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HtrA, a Temperature- and Stationary Phase-Activated Protease Involved in Maturation of a Key Microbial Virulence Determinant, Facilitates *Borrelia burgdorferi* Infection in Mammalian Hosts

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High-temperature requirement protease A (HtrA) represents a family of serine proteases that play important roles in microbial biology. Unlike the genomes of most organisms, that of *Borrelia burgdorferi* notably encodes a single HtrA gene product, termed BbHtrA. Previous studies identified a few substrates of BbHtrA; however, their physiological relevance could not be ascertained, as targeted deletion of the gene has not been successful. Here we show that Bb*htrA* transcripts are induced during spirochete growth either in the stationary phase or at elevated temperature. Successful generation of a Bb*htrA* deletion mutant and restoration by genetic complementation suggest a nonessential role for this protease in microbial viability; however, its remarkable growth, morphological, and structural defects during cultivation at 37°C confirm a high-temperature requirement for protease activation and function. The Bb*htrA*-deficient spirochetes were unable to establish infection of mice, as evidenced by assessment of culture, PCR, and serology. We show that transcript abundance as well as proteolytic processing of a borrelial protein required for cell fission and infectivity, BB0323, is impaired in Bb*htrA* mutants grown at 37°C, which likely contributed to their inability to survive in a mammalian host. Together, these results demonstrate the physiological relevance of a unique temperature-regulated borrelial protease, BbHtrA, which further enlightens our knowledge of intriguing aspects of spirochete biology and infectivity.

Lyme disease is a common vector-borne infectious disease in the Northern Hemisphere, posing a serious health threat to humans and animals (1–3). Newly revised estimates from the Centers for Disease Control and Prevention suggest that there are likely to be over 300,000 new cases per year in the United States alone, and a vaccine to prevent human infection is currently unavailable. The disease can be transmitted via the bite of the infected arthropod vector, the *Ixodes scapularis* tick, harboring the spirochete pathogen *Borrelia burgdorferi*, often resulting in serious illness in susceptible hosts (2, 3). While most Lyme disease cases can be treated with an antibiotic, a minority of patients will have persistent or relapsing nonobjective symptoms that vary in intensity, are nonresponsive to further antibiotic therapy, and are collectively termed chronic Lyme disease or posttreatment Lyme disease syndrome, the underlying mechanism and pathogenesis of which remain highly controversial (4). Detection of early Lyme disease, when antibiotic treatment is most effective, also remains challenging, as the infection reflects many nonspecific symptoms that are shared by many other febrile or influenza-like diseases (5, 6). Thus, the development of efficient diagnostics, vaccines, or new effective drugs is a high-priority goal that requires a thorough understanding of the unusual biology and infection process of this pathogen.

The pathogen of Lyme disease exhibits evolutionary divergence from other bacteria, even related pathogenic spirochetes, and persists in a complex enzootic infection cycle. Thus, it is perhaps not surprising that a great majority of the proteins encoded by the *B. burgdorferi* genome have unknown functions relevant only to the special biology of spirochetes (7–10). The genome also shows notable structural and functional redundancy, reflected by the presence of a significant number of paralogous gene clusters, as well as many genes that are not required for infectivity in mammals and/or ticks (2, 7–11). Although various aspects of spirochete infectivity are well studied, many questions remain unanswered, particularly regarding mechanisms that dictate the remarkable ability of the pathogen to adapt to and persist in diverse hosts, including the multifactorial processes that influence the inflammatory outcome in susceptible hosts. Maintenance of *B. burgdorferi* in the enzootic cycle requires successful persistence in the arthropod and reservoir hosts, as well as efficient transmission between the tick and its host (11). Therefore, in order to survive transitions between diverse vector and host environments, *B. burgdorferi* must be able not only to detect changes in its environ-

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ment but also to generate suitable response to these changes. As a result, gene products playing roles in adaptation to cellular stress, including temperature, oxidative stress, pH, etc., must be critical for the life cycle of the pathogen.

The high-temperature requirement A (HtrA) family of serine proteases can be found in all forms of life, from prokaryotes to mammals (12–14). HtrA proteases function primarily in protein homeostasis and quality control, acting as proteases and chaperones that stabilize specific proteins and modulate signaling pathways (12). Similarly, HtrA proteases have been reported to play an essential role in the virulence of a number of pathogens, such as *Mycobacterium tuberculosis* (15), *Salmonella enterica* (16, 17), *Helicobacter pylori* (18), *Streptococcus pneumoniae* (19), *Bacillus anthracis* (20), and *Staphylococcus aureus* (21), and have also been associated with several human diseases (21–23). While the predominant mechanism of loss of virulence lies in the substrate proteins that are stabilized or processed by HtrA, a direct role for HtrA in pathogen invasiveness has also been reported (18, 24). The unique feature of HtrA is the presence of a protease domain containing a highly conserved active-site catalytic triad (Ser-His-Asp) and one or two PDZ domains toward the carboxyl terminus (12, 13). The overall structures of HtrA proteases are similar; however, their functions and substrates are remarkably quite diverse. While the common structural building unit is a trimer, upon recognizing substrates, HtrA monomers form higher-order oligomeric structures ranging from 6- to 24-mers (13, 25). These oligomers form a cage with the proteolytically active site facing the interior and the PDZ domains protruding outside. The involvement of HtrA proteases in the processing of critical substrates supporting microbial virulence, as well as their pathogen-specific diversification, makes them an attractive target for therapeutic intervention (23, 26).

Most of the metazoan or bacterial genomes encode multiple HtrA homologs that are implicated in diverse physiological functions (27). Notably, the genome of *B. burgdorferi* contains a single *htrA* gene (locus *bb0104*) (28) that encodes BbHtrA, which has been biochemically characterized and shown to possess conserved, catalytic, serine-dependent proteolytic activity (28–32). BbHtrA possesses an N-terminal signal peptide sequence, and apart from its periplasmic and cytosolic fractions, it also localizes in outer membrane vesicles and is secreted into growth media (29, 32). Few interacting partners and substrates of BbHtrA have also been identified (28–32). For instance, BbHtrA has been shown to cleave basic membrane protein D (BmpD) and chemotaxis signal transduction phosphatase (CheX) (29). In addition, previous *in vitro* studies have suggested that BbHtrA also cleaves BB0323 (28), a borrelial protein important for cell growth, fission, and infection (33, 34). Also, it has been shown that BbHtrA degrades the extracellular matrix proteoglycan aggrecan, fibronectin, and numerous other proteoglycans (32), and recently, the inhibition of this protease by zinc has been reported (35). However, despite these seminal studies, the precise biological significance of BbHtrA remains enigmatic, as efforts aimed at targeted deletion of the gene have thus far not been successful (28–30). Here we report the successful generation and characterization of a Bb*htrA* deletion mutant and complemented isolates of an infectious isolate of *B. burgdorferi*, which highlight a critical role for this protease in spirochete biology and infectivity.

MATERIALS AND METHODS

***B. burgdorferi* strains and mice.** Low-passage-number *B. burgdorferi* strain 297 clone AH130 (36) was used in this study. Bacteria were cultivated in Barbour-Stoenner-Kelly II (BSK-II) medium at 34°C or 37°C. Bb*htrA* mutant strains were maintained under antibiotic selection with gentamicin (40 mg/ml). Four- to 6-week-old C3H/HeN mice were purchased from the National Institutes of Health. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the University of Maryland, College Park.

Generation of Bb*htrA* deletion mutant and complemented strains. Bb*htrA*-deficient *B. burgdorferi* clones were generated by homologous recombination, replacing the *bb0104* gene with a gentamicin resistance cassette. Briefly, the 1.5-kb upstream region and the 1.5-kb downstream region of *bb0104* (Bb*htrA*) were PCR amplified from the *B. burgdorferi* genomic DNA with the primers indicated in Table S1 in the supplemental material. The DNA amplicons were then cloned upstream and downstream of a gentamicin resistance cassette in a pUC18-derived plasmid (pMP016) at the SacI/MluI and SacII/PstI restriction sites, respectively. The resulting suicide vector was confirmed by sequencing and designated pMP007. The pMP007 plasmid DNA was then transformed into an infectious clone of *B. burgdorferi* strain 297, AH130 (37). Gentamicin-resistant transformants were analyzed by PCR, reverse transcription (RT)-PCR, and immunoblotting to confirm the loss of *bb0104*. Endogenous plasmid profiles of the *bb0104* mutant clones were determined by PCR on the basis of recently published sequences (10). Two clones with a plasmid profile identical to that of AH130 were chosen for further study.

For genetic complementation of the Bb*htrA* mutant, we used *B. burgdorferi* shuttle plasmid pBSV2-derived shuttle vector pYY003 (38) to deliver the wild-type (WT) copy of the Bb*htrA* gene. Since the intergenic region upstream region of the Bb*htrA* open reading frame (ORF) does not seem to harbor a discernible promoter region, we fused the Bb*htrA* ORF with the *B. burgdorferi flaB* promoter. The DNA fragment encompassing the Bb*htrA* gene downstream of the *flaB* promoter was cloned into the NdeI and BglII sites of pYY003. About 10 μg of the recombinant plasmid, designated pXW003, was electroporated into the Bb*htrA*-deficient *B. burgdorferi* strain, and complemented clones were selected with 350 μg/ml kanamycin in the growth medium. PCR analyses were further performed to confirm that the Bb*htrA*-complemented clones retained the same plasmid profile as the WT spirochetes.

Our repeated attempts to attain stable in *cis* complementation of Bb*htrA* isolates were unsuccessful. Therefore, the above-mentioned Bb*htrA*-complemented isolate, which contained a WT copy of the Bb*htrA* gene in the shuttle plasmid, was used in all *in vitro* studies in the presence of kanamycin. For phenotypic analysis *in vivo*, two isogenic and independent Bb*htrA* mutant clones (designated M1 and M2) were included in all of our experiments.

Western and far-Western blotting. Immunoblotting assays were performed by standard procedures as detailed elsewhere (28). Briefly, whole-cell lysates of *B. burgdorferi* grown in BSK-II medium at 34°C and 37°C were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-BB0323 (1:3,000), anti-BB0238 (1:3,000), anti-BB0365 (1:1,000), and anti-BbHtrA (1:3,000) antibodies (28, 39). Immunoblot assays were developed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000 to 10,000) and enhanced chemiluminescence as previously described (28). For far-Western analyses, the nitrocellulose membrane was incubated with His-BB0323 (2 μg) overnight at 4°C in blocking buffer (2% skim milk in Tris-buffered saline, pH 7.5, with 0.05% Tween 20) and washed with Tris-buffered saline with Tween 20, and bound proteins were detected by immunoblotting with an anti-BB0323 antibody and an HRP-conjugated secondary antibody. Quantitative densitometry was performed with ImageJ software (NIH).

PCR assays. The primers used in PCR and quantitative RT-PCR (qRT-PCR) assays are shown in Table S1 in the supplemental material.

Total RNA from *in vitro*-grown *B. burgdorferi* or from infected mouse tissues was isolated with TRIzol reagent (Invitrogen), treated with DNase I (NEB), reverse transcribed to cDNA (SuperScript VILO; Invitrogen), and analyzed by real-time PCR with iQ SYBR green Supermix (Roche Diagnostics) as detailed previously (40). For quantitative analysis of gene expression, the target transcripts were normalized to the number of *flaB* transcripts, whereas for quantitative measurement of *B. burgdorferi* burdens in infected tissues, *flaB* transcripts were normalized to mouse β-actin levels as previously detailed (40).

Growth analysis of spirochetes. WT, Bb*htrA* mutant, and complemented spirochetes were assessed for growth *in vitro* and further analyzed by electron microscopy. Spirochetes were grown at 34°C in BSK-II medium containing 6% rabbit serum to early log phase and repassaged (10^5 spirochetes/ml) into new medium. The spirochetes were cultivated in triplicate at 34°C and 37°C and counted every 24 h with a Petroff-Hausser cell counter (Fisher Scientific) under a dark-field microscope as previously detailed (41).

Electron microscopy. Electron microscopic analyses were performed as previously detailed (42), with minor modifications. The WT, Bb*htrA* deletion mutant, and complemented strains were grown at 34°C or 37°C to a density of 10^7 cells/ml. For transmission electron microscopy, spirochetes were washed four times with phosphate-buffered saline (PBS) and fixed in 2% glutaraldehyde–PBS for 60 min, followed by 1% osmium tetroxide for 30 min, at room temperature. The samples were dehydrated, embedded, sectioned, and then collected on copper grids. Samples were poststained with 2.5% uranyl acetate and lead citrate and finally analyzed with two transmission electron microscopes (Zeiss 10CA and JEOL 100CXII). Scanning electron microscopy was performed by a similar procedure, except that the samples were subjected to critical-point drying with liquid carbon dioxide after the dehydration step. Dried samples were mounted on stubs coated with a gold-palladium alloy and analyzed under a scanning electron microscope (Hitachi S-4700).

Infection studies. Groups of three C3H/HeN mice were injected intradermally with the same number (10^5 cells/per mouse) of WT spirochetes or two independent clones of Bb*htrA*-deficient spirochetes (designated M1 and M2). Samples of skin, heart, and bladder were isolated at 14 days after infection, and *B. burgdorferi* levels were assessed by qRT-PCR analysis of *flaB* transcripts normalized to murine β-actin levels as described previously (40). Additionally, skin and spleen tissues were cultured in BSK-II medium and analyzed weekly for 4 weeks with a dark-field microscope (43).

Statistical analysis. Results are expressed as the mean ± the standard error of the mean (SEM). The statistical significance of differences observed between experimental and control groups was analyzed with GraphPad Prism version 4.0 (GraphPad Software, CA). Student's *t* test was used to compare the mean values, and $P < 0.05$ was considered statistically significant.

RESULTS

BbHtrA is a conserved spirochete protein induced in the stationary growth phase and at elevated temperatures. The primary sequence of BbHtrA, which possesses typical serine protease activity (28–32), is highly conserved within diverse *B. burgdorferi sensu lato* strains. To further confirm the conservation of BbHtrA and to assess whether diverse isolates and strains of the species *B. burgdorferi sensu lato* produce HtrA orthologs, whole-cell lysates from different strains were probed with BbHtrA antibody raised against isolate B31. As shown in Fig. 1A (top), an antibody raised against isolate B31 recognized HtrA orthologs in all of the strains, which produced the antigen at comparable levels. As HtrA belongs to a family of proteases that are activated under various stress conditions, including high temperature, we examined the levels of Bb*htrA* expression at different temperatures and in different phases of growth. *B. burgdorferi* bacteria were cultured at the optimal laboratory growth temperature (34°C) or at an elevated, mammal-specific temperature (37°C). Samples were collected in the early (10^5 cells/ml) and late (10^8 cells/ml) growth phases and processed for assessment of Bb*htrA* transcript and protein levels by qRT-PCR and immunoblotting, respectively. Notably, as shown in Fig. 1B, Bb*htrA* transcripts were more abundant in the stationary phase than in the early growth phase, irrespective of the culture temperature. There was also an overall higher Bb*htrA* transcript level at 37°C than at 34°C, especially in the late phase of growth. To further confirm whether increased mRNA levels also resulted in an increase in corresponding protein expression levels, samples from the different growth phases were subjected to immunoblotting with anti-BbHtrA antiserum. In accordance with the transcript level, BbHtrA protein levels were higher in the stationary phase (Fig. 1C and D) than in the early growth phase, while the level of FlaB remained constant throughout the growth of spirochetes *in vitro*. Taken together, these data suggest that the expression of Bb*htrA* is induced under various stress conditions, such as higher temperatures, as mimicked by growth at 37°C and spirochete growth in the stationary phase.

BbHtrA deficiency results in impairment of spirochete growth and cellular organization at a mammalian-phase-specific temperature. Previous attempts by several groups to generate a Bb*htrA* deletion mutant of *B. burgdorferi* were unsuccessful (28–32). This observation, together with the occurrence of Bb*htrA* as a single-copy gene in the spirochete genome, led to speculation that BbHtrA is likely involved in an essential housekeeping function and thus attempts to delete it would incur a lethal mutation. However, unlike our previous mutagenesis attempts involving the *B. burgdorferi* B31 isolate, we were successful in generating a Bb*htrA* deletion mutant of infectious and low-passage-number 297 isolate AH130 (37). The mutant was generated by replacing the ORF of Bb*htrA* (*bb0104*) with the gentamicin resistance cassette via homologous recombination as illustrated in Fig. 2A. Two independent gentamicin-resistant clones (designated M1 and M2) with the same endogenous plasmid profile as AH130 were used for subsequent analysis. The desired integration was confirmed by amplification of the product in mutants with primers specific to the antibiotic resistance gene, as opposed to the WT (Fig. 2B). qRT-PCR analysis confirmed the absence of a Bb*htrA* transcript in the mutants and its restoration in the complemented strain (Fig. 2C) and ruled out the polar effects of mutation on adjacent transcripts *bb0103* and *bb0105*, which were expressed at levels similar to those of the WT. Finally, the loss of the BbHtrA protein was verified by immunoblotting whole-cell extracts from WT and mutant spirochetes with anti-BbHtrA antibody (Fig. 2E). To rule out the possibility of anomalous effects of genetic manipulation, we sought to complement the Bb*htrA*-deficient *B. burgdorferi* strain with a WT copy of the Bb*htrA* gene in *cis*. As the Bb*htrA* gene lacks an obvious upstream promoter and is probably part of an operon, we fused the ORF of Bb*htrA* with the *B. burgdorferi flaB* promoter. Although our exhaustive efforts to generate a stable and *cis*-complemented strain were unsuccessful, we were able to complement, in *trans* via a shuttle plasmid, the Bb*htrA*-deficient spirochetes with a WT copy of the gene under the control of the *flaB* promoter (Fig. 2D), which restored the production of BbHtrA in the complemented isolate (Fig. 2E).

To assess the role of BbHtrA in the growth of *B. burgdorferi*, WT, Bb*htrA* mutant, and complemented spirochetes were cultured at laboratory (34°C) and elevated (37°C) temperatures. As

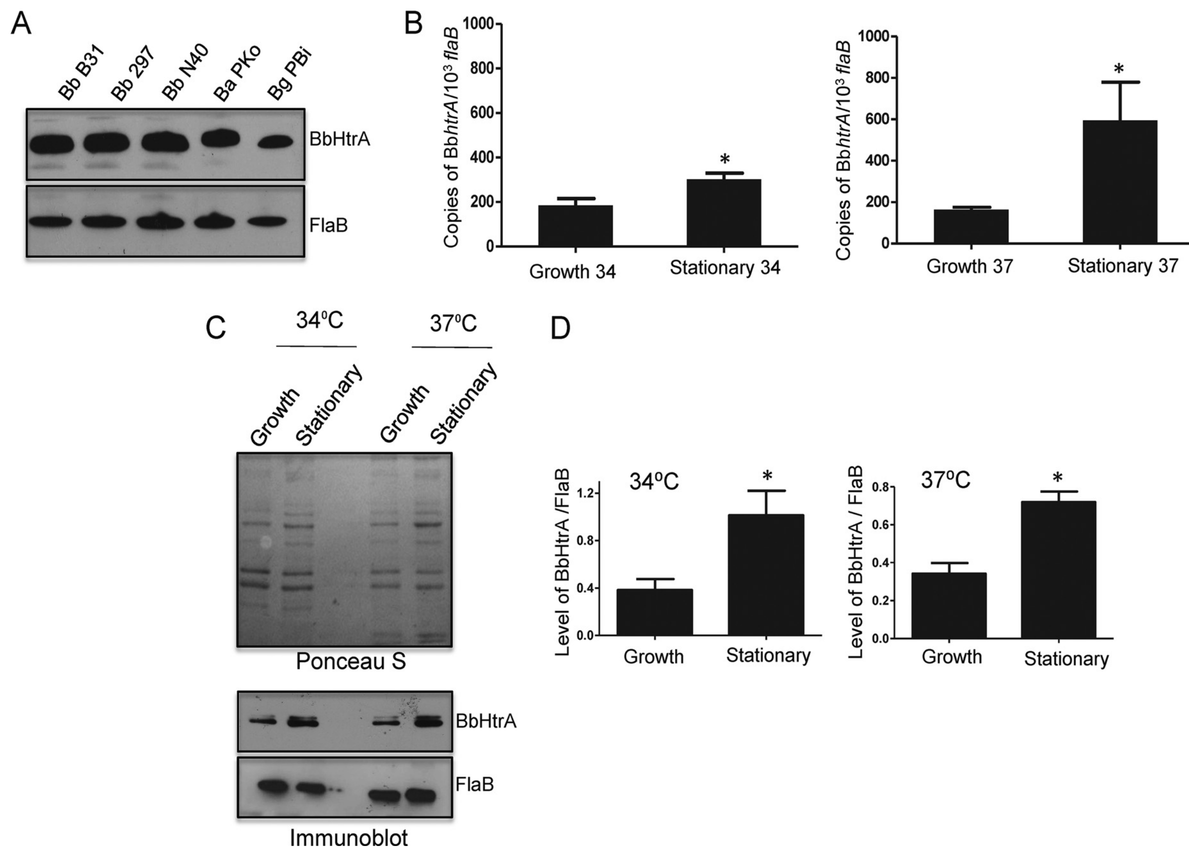

FIG 1 BbHtrA is conserved in infectious *B. burgdorferi sensu lato* isolates and highly expressed in the stationary phase or at an elevated temperature. (A) Production of BbHtrA in representative isolates of infectious *B. burgdorferi sensu lato*. Whole-cell extracts from *B. burgdorferi* isolates B31, 297, and N40; *B. afzelii* isolate PKo; and *B. garinii* isolate PBi were immunoblotted with BbHtrA antiserum generated against the recombinant HtrA protein of *B. burgdorferi* B31. The expression of FlaB in different isolates is shown at the bottom. (B) Bb*htrA* is induced in the stationary growth phase and at an elevated temperature. Spirochetes were grown at 34°C (left) and 37°C (right), and samples were harvested in the growth (10^5 cells/ml) and stationary (10^8 cells/ml) phases. The Bb*htrA* transcript level was measured by qRT-PCR and normalized to the number of copies of the *flaB* transcript. The bars represent the mean values of three independent experiments, and the error bars represent the SEM. The levels of Bb*htrA* mRNA are elevated in the stationary phase and at 37°C. *, $P < 0.05$. (C) BbHtrA protein expression levels in the log and stationary growth phases of spirochetes in culture. Bacterial lysates at the growth phases and temperatures indicated were either assessed by Ponceau S staining (top) or subjected to immunoblotting with anti-BbHtrA (middle) and anti-FlaB (bottom) antisera. (D) Levels of BbHtrA relative to FlaB from panel C were determined by quantitative densitometry. The levels of BbHtrA are increased in the stationary phase. *, $P < 0.05$.

shown in Fig. 3A, the Bb*htrA* mutant grew comparably to the WT at 34°C but exhibited a slow-growth phenotype at 37°C (Fig. 3A) and formed unusually large clumps at all cell densities. Notably, the mutant isolates regained their normal morphology when shifted back to 34°C either during early log phase or following inoculation into fresh medium (data not shown). Unlike the Bb*htrA* mutant, the complemented isolate displayed a growth pattern similar to that of WT spirochetes, indicating that the defect was specific to the deletion of Bb*htrA* (Fig. 3A). These data also suggest that BbHtrA might not be essential for spirochete growth *in vitro* at 34°C but is important for maintaining normal cell growth and morphology at elevated temperatures.

Scanning and transmission electron microscopic studies revealed that the loss of Bb*htrA* results in striking alterations in cellular organization when spirochetes are grown at 37°C. In contrast to WT organisms that grew as single replicating cells, Bb*htrA* mutant organisms appeared as clumped cells or intertwined masses with spherical blebs (Fig. 3B). While the WT and complemented spirochetes showed distinct outer and inner membrane sandwiching the periplasmic space harboring flagella, Bb*htrA* mutant spirochetes exhibited several large, predominantly membranous, irregularly shaped, hollow structures containing round vesicles, protoplasmic cylinders revealing flagella beneath the outer membrane (Fig. 3B).

BbHtrA function is critical for infection of mice. To examine the role of BbHtrA in infectivity, separate groups of C3H/HeN mice were inoculated intradermally with WT and genetically manipulated spirochetes (10^5 cells/mouse). As our complemented isolate was generated with a shuttle plasmid that uses the antibiotic kanamycin, which has a shorter half-life in mice, this isolate cannot be used for *in vivo* infection studies. In fact, our efforts to maintain the shuttle plasmid in mice via intermittent administration of various doses of the antibiotic remained unsuccessful, perhaps because of faster clearance or an insufficient concentration of kanamycin *in vivo*. Therefore, for the infection studies, we used two independent Bb*htrA* mutant clones, designated M1 and M2. Samples of skin, heart, bladder, and serum were collected at day 14 and tested for *B. burgdorferi* infection by serology, qRT-PCR, and culture. As shown in Fig. 4A, unlike the WT, which showed a strong serological response, both of the mutants had a severely

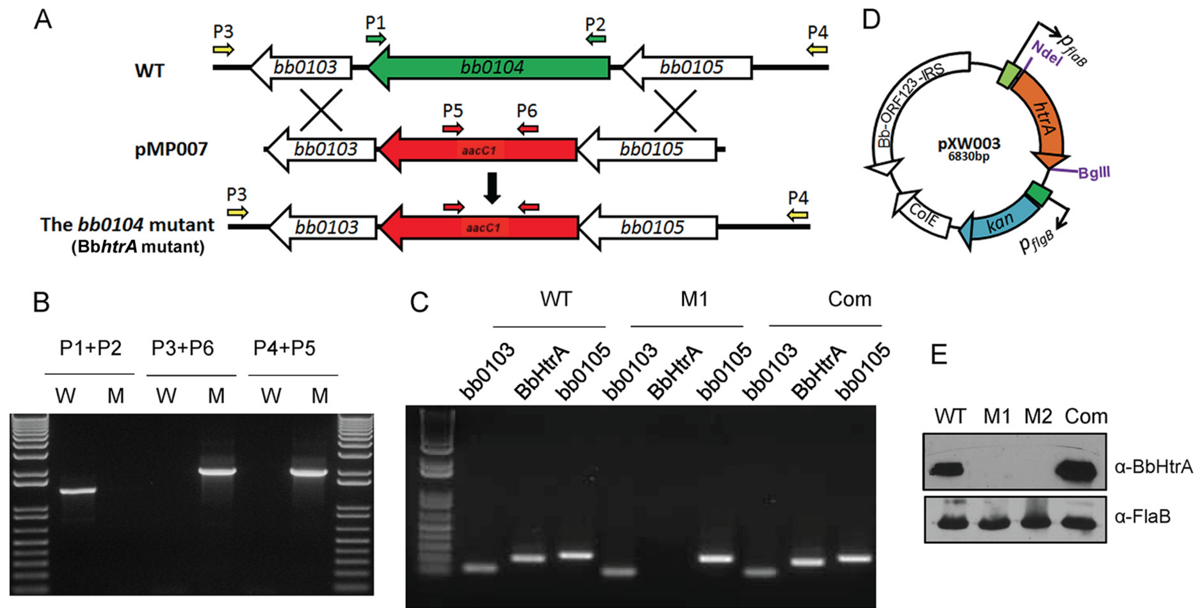

FIG 2 Construction and analysis of *bb0104* (Bb*htrA*) mutant and complemented strains. (A) Schematic representation of WT and Bb*htrA* mutant isolates (M1 and M2) at the *bb0104* (*hrtA_Bb*) locus of *B. burgdorferi* 297. The *bb0103*, *bb0104*, and *bb0105* genes and the gentamicin cassette (*aacC1*) replacing the native *bb0104* ORF are indicated. (B) Integration of the gentamicin resistance cassette at the intended locus in the genome. Primer pairs P1-P2, P4-P5, and P3-P6 were used to amplify the native *bb0104* gene and the inserted gentamicin cassette at the *bb0104* locus, respectively. PCR was performed with the genomic DNA of WT (lanes W) and mutant (lanes M) *B. burgdorferi*, and the amplified products were checked on a 1% agarose gel. The migration of a DNA molecular size ladder is shown in the leftmost and rightmost lanes. (C) qRT-PCR analysis of Bb*htrA* deletion and complementation and polar effects on cognate *bb0103* and *bb0105* transcripts. Total RNA was isolated from WT, mutant (M1), and complemented (Com) *B. burgdorferi*, converted to cDNA, and further used to amplify the regions with Bb*htrA* and the adjacent *bb0103* and *bb0105* genes. The amplified products were visualized on a 1% agarose gel. (D) Diagram of the shuttle vector pXW003 used for complementation. pXW003 is a pBSV2-derived shuttle vector carrying the *bb0104* gene under the control of the *flaB* promoter. (E) Protein expression analyses of WT, Bb*htrA* mutant (M1 and M2), and complemented (Com) *B. burgdorferi*. Equal amounts of bacterial extracts were subjected to SDS-PAGE, transferred to a membrane, and immunoblotted with anti-BbHtrA (top) and anti-FlaB (bottom) antisera.

attenuated ability to induce an antibody response to spirochete proteins, suggesting their clearance during early mammalian infection. The qRT-PCR analyses further revealed that Bb*htrA* mutant clones were undetectable in all of the tissues tested (Fig. 4B). Finally, culture analysis confirmed that the Bb*htrA* mutants remained undetectable in the samples collected from all six mice infected with either of the two mutant clones, whereas WT spirochetes could be readily detected in corresponding mice (see Table S2 in the supplemental material). Together, studies described above suggest an essential role for BbHtrA in the establishment of *B. burgdorferi* infection in murine hosts.

The borrelial protein BB0323 is processed by BbHtrA. We previously demonstrated that BbHtrA cleaves BB0323, one of the proteins required for infectivity, in a manner dependent upon its catalytic serine residue (28). The BB0323 protein is required for normal organization of the outer membrane, cell fission, persistence in mice, and the transition between the host and the vector *in vivo* (34). Recent *in vitro* studies using recombinant BbHtrA have led to the identification of additional substrates that include borrelial virulence determinants like P66 and host proteins that are important for infectivity or pathogenesis (28–32). Specifically, our previous studies suggest that recombinant BbHtrA interacts with and specifically cleaves BB0323 into a larger N-terminal polypeptide and a shorter C-terminal polypeptide (28). As morphological abnormalities and the defect in virulence exhibited by BbHtrA are analogous to those of BB0323 deletion mutants (34), we sought to confirm whether maturation of BB0323 is dysregulated in the absence of BbHtrA. To test this, we measured the BB0323 mRNA and protein levels in the Bb*htrA* mutant and complemented isolates. As shown in Fig. 5A, no discernible difference in the BB0323 transcript or protein levels of the WT, Bb*htrA* mutant, and complemented strains were noted at 34°C. Interestingly, a less BB0323 protein expression was noticed in Bb*htrA* mutant clones than in the WT or complemented strain at 37°C (Fig. 5B). Our earlier studies suggested that the immature full-length BB0323 protein and its mature C-terminal polypeptide are difficult to detect in *B. burgdorferi* by conventional immunoblotting assays with anti-BB0323 antibodies, most likely because of their lower stability or rate of occurrence; however, they can be detected by a far-Western assay with the BB0323 N-terminal polypeptide that binds the C-terminal polypeptide (28). Using the latter approach, we detected full-length BB0323 in the Bb*htrA* mutant isolates, which was absent from the WT at 37°C (Fig. 5C). The expression of full-length BB0323 was significantly reduced in the complemented strain, further suggesting that the processing of full-length BB0323 is impaired in the absence of BbHtrA. It has previously been established that BB0323 interacts with another virulence determinant, BB0238, and regulates its expression in *B. burgdorferi* (44). In order to test whether BbHtrA regulates BB0323 via BB0238, we monitored the level of BB0238 expression in the Bb*htrA* mutant. As shown in Fig. 5D, BbHtrA-deficient spirochetes expressed BB0238 at a level similar to that of the WT or complemented strain at the elevated mammalian host-specific (37°C) temperature, further suggesting the specificity of BB0323 processing by BbHtrA.

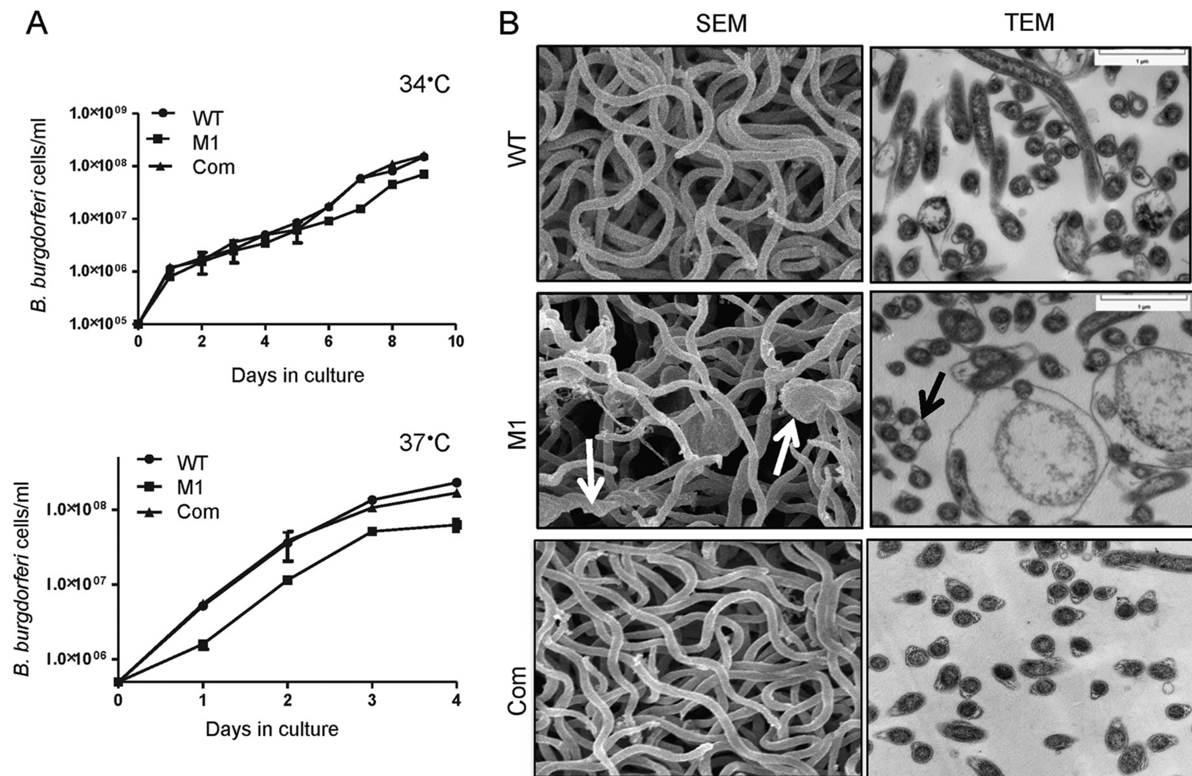

FIG 3 Growth and morphological analysis of WT and genetically manipulated spirochetes *in vitro*. (A) WT, Bb*htrA* mutant (M1), and complemented (Com) spirochetes were diluted to a density of 10^5/ml, grown at either 34°C (top) or 37°C (bottom) in BSK-H medium, and counted under a dark-field microscope every 24 h with a Petroff-Hausser cell counter. Note that Bb*htrA* mutant isolates grew as single cells in the early growth phase but started forming clumps in the late growth phase at 37°C. (B) Morphological analysis of WT, Bb*htrA* mutant, and complemented *B. burgdorferi* isolates. On the left is a scanning electron microscopic (SEM) analysis of spirochetes grown at 37°C. Masses of intertwined spirochetes and spherical blebs (arrows) are visible. On the right are transmission electron micrographs (TEM) of Epon-embedded spirochetes. Note the presence of large, nearly round structures enclosing two or more cells (arrow) in the mutant.

DISCUSSION

HtrA proteins are generally involved in cellular adaptation to various stress response events via their protease and chaperone functions, maintaining tight protein quality control in organisms, including microbes (12). These proteases-chaperones function in an ATP-independent manner; therefore, many prokaryotic and eukaryotic genomes encode multiple homologs of these highly efficient proteins that are involved in diverse cellular functions (12, 13). Interestingly, HtrA has been shown to play an important role in the pathogenesis of several Gram-positive, as well as Gram-negative, bacteria (27, 45). Owing to their important roles in cellular physiology, several pathogens harbor multiple homologs of HtrA. However, *B. burgdorferi* encodes a single HtrA, termed BbHtrA, which is pivotal in the establishment of infection in the mammalian host. Recent studies have suggested that BbHtrA is responsible for the proteolytic processing of a number of key virulence determinants, such as BB0323, P66, BmpD, and CheX (31), indicating its role in diverse cellular processes. For instance, the cleavage of the phosphatase CheX by BbHtrA suggests that it has a role in regulating bacterial motility and chemotaxis, the key cellular processes that contribute to the invasiveness of *B. burgdorferi* and play important roles in the progression of the disease (29). On the other hand, the identification of outer membrane proteins such as BB0323, P66, and BmpD as the substrates of BbHtrA implies that it has a role in the regulation of key virulence determinants that are important for the biogenesis or organization of the membrane, transport across the membrane, or host-pathogen interaction (28–30). Although studies using *B. burgdorferi* stains overexpressing Bb*htrA* have shed new light on the function of the protease it encodes and its potential roles in virulence, previous efforts to generate Bb*htrA* deletion mutants have been unsuccessful (28–30), which has also limited our understanding of its role in spirochetal biology and infection. The study described here shows that the successful deletion of Bb*htrA* in low-passage-number strain 297 of *B. burgdorferi* results in a severely distorted morphology of the spirochetes at elevated temperatures, thereby attenuating their ability to infect mice. The aberrant cell morphology of Bb*htrA* mutants grown at the higher, mammalian host-specific temperature included striking alterations in their cellular structures and occasional fission defects, as well as the formation of blebs within a nearly round sac-like structure. The restoration of normal morphology upon the reintroduction of the gene in *trans* further reinforced the idea that the effect was specifically mediated by BbHtrA. Similar structurally heterogeneous entities have previously been recorded and are known to occur in spirochete populations that encounter stress, such as in unfed ticks or upon exposure to borreliacidal agents (46–49). Here, we propose a critical role for BbHtrA, a high-temperature-induced protease, in the cellular regulation and stress tolerance of Lyme disease spirochetes. A similar high-temperature sensitivity of HtrA deletion mutants has

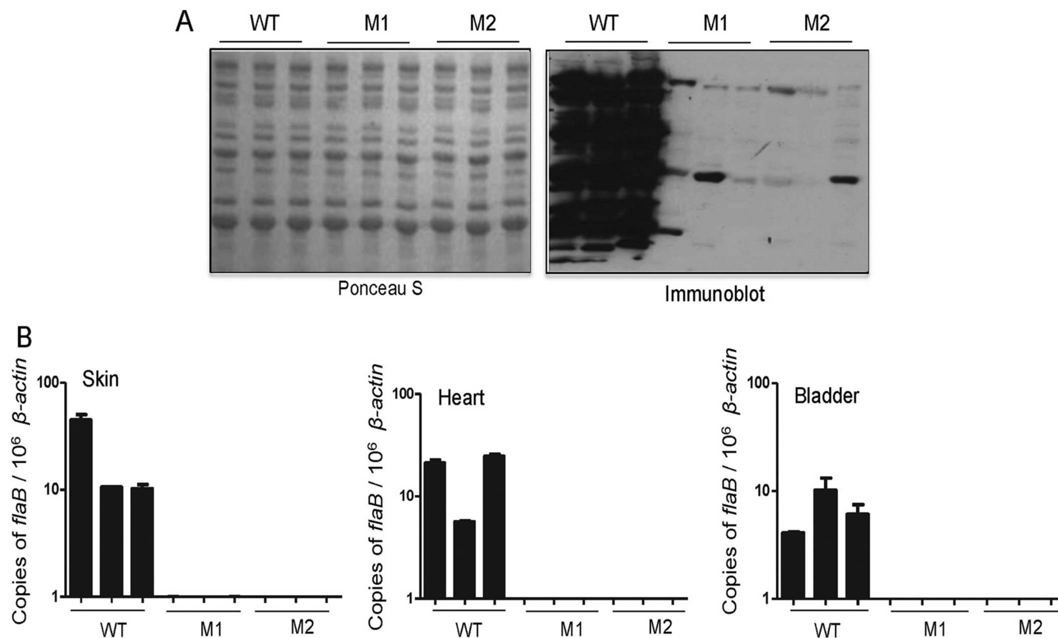

FIG 4 Bb*htrA* is required for infection of mice. (A) Immunoreactive profiles of mice infected with WT and Bb*htrA* mutant spirochetes. Groups of three C3H/HeN mice were infected with WT and Bb*htrA* mutant isolates (M1 and M2). Serum samples collected 2 weeks postinoculation were used to screen an immunoblot of *B. burgdorferi* cell lysate. (B) Spirochete burdens in infected mice were assessed by qRT-PCR by measuring the number of copies of the *B. burgdorferi flaB* gene normalized to mouse β-*actin* levels in the skin, heart, and bladder. Error bars represent the SEM of three independent experiments. Bb*htrA* mutants M1 and M2 remained undetectable in the murine tissues tested.

also been observed in other organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* (50, 51). Interestingly, despite the presence of Bb*htrA* as a single gene, the generation of deletion mutants with growth kinetics similar to those of WT spirochetes, especially at 34°C, suggests that its role is nonessential, at least in the *in vitro* growth of the organism.

While determination of the precise cellular function of BbHtrA awaits future studies, genetic manipulation of its expression in *B. burgdorferi* affected the levels of several spirochete proteins, such as P66 (30) and BB0323, but not those of several other proteins tested, suggesting that the protease is involved in the regulation of a specific set of borrelial proteins performing diverse functions in spirochete biology. As both the transcript levels and posttranslational maturation of target proteins are affected by Bb*htrA* deficiency or overexpression, as shown for BB0323 and P66 (30), it is likely that this protein could affect the processing of an unknown gene regulator, as speculated earlier (30). Additionally, BbHtrA could also act as a chaperone by providing an extended protein conformation in the periplasm before folding and insertion into the membrane or may promote the degradation of misfolded proteins prior to translocation in the membrane (12, 13). These observations are supported by the localization of BbHtrA in the various cellular compartments, including the periplasmic fraction, of *B. burgdorferi* (28). Apart from its role in the intracellular regulation of spirochete proteins, the secreted form of BbHtrA, which is potentially released into the mammalian host during infection and degrades the host extracellular matrix and proteoglycan (32), is directly involved in host infection and pathogenesis. One caveat regarding our study is that we did not perform infection experiments with the *trans*-complemented clone. However, two independent mutants that have all of the plasmids described here showed the same phenotype, strongly indicating that the BbHtrA-deficient spirochete is not infectious. While the precise reason why Bb*htrA* mutants lost virulence in mammalian hosts remains enigmatic, we speculate that multiple mechanisms could be responsible. To persist in a higher-temperature environment such as a mammalian host, spirochetes would require the critical functional support of BbHtrA, the absence of which renders mutants impaired for optimal growth and successful dissemination through host tissues.

Previously, we identified a virulence determinant, BB0323, that plays an important role in infectivity (34), possibly because of its critical involvement in spirochete fission and outer membrane integrity (33, 34), including other unknown functions. The recombinant form of BbHtrA specifically cleaved BB0323 into N- and C-terminal polypeptides with sizes that roughly corresponded to the mature polypeptides in spirochetes, suggesting that BB0323 is the specific substrate of BbHtrA (28). These initial results and the similarity of the morphological aberrations associated with the deletion of both BB0323 (34) and BbHtrA in the present study underscore the important role of the protease in the processing of BB0323 in *B. burgdorferi*. Interestingly, we not only noticed a reduction in cleaved and mature BB0323 but also detected uncleaved BB0323 in the Bb*htrA* deletion mutant at 37°C, which was further reduced upon complementation of the gene in *trans*. The processing of BB0323 remains unaffected at the normal growth temperature (34°C), raising the possibility that another cleavage mechanism or an unknown protease with a function similar to that of BbHtrA exists in spirochetes. It is noteworthy that the C-terminal protease CtpA also plays a role in the final processing of BB0323 polypeptides (52), and therefore, it might be possible that multiple proteases cleave BB0323 at the various temperatures and environments the spirochetes encounter in mammals and in the arthropod vector. At least two mechanisms could be

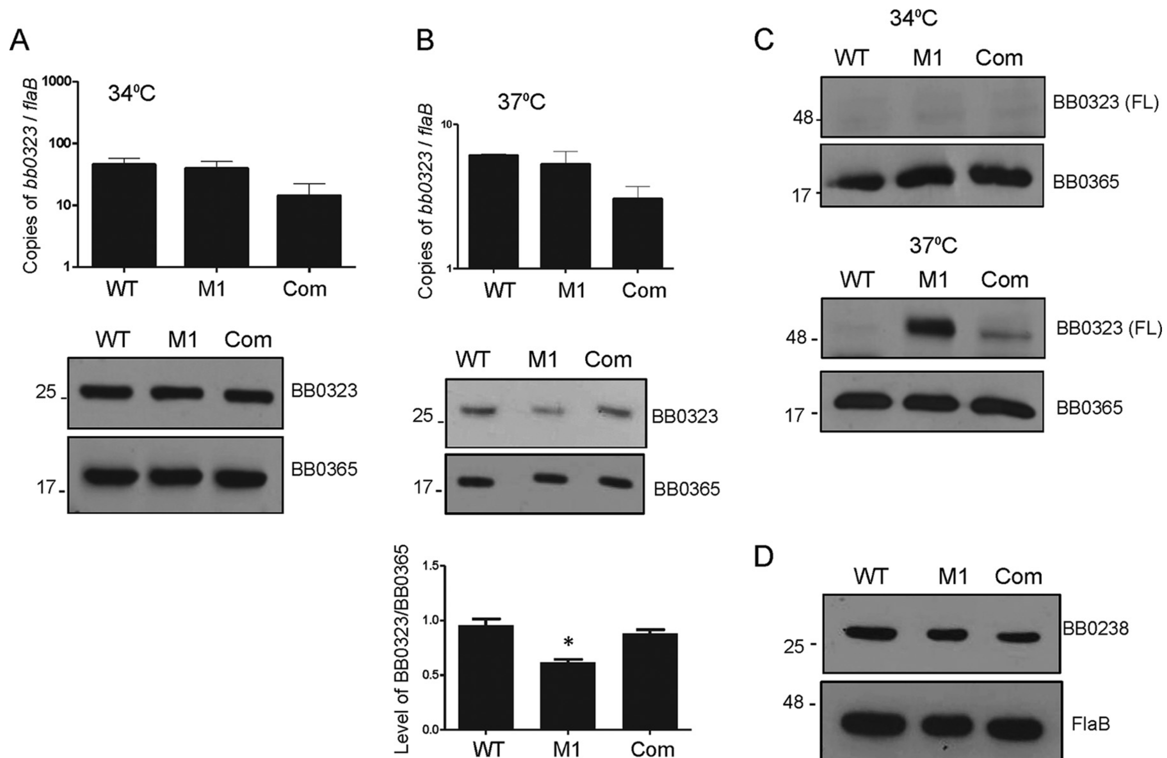

FIG 5 BbHtrA plays a role in the proteolytic processing of the borrelial virulence determinant BB0323. (A) Expression of BB0323 in the WT, Bb*htrA* mutant, and complemented strains at 34°C. WT, Bb*htrA* mutant (M1), and complemented (Com) isolates were grown at 34°C, and samples were harvested at stationary (10^8 cells/ml) phase. Expression of *bb0323* was monitored either at the transcription level by qRT-PCR and normalized to copies of the *flaB* transcript (top) or at the protein level after immunoblotting (bottom) with anti-BB0323 or anti-BB0365 antiserum. The bars represent the mean values from three independent experiments, and the error bars represent the SEM. The levels of Bb*htrA* mRNA are similar in all of the isolates ($P > 0.05$). (B) Production of BB0323 in the WT, Bb*htrA* mutant, and complemented strains at 37°C. Expression of *bb0323* mRNA (top) or protein (middle and bottom) was determined in WT, Bb*htrA* mutant, and complemented spirochetes at 37°C. Extracts from WT, Bb*htrA* mutant, and complemented isolates were resolved by 12% SDS-PAGE and immunoblotted with anti-BB0323 and anti-BB0365 antisera. Levels of BbHtrA relative to those of BB0365 (bottom) were determined by quantitative densitometry. The levels of BB0323 are reduced in the Bb*htrA* mutant. *, $P < 0.05$. The bars represent the mean values from three independent experiments, and the error bars represent the SEM. The levels of *bb0323* mRNA are similar in all of the isolates ($P > 0.05$). (C) Detection of full-length (FL) BB0323 in the Bb*htrA* mutant by far-Western blotting. Extracts from WT, Bb*htrA* mutant, and complemented isolates grown at either 34°C (top) or 37°C (bottom) were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, incubated overnight with recombinant His-BB0323 purified from *E. coli*, and immunoblotted with anti-BB0323 or anti-BB0365 antisera. (D) Expression of BB0238 in WT, Bb*htrA* mutant, and complemented spirochetes at 37°C. Extracts from WT, Bb*htrA* mutant, and complemented isolates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-BB0238 antiserum (top) or anti-FlaB antiserum (bottom) as a loading control. The values to the left of the blots are molecular sizes in kilodaltons.

attributed to the role of BbHtrA in the processing of BB0323 at the mammal-specific temperature. First, BbHtrA directly processes BB0323, and precise cleavage of BB0323 might be essential for cell fission or infectivity; therefore, absence of BbHtrA results in accumulation of the uncleaved polypeptide, leading to altered cell morphology, as seen in Bb*htrA* deletion mutants, and subsequent loss of virulence. Second, BbHtrA might act as a chaperone for the cleaved N terminus, and the loss of BbHtrA increased the susceptibility of the N terminus to degradation, thereby resulting in a defect in fission and hence infectivity. At this point, it cannot be confirmed that the reduction in the processed or cleaved BB0323 polypeptide was due to its increased susceptibility to degradation in the absence of BbHtrA at the elevated temperature or to impaired cleavage of the full-length protein. Nevertheless, our data strongly argue that BbHtrA is involved in the processing and regulation of BB0323 in *B. burgdorferi*, especially at an elevated, mammalian host-specific temperature.

In summary, we demonstrated that targeted deletion of Bb*htrA* results in specific spirochete phenotypes, preferentially at an elevated temperature, including slower growth, altered morphology and cellular architecture, and attenuated infection of mice. Although the loss of virulence could not be reversed in the complemented isolate because of technical constraints, most of the defects exhibited by Bb*htrA* mutants *in vitro* can be cured by the introduction of a copy of the gene in *trans*. The transcription and posttranslational processing of at least one key virulence determinant, BB0323, are also affected. These studies open a new line of investigation in understanding the regulation of proteins involved in key cellular processes and infection by BbHtrA. Further studies are warranted to investigate the role of BbHtrA in the tick phase of the cycle of *B. burgdorferi* and further elucidate its involvement in the global regulation of important cellular proteins.

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