.*, 2011b). Transformants were selected for their resistance to streptomycin and screened by PCR to confirm the deletion of *bba33* using primers listed in Table 3 and as depicted in Fig. 1. Candidates were screened for borrelial plasmid content as before (Labandeira-Rey and Skare, 2001), and those that mirrored strain ML23 were transformed with pBBE22*luc* and transformants were selected for resistance to kanamycin (Hyde *et al.*, 2011a). Putative transformants were screened for the presence of pBBE22*luc* and total

borrelial plasmids using methods described previously (Labandeira-Rey and Skare, 2001; Hyde *et al.*, 2011a).

For genetic complementation, *bba33* and 210 bp 5′ to the translation start site were cloned into the *Bam*HI and *Sal*I sites in the pBBE22*luc* shuttle vector to yield the final construct, pHZ300, using the oligonucleotides used are listed in Table 3. HZ001 was then made competent and electroporated with pHZ300 as described (Samuels, 1995). Transformants were selected for resistance to kanamycin, and candidates were confirmed by PCR as indicated in Fig. 2. As before, borrelial plasmid DNA was accounted for using previously published methods (Labandeira-Rey and Skare, 2001). Only strains that contained the DNA profile of transformed ML23 were used for subsequent analyses.

Infectivity studies and bioluminescent imaging

Infectivity studies were performed as previously described (Hyde *et al.*, 2009). Described briefly, 8-week-old C3H/HeN mice were inoculated with either 10^3 or 10^5 organisms of the *B. burgdorferi* parent strain ML23/pBBE22*luc*, the Δ*bba33* strain HZ001/pBBE22*luc*, or the genetic complement strain HZ001/pHZ300 by intradermal injection. For each dose and strain used, four mice were infected per group. Imaging of infected mice to detect bioluminescence in the aforementioned *B. burgdorferi* strains was done as described in Hyde *et al.* (Hyde *et al.*, 2011a). After 21 days, the mice were sacrificed, and inguinal lymph node, skin, heart, spleen, bladder and tibiotarsal joint tissues from each mouse were aseptically collected for *in vitro* cultivation and qPCR analysis of *B. burgdorferi* burden as described (Weening *et al.*, 2008; Hyde *et al.*, 2011a). All animal experiments were performed in accordance to the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. The Texas A&M University Institutional Animal Care and Use Committee (IACUC) approved all animal procedures used in this study.

DNA extraction of *B. burgdorferi* from infected tissues and qPCR analysis

Total DNA was isolated from mice skin, lymph node, heart and tibiotarsal joint samples using Roche High Pure PCR template preparation kit as previously described (Weening *et al.*, 2008). Approximately 100 ng of total DNA was used for each qPCR reaction. Quantitative real-time PCR analysis was conducted using the Applied Biosystems ABI 7900 HT system. *B. burgdorferi* genome copies and mammalian cell equivalents were determined using either the oligonucleotides nTM17FRecA and nTM17RRecA (Liveris *et al.*, 2002; Weening *et al.*, 2008) and primer set βactin-F and βactin-R (Pal *et al.*, 2008) respectively. The bacterial burden was depicted as the number of *B. burgdorferi recA* per 10^6 β-actin copies.

Purification of rBBA33

To overproduce and purify rBBA33, a construct lacking the first 51 bp of the *bba33* open reading frame (corresponding to the leader peptide) was amplified from *B. burgdorferi* genomic DNA using primer set A33-NdeI-5′F and A33-BamHI-3′R (Table 3) and cloned in-frame into pET15b following its digestion with *Nde*I and *Bam*HI. The resulting construct, designated pSS008, encodes an amino terminal His-tagged version of BBA33. To affinity purify His-tagged BBA33, pSS008 was transformed into BL21 Star™(DE3) pLysS, grown to

early log phase and the His-tagged BBA33 produced following induction with 0.2 mM IPTG for 16 h at 15°C. The cells were harvested by centrifugation, the pellet frozen and lysed via French pressure cell treatment. The soluble fraction, containing the His-tagged BBA33 protein, was applied to HisPur Cobalt resin (Thermo scientific) and affinity purified as outlined by the manufacturer.

Rabbit polyclonal antiserum to BBA33 was obtained following immunization of New Zealand white rabbits via an IACUC approved regimen at the Comparative Medicine Program, Texas A&M University.

SDS-PAGE and immunoblotting

Borrelia burgdorferi protein lysates were resolved by SDS-PAGE (Laemmli, 1970), and gels were either stained with Coomassie Brilliant Blue R-250 (Sigma Aldrich, St. Louis, MO, USA) or transferred to PVDF membranes and immunoblotted as described (Weening *et al.*, 2008). Primary antibodies were used at the following dilutions: anti-BBA33 at 1:2000; anti-His_6 (GE Healthcare) at 1:6000; anti-OspC at $1:4 \times 10^6$ (a kind gift from R. Gilmore); anti-PncAat 1:1500 (generously provided by J. Seshu); anti-P66 at 1:1000 (kindly provided by Sven Bergström); or anti-FlaB at 1:20,000 (Affinity Bioreagent, Golden, CO, USA). Prior to its use for Western immunoblotting, the BBA33 antiserum was adsorbed against HZ001 borrelial lysates to reduce the amount of nonspecific antibodies, as reported previously (Skare *et al.*, 1999). Appropriate secondary antibodies, with horseradish peroxidase (HRP) conjugates [anti-mouse HRP (Invitrogen, Carlsbad, CA, USA) or anti-rabbit HRP (Amersham, Piscataway, NJ, USA), both diluted 1:4000] were used to detect immune complexes, the membranes washed extensively in PBS, 0.2% Tween-20, and developed using the Western Lightning Chemiluminescent Reagent plus system (Perkin Elmer, Waltham, MA, USA).

Proteinase K accessibility assay

Borrelia burgdorferi strain ML23 pBBE22*luc* was grown under inducing conditions (37°C, 5% CO_2, pH 6.8) and harvested by centrifugation at 5,800 × *g*, and washed twice with PBS. The cell pellet was resuspended in 0.5 ml of either PBS alone, PBS with proteinase K (to a final concentration of 200 μg ml^{-1}) or PBS/proteinase K with 0.05% Triton X-100. All samples were incubated at 20°C for 40 min. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM. Cells were again pelleted by centrifugation (9,000 × *g* for 10 min at 4°C), washed twice with PBS containing 1 mM PMSF and resuspended in Laemmli sample buffer (Laemmli, 1970). Samples were run on SDS-PAGE gel and probed with anti-BBA33, anti-P66 and anti-FlaB antibodies respectively.

Triton X-114 phase partitioning

Borrelia burgdorferi strain ML23 pBBE22*luc* was grown under inducing conditions (37°C, 5% CO_2, pH 6.8) and subjected to Triton X-114 phase partitioning as previously described (Skare *et al.*, 1995).

Solid-phase phage display screening

A phage library displaying random cyclic 8-mer peptides (CX_8C; X is any amino acid) was used for the screening against rBBA33 immobilized onto microtiter wells overnight at 4°C (at 1 μg per well in PBS). After the wells were washed twice with PBS and blocked with PBS containing 3% BSA for 1 h at 23°C, 10^9 PFU in 50 μl PBS containing 2% BSA were added to the wells (Barbu *et al.*, 2010). After 2 h at 23°C, the wells were washed six times with PBS containing 0.1% Tween 20 and three times with PBS, and phage were recovered by bacterial infection as described (Smith and Scott, 1993). Phage recovered after the second and third round of selection were subjected to dideoxy sequencing and the resulting deduced amino acid sequences compared in the existing NCBI protein database.

In vitro ECM binding assay

Maxinunc microtiter plates (eBiosciences) were coated with 0.5 μg human collagen I (Corning), human collagen VI (Abcam), bovine fibronectin (Corning), mouse collagen IV (Corning) and mouse laminin (Corning) at 4°C for overnight, and blocked with 3% BSA for 1 h at 37 °C. Recombinant His-tagged BBA33 protein was serially diluted 1:2 starting from 4 μM down to 62.5 nM in PBS, 0.1% Tween-20, and added to coated wells in triplicate and incubated for 1 h at 37 °C. After five washes in PBS, 0.1% Tween-20, a 1:6000 dilution of monoclonal antibody directed against poly-His (His_6) was added to each well and incubated for 1 h at 37°C. Following five washes in PBS, 0.1% Tween-20, a 1:4000 dilution of HRP conjugated antimouse IgG was added to each well and incubated for 1 h at 37°C. The wells were then washed in PBS, 0.1% Tween-20, after which 3,3′,5,5′-tetramethylbenzidine was added as substrate. The enzymatic reaction was stopped after 3 min using 0.16 M sulfuric acid (Thermo scientific), and the absorbance at 450 nm was determined.

B. burgdorferi whole cell adherence assay

Poly-D-lysine precoated coverslips (Corning Biocoat) were coated with 2 μg human collagen VI or mouse laminin and incubated at 4°C overnight. The coverslips were washed thoroughly in PBS to remove excess unbound proteins. The coverslips were then blocked with 3% BSA at 37°C for 1 h. The *B. burgdorferi* parent strain ML23 pBBE22*luc*, the Δ*bba33* strain HZ001/pBBE22*luc* and the genetic complement strain HZ001/pHZ300 were all grown to exponential phase at either 32°C, 1% CO_2, pH 7.6 (noninducing conditions) or 37°C, 5% CO_2, pH 6.8 (inducing conditions). The resulting cells were then harvested by centrifugation at 5,800 × *g* and washed with BSK-II medium without serum three times, and diluted to 10^7 organisms per ml in BSK-II medium without serum. The resulting *B. burgdorferi* cells, in 0.1 ml volumes, were applied onto the coverslips and incubated for 2 h at 32°C. Unbound bacteria were removed from the coverslips by gentle washing with PBS; this wash step was repeated 10 times. The coverslips were applied to a glass slide, and attached bacteria were counted by dark field microscopy. Binding was scored as the average number of bacteria bound per field based on 10 random fields per slide.

Statistical analysis

For real-time qPCR analysis, a one-tailed Mann–Whitney *t*-test was performed between the strains indicated. For ELISA and bioluminescent assays, two-way analyses of variance

(ANOVA) was performed respectively among variables. Tukey's post-test was used to determine *P* values between coated proteins. Statistical significance was accepted when the *P* values were less than 0.05 for all statistical analyses employed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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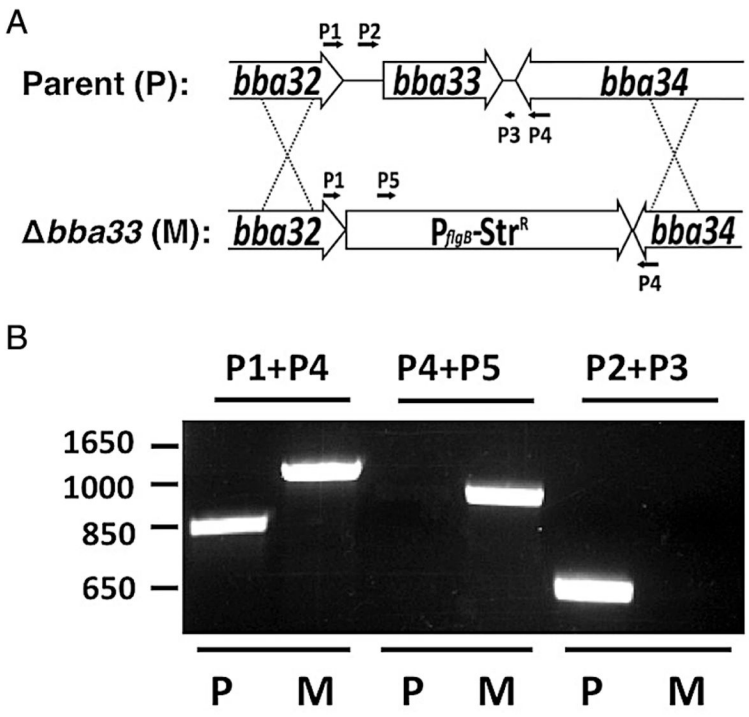

Fig. 1.

A. Schematic representation of the *bba33* deletion strategy. The *bba33* locus and its flanking region were replaced by a P_{flgB}-Str^R cassette by homologous recombination.

B. Primer pairs P1/P4 and P4/P5 (Table 3) were used to confirm the presence of P_{flgB}-Str^R cassette in the *bba33* deletion mutant strain HZ001 (M, Δ*bba33* mutant) relative to its parent strain ML23 (P, parent) by PCR. Likewise, PCR with the primer pair P2/P3 (Table 3) confirmed that ML23 alone carried intact *bba33* (P).

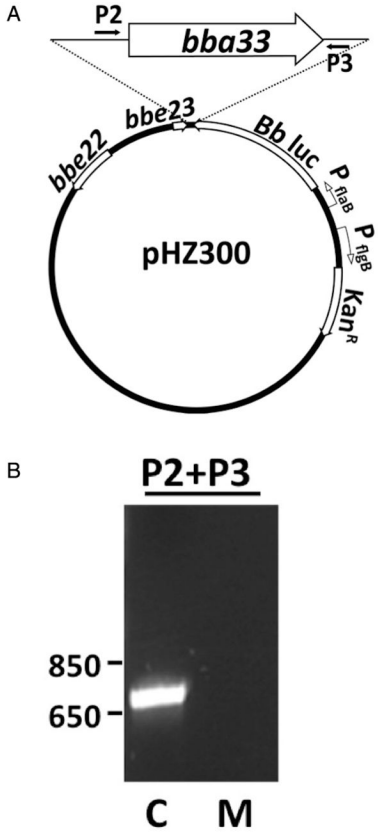

Fig. 2.

A. Schematic of the *bba33* complementation construct pHZ300.

B. PCR using primer pairs P2/P3 confirmed the presence of *bba33* in the complemented strain HZ001 pHZ300 (C; complement) relative the Δ*bba33* strain HZ001 pBBE22*luc* (M; Δ*bba33* mutant).

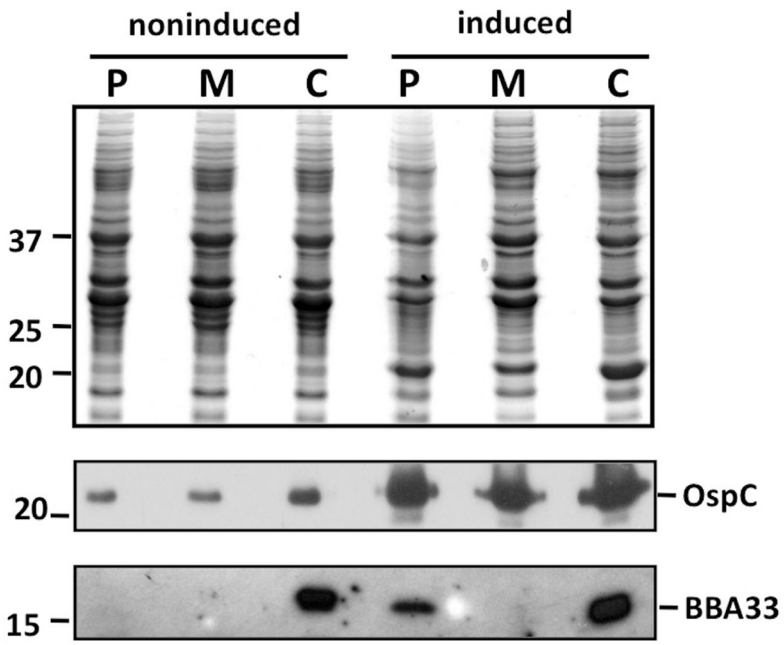

Fig. 3.

BBA33 is produced under conditions that mimic the mammalian-like environment. Equivalent amount of protein were loaded for the *B. burgdorferi* parent strain ML23 pBBE22*luc* (P for parent), the Δ*bba33* strain HZ001 pBBE22*luc* (M for mutant) and the genetic complement HZ001 pHZ300 (C for complement) from cells that were either grown in media under 'noninduced' (32°C, 1% CO_2, pH 7.6; left 3 lanes) or 'induced' conditions (37°C, 5% CO_2, pH 6.8; right 3 lanes), separated by SDS-PAGE, and stained with Coomassie blue (top panel) or immunoblotted with anti-OspC (middle panel), or anti-BBA33 serum (bottom panel). Note that the only condition where the parent strain made BBA33 is shown; no other combination of temperature, CO_2 content, or pH resulted in the detection of BBA33. The numbers on the side panel depict the molecular mass of protein markers (in kDa).

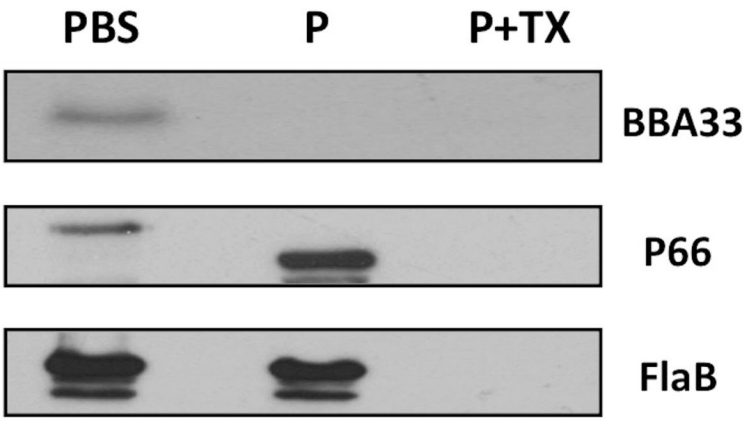

Fig. 4.

BBA33 is surface exposed. *Borrelia burgdorferi* strain ML23 pBBE22*luc* was cultivated at 37°C, 5% CO_2, pH 6.8 (inducing conditions) and harvested. The cells were then either resuspended in buffer (PBS), incubated with Proteinase K (P) or treated with both Proteinase K and Triton X-100 (P + TX). Following processing, the resulting samples were subjected to SDS-PAGE and immunoblotted with antiserum directed against either BBA33, the outer membrane P66 protein, or the subsurface FlaB protein.

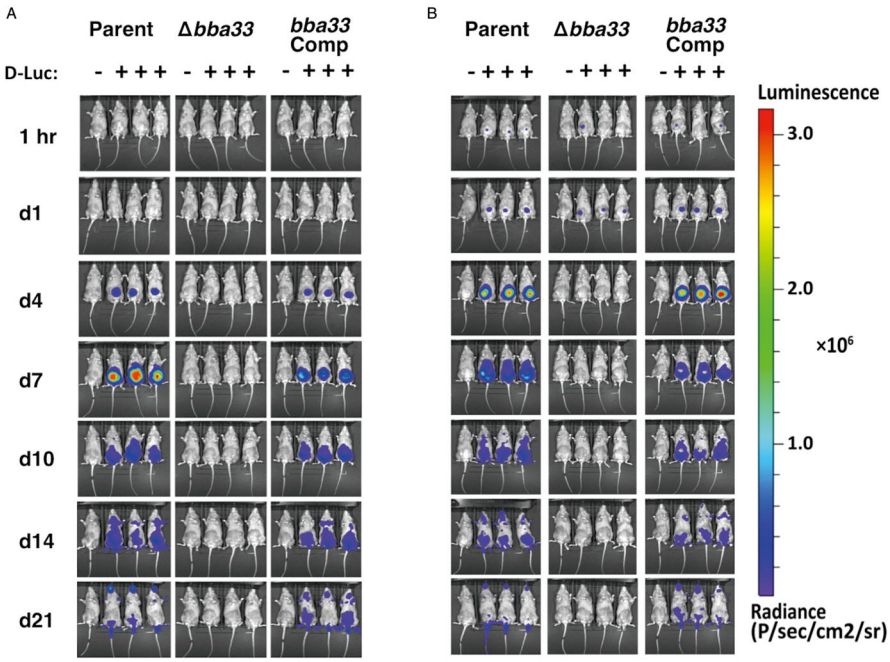

Fig. 5.

Temporal and spatial tracking of *Borrelia burgdorferi* strains following infection with 10^3 and 10^5 spirochetes. C3H mice were infected with the parent (Parent; ML23 pBBE22*luc*), the Δ*bba33* mutant (Δ*bba33*; HZ001 pBBE22*luc*) or the *bba33* complemented strain (*bba33* Com., HZ001 pHZ300) at a dose of 10^3 (A) and 10^5 (B). Mice denoted with '+' were treated with D-luciferin and imaged at the times listed on the left. For each image shown, the mouse on the far left (denoted as '−') was infected with *B. burgdorferi* but did not receive D-luciferin to serve as a background control. All images were normalized to the same scale (shown on the right).

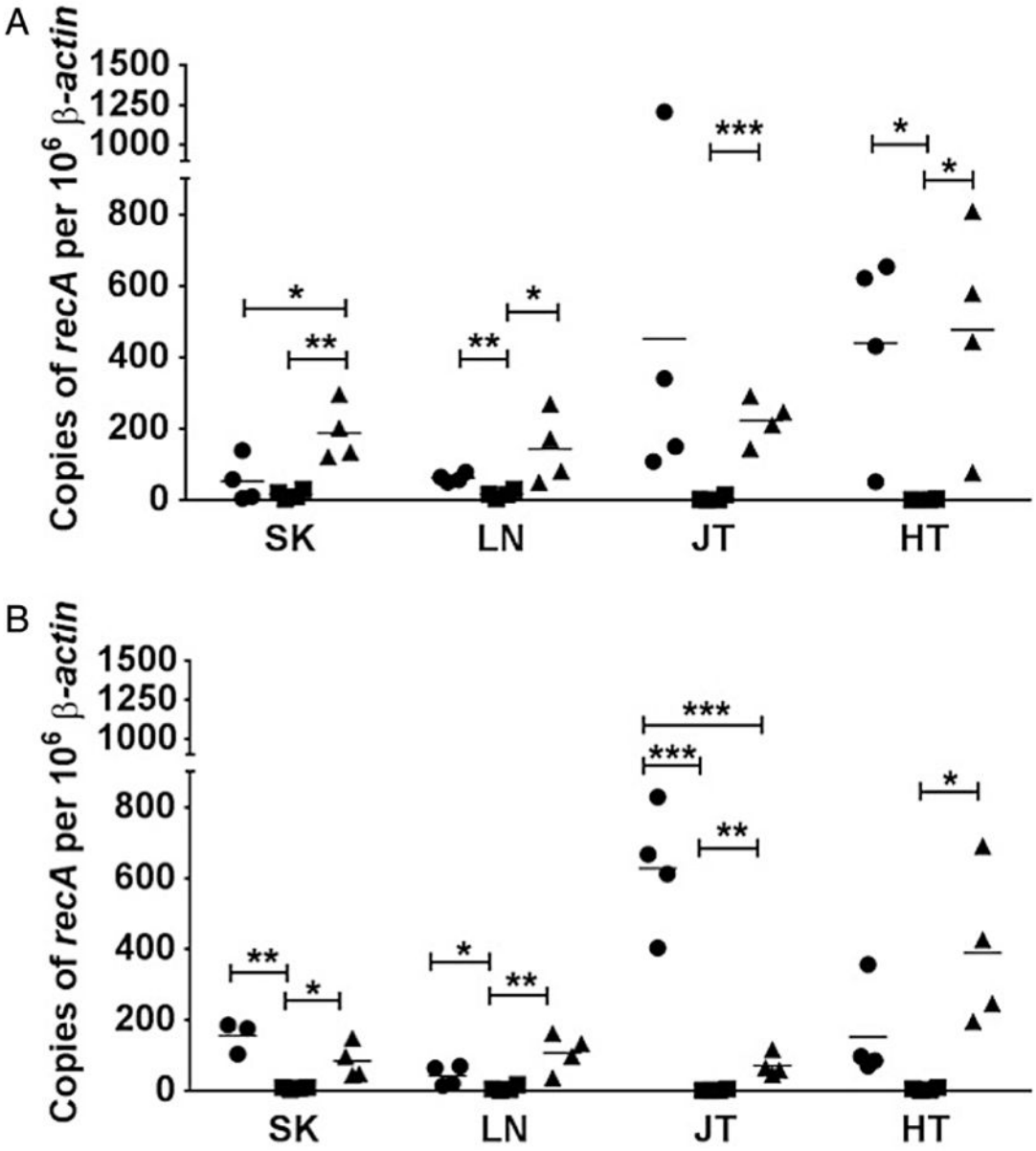

Fig. 6.

Quantitative assessment of *Borrelia burgdorferi* infection in C3H mice. Real-time PCR (qPCR) of the parental strain *B. burgdorferi* ML23 pBBE22*luc* (circles), the Δ*bba33* derivative HZ001 pBBE22*luc* (squares) and the genetic complement HZ001 pHZ300 (triangles) was used to enumerate borrelial genomic equivalents from mouse tissues. Mice were infected with either 10^3 (A) or 10^5 (B) *B. burgdorferi* strains for 21 days before total DNA was obtained from skin (SK), lymph nodes (LN), joints (JT) and heart (HT) tissues. The results are represented as the number of borrelial genome copies per 10^6 mouse β-actin copies. The horizontal line depicts the mean value. Each data point shown represents an independent sample from a single mouse tissue assayed in triplicate and averaged. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

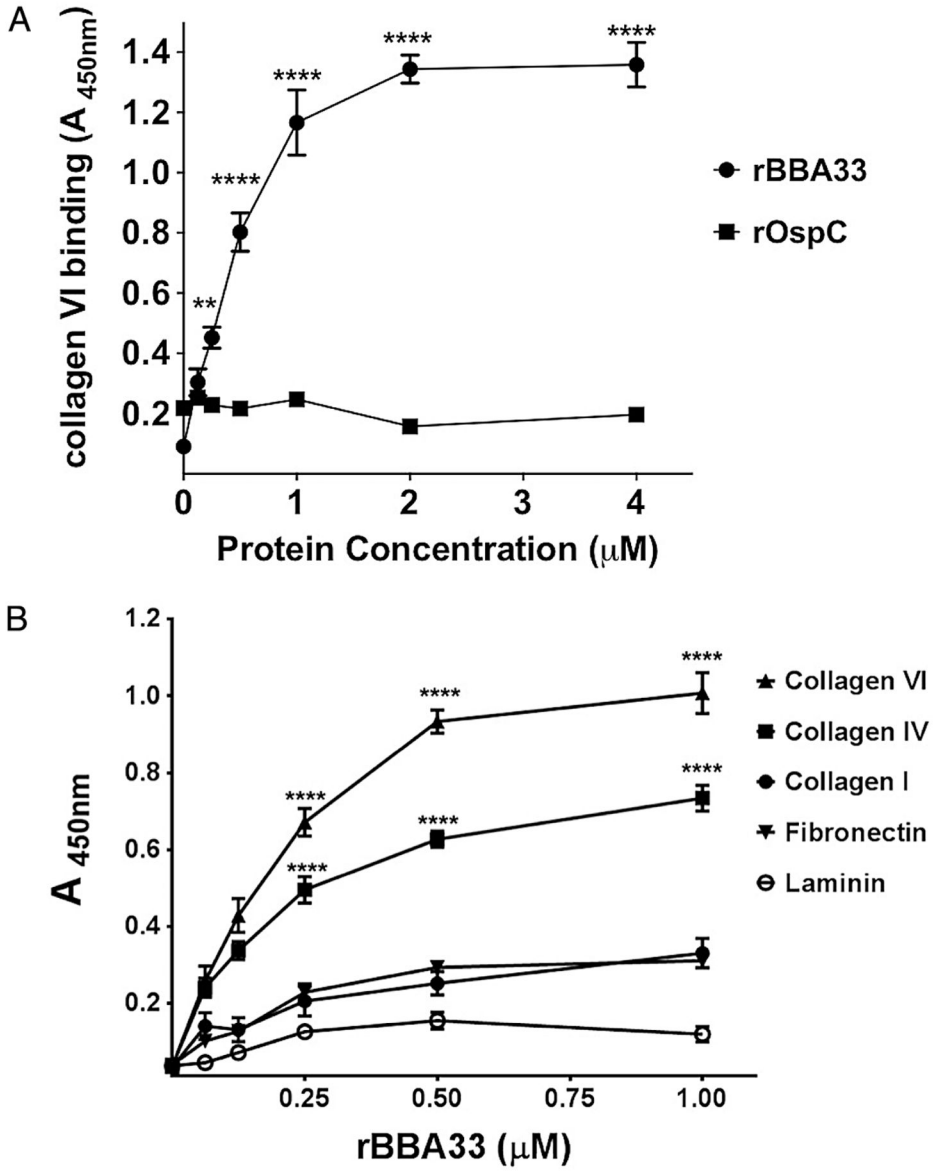

Fig. 7. BBA33 binds to type VI collagen and type IV collagen in a dose-dependent manner

A. Various concentrations of purified BBA33 were incubated with type VI collagen. Identical concentrations of OspC were tested for type VI collagen interaction as well and served as a negative control.

B. Binding of BBA33 to collagens type I, IV, and VI, as well as fibronectin and laminin, was evaluated as in panel A. Binding to collagens type VI and IV was dose dependent, whereas binding to fibronectin and laminin did not demonstrate specific binding to BBA33. All assays were done independently three times, each time in duplicate. $^{**}P < 0.01$; $^{****}P < 0.0001$.

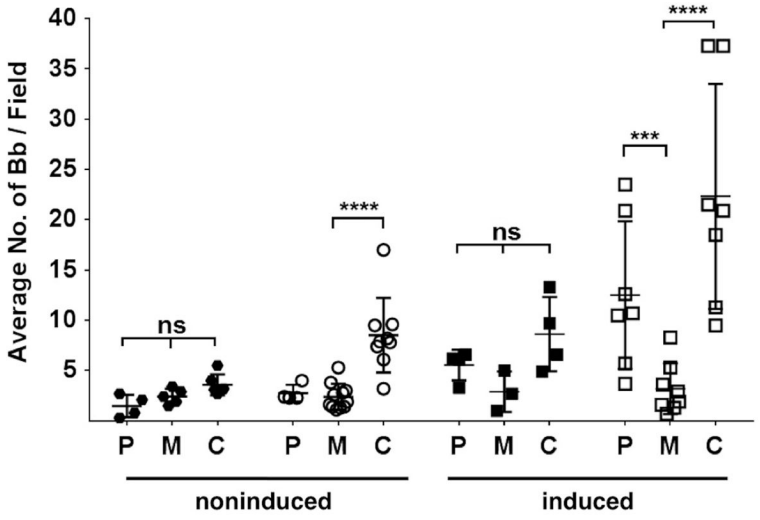

Fig. 8. Borrelia burgdorferi

whole cells that produce surface exposed BBA33 mediate specific binding to type VI collagen. *B. burgdorferi* strain ML23 pBBE22*luc* (P; parent), HZ001 pBBE22*luc* (M; Δ*bba33* mutant) and HZ001 pHZ300 (C; complement) were grown either under 'noninducing' conditions (32°C, 1% CO_2, pH 7.6; left side) or 'inducing' conditions (37°C, 5% CO_2, pH 6.8; right side) and incubated on cover slips containing either laminin (filled circles or squares) or type VI collagen (open circles or squares). *B. burgdorferi* cells binding was scored via dark field microscopy. Each data point represents the average number of spirochetes visualized in 10 random fields. Each strain was tested a minimum of three times, with each sample assayed in duplicate. ***$P < 0.001$; ****$P < 0.0001$; ns, nonsignificant difference.

Table 1

Infectivity of the Δ*bba33* mutant strain relative to its parent and genetic complement.

	Number of culture positive/total number							
Strain	**Inoculum dose**	**Lymph node**	**Skin**	**Heart**	**Spleen**	**Bladder**	**Joint**	**All sites**
ML23 pBBE22*luc* (parent)	10^3	4/4	4/4	4/4	4/4	4/4	4/4	24/24
	10^5	4/4	4/4	4/4	4/4	4/4	4/4	24/24
HZ001 pBBE22*luc* (Δ*bba33*)	10^3	0/4	0/4	0/4	0/4	0/4	0/4	0/24
	10^5	0/4	0/4	0/4	0/4	0/4	0/4	0/24
	10^7	0/3	0/3	0/3	0/3	0/3	0/3	0/18
HZ001 pHZ300 (Δ*bba33*/*bba33* Comp)	10^3	4/4	4/4	4/4	4/4	4/4	4/4	24/24
	10^5	4/4	4/4	4/4	4/4	4/4	4/4	24/24

Table 2

Strains and plasmid constructs used in this study.

Strain or plasmid	Genotype and/or characteristics	Source
E. coli strains		
Mach-1™-T1^R	φ80*lacZ*ΔM15 Δ*lacX*74 *hsdR* (r_K^-, m_k^+) Δ*recA1498 endA*1 *tonA*	Invitrogen
BL21 Star™ (DE3)	F$^-$ *ompT hsdSB* (rB$^-$mB$^-$) *gal dcm rne131* (DE3)	Invitrogen
BL21 Star™ (DE3) pLysS	F$^-$ *ompT hsdSB* (rB$^-$mB$^-$) *gal dcm rne131* (DE3) pLysS (Cam^R)	Invitrogen
Top10	F$^-$ *mcr*A Δ (*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139*, Δ (*ara leu*)7697 *galU galK rps*L (Str^R) *end*A1 *nup*G	Invitrogen
B. burgdorferi strains		
ML23	*B. burgdorferi* strain B31 clonal isolate missing lp25	Labandeira-Rey and Skare (2001)
ML23 pBBE22*luc*	Clonal isolate of strain B31 lacking lp25 containing *bbe22* and *B. burgdorferi* codon-optimized *luc* gene under the control of a strong borrelial promoter (P$_{flaB}$-*luc*); parent strain	Hyde *et al.* (2011a)
HZ001	ML23 Δ*bba33*::Str^R	This study
HZ001 pBBE22*luc*	ML23 Δ*bba33*::Str^R, containing *bbe22* and the *B. burgdorferi* codon-optimized *luc* gene under the control of a strong borrelial promoter (P$_{flaB}$-*luc*)	This study
HZ001 pHZ300	ML23 Δ*bba33*::Str^R, containing *bbe22, bba33*, both under the control of their native promoters, and the *B. burgdorferi* codon-optimized *luc* gene under the control of a strong borrelial promoter (P$_{flaB}$-*luc*)	This study
Plasmids		
pCR2.1	pCR™2.1-TOPO® vector; Amp^R, Kan^R	Invitrogen
pKFSS1	*B. burgdorferi* shuttle vector containing P$_{FlgB}$-Str^R cassette; Spc^R in *E. coli*, Str^R in *B. burgdorferi*	Frank *et al.* (2003)
pBBE22Gate	pBBE22 modified to be a gateway destination vector containing *attL* and *attR* sites; Cam^R, Kan^R	Weening *et al.* (2008)
pBBE22*luc*	Borrelial shuttle vector containing *bbe22* and *B. burgdorferi* codon-optimized *luc* gene under the control of a strong borrelial promoter (P$_{flaB}$-*luc*)	Hyde *et al.* (2011a)
pCR2.1Bactin	β-actin gene cloned into pCR2.1 vector; Kan^R	Hyde *et al.* (2011a)
pCR2.1*recA*	1119 bp fragment, containing the *recA* gene, cloned into pCR2.1 vector; Kan^R	Hyde *et al.* (2011a)
pET15b	Cloning vector for overexpression of genes to generate His-tagged recombinant protein; Amp^R	EMD Millipore
pSS008	*bba33* lacking its leader peptide (including the cysteine residue) was cloned into the *Nde*I and *Bam*HI sites of pET15b in order to produce His-tagged recombinant BBA33	This study
pHZ001	PCR amplicons containing sequences 1526 bp upstream of the start of *B. burgdorferi bba33*, the P$_{flgB}$-Str^R cassette from pKFSS1 and sequences 1527 bp downstream of the *bba33* stop codon, as well as pCR2.1, were specifically configured using Gibson assembly	This study
pHZ300	Intact *bba33* containing sequences 210 bp upstream and 129 bp downstream were cloned into the *Bam*HI and *Sal*I sites of pBBE22*luc*	This study

Table 3

Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′ to 3′)	Description
BamHI-A33us-recF	CCAAGCTTGGTACCGAGCTCGGATCCACACGCGCTAGCATCCTTTAAATT	Primer pair used to amplify 1526 bp region upstream of *bba33* with a 22 bp 5′ flanking region homologous to pCR2.1 upstream of the *Bam*HI site and a 3′ flanking region homologous to the 5′ region of the *flgB* promoter (P_{flgB})
flgB-A33us-recR	TAGGAAATCTTCCACGCCAATTGTTAAAGCTTTCTGTTTCTACGATATCG	
aadA-A33ds-recF	GTGAAAAAGTTTAAAAATCAGTTATTCTTCTATAGGTTTTATTTCTG	Primer pair used to amplify the 1527 bp region downstream from *bba33* with a 21 bp 5′ flanking region homologous to the *aadA* 3′ end and a 32 bp 3′ flanking region homologous to sequences downstream of the *Xho*I site of pCR2.1
A33ds-XhoI-recR	CGAATTGGGCCCTCTAGATGCATGCTCGAGCGGGTGTTGGAATTGGAAACGAACC	
flgB-F	CAATTGGCGTGGAAGATTTCC	Primer pair used to amplify P_{flgB}-Str^R cassette from pKFSS1
aadA-R	CTGATTTTTAAACTTTTTCACAAATAGG	
P1	GCCAGAGAGCGATATCGTAGAAAC	Primers P1 through P5 were used to confirm the *bba33* deletion in *B. burgdorferi* as well as complementation construct (see Figs 1 and 2).
P2	AATAATAGGTTCATGAAAAATTTGTATG	
P3	CACAAATAGGGTCAAAATTTTGACC	
P4	TGCCCTATCAGAAATAAAACCTATAGAAG	
P5	GAAGTGCCTGGCAGTAAGTTG	
A33-NdeI-5′F	ACGCCATATGTATTTAAATGATTTTTCTGGTATG	Primer pair used to amplify *bba33* lacking sequences that encode its leader peptide through the stop codon with *Nde*I (underlined) and *Bam*HI (bold) sites engineered for cloning into pET15b expression vector
A33-BamHI-3′R	ACGC**GGATCC**TTATTTTTGAATTAGTGCTAAAGCAC	
A33up210-BamHI-F	ACGC**GGATCC**GCCAGAGAGCGATATCGTAGAAAC	Primer pair used to amplify a region from 210 bp upstream and 129 downstream of *bba33* with BamHI (bold) and SalI (underlined) sites; cloned into pBBE22*luc*
A33dn129-SalI-R	ACGCGTCGACAAATGGCTAGGGTGGAATACAAAC	
b-Actin-F	ACGCAGAGGGAAATCGTGCGTGAC	Primer pair used for enumerating copies of mouse β-actin via qPCR (Pal *et al.*, 2008)
b-Actin-R	ACGCGGGAGGAAGAGGATGCGGCAGTG	
nTM17FrecA	GTGGATCTATTGTATTAGATGAGGCT	Primer pair used for enumerating copies of *B. burgdorferi recA* via qPCR (Liveris *et al.*, 2002; Weening *et al.*, 2008)
nTM17RrecA	GCCAAAGTTCTGCAACATTAACACCT	