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Proteolysis of BB0323 results in two polypeptides that impact physiologic and infectious phenotypes in *Borrelia burgdorferi*

Toru Kariu¹, Xiuli Yang¹, Carolyn B. Marks^{2,a}, Xinyue Zhang¹, and Utpal Pal¹

¹ Department of Veterinary Medicine and Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, Maryland 20742, USA

² Department of Biology, University of Richmond, Richmond, Virginia 23173, USA

Summary

Borrelia burgdorferi gene product BB0323 is required for cell fission and pathogen persistence *in vivo*. Here, we show that BB0323, which is conserved amongst globally prevalent infectious strains, supports normal spirochete growth and morphology even at early phases of cell division. We demonstrate that native BB0323 undergoes proteolytic processing at the C-terminus, at a site after the first 202 N-terminal amino acids. We further identified a periplasmic BB0323-binding protein in *B. burgdorferi*, annotated as BB0104, having serine protease activity responsible for the primary cleavage of BB0323 to produce discrete N- and C-terminal polypeptides. These two BB0323 polypeptides interact with each other, and either individually or as a complex, are associated with multiple functions in spirochete biology and infectivity. While N-terminal BB0323 is adequate to support cell fission, the C-terminal LysM domain is dispensable for this process, despite its ability to bind *B. burgdorferi* peptidoglycan. However, the LysM domain or the precisely processed BB0323 product is essential for mammalian infection. As BB0323 is a membrane protein crucial for *B. burgdorferi* survival *in vivo*, exploring its function may suggest novel ways to interrupt infection while enhancing our understanding of the intricate spirochete fission process.

Keywords

Borrelia burgdorferi; Lyme borreliosis; Proteolytic processing; Cell fission; Microbial pathogenesis

Introduction

Lyme disease (Steere *et al.*, 2004), caused by infection with *B. burgdorferi* sensu lato, remains a globally prevalent tick-borne illness (Piesman & Eisen, 2008). The Lyme disease pathogen possesses a number of unique qualities, including an unusually segmented genome, which encodes a vast array of proteins that lack homology to those of known functions (Casjens *et al.*, 2000, Casjens *et al.*, 2011, Fraser *et al.*, 1997, Schutzer *et al.*, 2011). Selective host and/or vector-specific expression of some of these proteins, which play indispensable roles in supporting spirochete infection (Kenedy *et al.*, 2012, Kung *et al.*, 2013), are controlled by a complex genetic regulatory network (Radolf *et al.*, 2012, Samuels, 2011). Given its evolutionary distance from other bacteria (Paster *et al.*, 1991), *B. burgdorferi*, a spirochete, features a distinct cellular structure. For example, the *B.*

Corresponding author: Utpal Pal, Department of Veterinary Medicine, Building 795, Room 1341, University of Maryland, College Park, MD 20742. Phone: 301-314-2118, Fax: 301-314-6855, upal@umd.edu.

^aCurrent address: Center for Nanoscale Systems, Harvard University, Cambridge, MA 02138

burgdorferi membrane has discrete structural organization (Bergström & Zückert, 2010). This includes the absence of phosphatidylethanolamine and canonical lipopolysaccharides, the presence of lower density membrane-spanning proteins in the outer membrane (OM), and a relatively large number of surface-exposed OM proteins, many of which are anchored to the membrane via an N-terminal lipid moiety. Spirochetes also possess distinct cellular organization including periplasmic flagella that run between the OM and protoplasmic cylinder; the latter is surrounded by an inner membrane and a peptidoglycan layer (Charon *et al.*, 2012). Although periplasmic flagella have been shown to contribute to the maintenance of the cellular architecture of *B. burgdorferi* (Motaleb *et al.*, 2000), precisely how the relatively fluid OM is organized over a flagellum and is physically linked to the peptidoglycan layer or the protoplasmic cylinder of spirochetes remains unknown.

In many Gram-negative bacteria such as *Escherichia coli*, a complex of five proteins, called the Tol-Pal protein complex (Bernadac *et al.*, 1998, Cascales *et al.*, 2002, Gerding *et al.*, 2007, Lazzaroni *et al.*, 1999), connects the OM to both the peptidoglycan and inner membrane layers via protein-peptidoglycan and protein-protein interactions. The trans-envelope Tol-Pal complex is also an integral part of the cell division machinery required for bacterial fission (Gerding *et al.*, 2007). Although the complex consists of multiple proteins, some of which perform redundant functions (demonstrated by a lack of phenotypic defects in targeted deletion mutants) (Bernadac *et al.*, 1998), none of the members have identifiable orthologs in the borrelial genome. In recent years, one of the chromosomally-encoded and conserved proteins of *B. burgdorferi*, annotated BB0323, has been shown to play a critical role in the organization of the spirochete OM, cell fission, and infectivity (Stewart *et al.*, 2004, Zhang *et al.*, 2009). Notably, BB0323 orthologs are absent in the sequenced genomes of other pathogenic spirochetes, such as *Leptospira* and *Treponema* species. In fact, other than possession of a single lysin motif (LysM) located at the extreme C-terminus of the protein, BB0323 is not related to any known protein. However, part of the phenotype of *bb0323* deficiency (impaired OM organization/cell fission) is analogous to the effects of the deletion of certain members of the bacterial Tol-Pal protein complex (Bernadac *et al.*, 1998, Cascales *et al.*, 2002, Llamas *et al.*, 2000, Yeh *et al.*, 2012). Given that BB0323 is an OM protein that is necessary throughout the tick-rodent infection cycle (Zhang *et al.*, 2009), understanding its function would not only provide novel insight into spirochete OM organization, cell fission, and the infection process, but also may aid in identifying possible therapeutic methods to combat Lyme disease. In this study, we show that BB0323 undergoes proteolytic processing and ultimately yields two distinct polypeptides - a larger N-terminus and a smaller C-terminus containing a LysM domain - that play distinct roles in cell morphology, fission, and murine infection.

Results

BB0323 is highly conserved in Lyme disease spirochetes and is constitutively expressed in cultured spirochetes during growth and division

Database searches indicated a high conservation of BB0323 across diverse *B. burgdorferi* sensu lato strains; for example, the amino acid sequence identities of BB0323 proteins between *B. burgdorferi* isolate B31 and *B. garinii* ZS7 and *B. afzelii* PKo are 94% and 92%, respectively. Their amino acid similarities are 95% and 94%. Accordingly, immunoblot analyses confirmed that the BB0323 antibody raised against the *B. burgdorferi* B31 antigen (Zhang *et al.*, 2009) readily recognized the orthologs in several other infectious strains of Lyme disease spirochetes (Fig. 1A). We further show that *bb0323* is consistently transcribed throughout spirochete growth in culture, albeit at slightly enhanced levels during the early phases of growth (Fig. 1B) at a time *bb0323* deletion mutants display cell fission defects (Fig. 1C). Together, these results suggest that BB0323 likely serves an important function across diverse species of Lyme disease spirochetes. These studies have also reinforced our

initial observation that BB0323 is required for proper cell division and maintenance of outer membrane integrity during *B. burgdorferi* growth *in vitro*.

BB0323 is proteolytically processed into distinct polypeptides in *B. burgdorferi*

In contrast to the molecular weight of recombinant BB0323 produced in *E. coli* (~42 kDa), as well as its predicted molecular weight (42 kDa), the native borrelial protein migrates at a mobility corresponding to a molecular weight of 27 kDa. To study its post-translational processing, we generated multiple *B. burgdorferi* mutants, lacking either the ~6 kDa LysM domain located at the C-terminal end (Δ LysM) or having a greater deletion of the C-terminus, at a randomly chosen amino acid site (Δ C, ~20 kDa). To accomplish this, a previously generated *bb0323* deletion mutant (Zhang et al., 2009) was complemented with two truncated versions of the *bb0323* open reading frame (Fig. S1A and S1B), as detailed in the experimental procedures section. RT-PCR analyses showed that wild-type spirochetes and *bb0323* Δ LysM and *bb0323* Δ C mutants produced appropriate-sized *bb0323* transcripts (Fig. S1C). Control RT-PCR reactions for deleted regions did not yield any products from the truncation mutants but did produce the expected-sized products from either the wild-type spirochetes or a previously generated full *bb0323*-complemented strain (data not shown). An immunoblot analysis using BB0323 antiserum further indicated that *bb0323* Δ LysM mutants produced a similar-sized BB0323 (27 kDa) as in wild-type *B. burgdorferi* (Fig. S1D). However, *bb0323* Δ C mutation, which reflected an additional C-terminal truncation of BB0323, resulted in a polypeptide that migrated at a mobility consistent with a molecular weight of approximately 22 kDa (the predicted size of the truncated BB0323 protein expressed from the construct pXLF14301-p*bb0323* Δ C (21.6 kDa)). These observations indicate that the full-length BB0323 (42 kDa) is proteolytically processed at the C-terminus and that the N-terminus of BB0323 represents the predominant 27 kDa polypeptide previously detected in borrelial cells (Zhang et al., 2009). These studies also suggest that cleavage of BB0323 occurs at a site after the first 202 N-terminal amino acids of the predicted translated product of the *bb0323* open reading frame. Notably, although BB0323 antibody generated against full-length protein was unable to recognize the C-terminal polypeptide, likely due to its poor immunogenicity, other indirect evidence, such as identification of a C-terminal BB0323 peptide (encompassing amino acids 303-313) via LC-MS/MS analyses of spirochete lysates (data not shown), provided an initial clue to the existence of a BB0323 C-terminal polypeptide in borrelial cells. Despite our repeated attempts, we are unable to isolate and identify native BB0323 polypeptides using immunoprecipitation and mass spectrometry-based assays; therefore, the precise size of mature BB0323 polypeptides or exact cleavage sites remain unknown.

Processing of BB0323 polypeptide is a proteolytic maturation process potentially involving the periplasmic serine protease BB0104 (BbHtrA)

Although BB0323 is known to be a substrate for a borrelial C-terminal protease (CtpA) (Ostberg *et al.*, 2004), presence of a lower-molecular-weight BB0323 polypeptide in the *ctpA* mutant (Ostberg *et al.*, 2004) suggests that there are primary cleavage mechanism(s) for the proteolytic processing of the full-length protein. As BB0323 lacks identifiable protease domains, we also assessed whether the full-length protein exhibits autoproteolytic activity *in vitro*. No degradation of BB0323 was recorded when the 42 kDa recombinant protein was exposed to a variety of pH (2-10) or temperature (25-34°C) conditions *in vitro* (data not shown). We therefore speculated that primary cleavage of BB0323 could be mediated by a borrelial protease such as BB0104, a putative periplasmic serine protease that is a bacterial HtrA homologue, and we thus attempted to identify the protein using a BB0323 affinity chromatography approach. To accomplish this, soluble proteins from *B. burgdorferi* were loaded onto a recombinant BB0323-conjugated sepharose column. After extensive washing, bound materials were eluted, separated by SDS-PAGE, and stained, and

visible bands were excised and identified by a LC-MS/MS approach as detailed in our earlier publications (Promnares *et al.*, 2009, Yang *et al.*, 2011). The data indicated a predominance of BB0104 (herein referred to as BbHtrA), which bound to BB0323 (data not shown). A few other proteins were also identified, including BB0238, BB0713, and BBL34; however, their interactions with BB0323 within the borrelial cell envelope remains a subject of future investigation. Incidentally, our published data also suggested the co-existence of BbHtrA with BB0323 in a common OM protein complex (Yang *et al.*, 2011). We therefore examined whether BbHtrA binds BB0323 directly and whether it has specific cleavage activities. Recombinant BbHtrA was produced in *E. coli* and used for immunizing mice to generate polyclonal antibodies. As BbHtrA is likely to be a serine protease, we also created a mutated recombinant protein to assess its specific enzymatic activities. An active site mutated version of BbHtrA was generated with the critical replacement of the serine residue with an alanine (BbHtrA.S226A) at the predicted catalytic site (Loosmore *et al.*, 1998), which we identified via sequence alignment of BB0104 with bacterial HtrA family proteins. Direct interaction between recombinant BbHtrA and BB0323 was further demonstrated using a GST pull-down assay (Fig. 2A), indicating that BbHtrA could be responsible for proteolytic cleavage of BB0323. In subsequent digestion assays, recombinant BbHtrA, but not the mutated protein (BbHtrA.S226A), processed BB0323 into ~30 kDa N-terminal and ~15 kDa C-terminal polypeptides (Fig. 2B, top panels). Note that the BbHtrA-digested N-terminal polypeptide has a molecular weight approximately 3-4 kDa greater than the mature BB0323 polypeptide as detected in the *ctpA* mutant (Ostberg *et al.*, 2004), strongly suggesting that BbHtrA-derived BB0323 polypeptide(s) are further subjected to CtpA processing to ultimately yield mature polypeptides. Unlike the specific processing of BB0323 polypeptides, the protease activity of BbHtrA was absent when tested against another borrelial OM protein, BBA52 (Kumar *et al.*, 2011) (Fig. 2B, bottom panel). BbHtrA could be the periplasmic serine protease, as isolation of periplasmic proteins from *B. burgdorferi* using a commercially available kit and subsequent immunoblot analyses using anti-BbHtrA and anti-BB0323 indicated that BB0323 polypeptides and BbHtrA are detectable in the periplasm (Fig. 2C). However, BB0323 is also detectable in the OM (Zhang *et al.*, 2009), and similar to most lipoproteins, the N-terminal BB0323 fragment that possesses an amino-terminal lipoprotein sequence also partitions into the Triton X-114 detergent phase (Fig. S2).

BB0323 polypeptides interact with each other

As the phenotype of *bb0323* deficiency was comparable to the effects of the deletion of certain members of the bacterial Tol-Pal protein complex, we next examined whether BB0323 N- and C-terminal polypeptides also form a complex by interacting with each other. First, to assess the possible interaction of BB0323 polypeptides directly *in vitro*, we made recombinant truncated BB0323 proteins representing the N- and C-terminal polypeptides. As a cleavage product of BB0323 is around 27 kDa (Fig. S1D), three N-terminal polypeptides (N1, N2, and N3 with molecular weights of 24.1, 26.1, and 28.4 kDa) and a C-terminal polypeptide (C, 15.8 kDa) were produced (Fig. 3A). An additional C-terminal polypeptide that encompasses the LysM domain only (LysM, ~6 kDa) was also generated (Fig. 3A). These polypeptides were produced with or without a GST tag and were assessed for protein-protein interaction using GST pull-down and far-Western blot assays. The results showed that N2 and N3 strongly interact with GST-C in a GST-pull down assay (Fig. 3B). As the N1 fragment failed to bind GST-C, our results also indicated that the sequence between 226 and 242 is essential for this heteromer formation. Next, we performed a far-Western blot analysis to further verify the interaction of recombinant N- and C-terminal polypeptides with the respective native BB0323 polypeptides. To accomplish this, lysates from wild-type spirochetes or *bb0323* mutants were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with recombinant GST-tagged N- or C-

terminal polypeptides. Finally, bound proteins were detected using GST antibodies. In accordance with interactions between recombinant N- and C-terminal polypeptides (Fig. 3B), our results revealed that these polypeptides also bind to native protein/polypeptides migrating as with molecular masses comparable to those of the respective C- and N-terminal polypeptides (Fig. 3C). Notably, the polypeptide encompassing only the LysM domain (LysM) failed to exhibit such binding (Fig. 3C), suggesting that sequences before it are involved in the BB0323 interaction.

BB0323 LysM domain binds peptidoglycan but lacks detectable cell wall lytic activities

Although BB0323 is processed into separate polypeptides that encompass the N- and C-terminal regions of BB0323 (Fig. 2), it remained unclear whether or how both polypeptides contributed to the mutant phenotype of improper cell fission and poor infectivity. A possible clue to the function of BB0323 was the presence of a putative LysM domain located at the extreme C-terminus of the protein. As LysM domains are involved in the binding of bacterial peptidoglycan (Buist *et al.*, 2008), we further examined whether the BB0323 LysM domain interacted with peptidoglycan purified from cultured *B. burgdorferi* cells. To test this, recombinant BB0323 was produced as a GST fusion protein encompassing either the full-length protein (BB0323), a truncated protein without the LysM domain (BB0323 Δ LysM) (Fig. 4A), or a polypeptide with only the LysM domain (LysM) (Fig. 3A). Native peptidoglycan was purified from *B. burgdorferi*, and a microtiter-based assay was used to assess its interaction with BB0323 or the LysM domain. Equivalent amounts of peptidoglycan sample were immobilized in wells and incubated with the recombinant GST-fused full-length or truncated BB0323 proteins (Fig. 4B). Bound proteins were detected using anti-GST antibodies and secondary antibodies. Full-length BB0323 and the LysM domain displayed higher peptidoglycan binding than BB0323 Δ LysM (Fig. 4B). A control GST-fused *B. burgdorferi* protein, BBA57, did not have detectable peptidoglycan binding activity (data not shown).

Additionally, using a different assay, we achieved a similar result that supports specific interactions between borrelial peptidoglycan and LysM. Purified *B. burgdorferi* peptidoglycan was separately incubated with equal amounts of recombinant GST-BB0323, BB0323 Δ LysM, or LysM. After incubation, peptidoglycan-bound proteins were pelleted by centrifugation, isolated from unbound and soluble proteins in the supernatant, and analyzed by immunoblotting using GST antibodies. Figure 4C demonstrates that both BB0323 and LysM bound to peptidoglycan and then precipitated in the insoluble pellet. Conversely, BB0323 Δ LysM was unable to bind to peptidoglycan and remained in the supernatant. Although BB0323 bound to peptidoglycan via the LysM domain, unlike other bacterial enzymes associated with peptidoglycan degradation, we could not detect cell wall lytic activity of BB0323 with an established zymographic procedure using *Micrococcus* cell wall (data not shown). However, there still remains a possibility that the recombinant protein used in our assays lacks the activity of native BB0323.

BB0323 N-terminal polypeptide can independently support normal cell fission or spirochete growth

Next we assessed whether the N-terminal or C-terminal (LysM domain) region of BB0323 contributed to normal borrelial growth and fission - defective in *bb0323* mutants. The wild-type and genetically-manipulated spirochetes (Δ LysM and Δ C mutants, Fig. S1) were diluted to a density of 10^5 cells ml⁻¹ and were allowed to grow at 34°C in culture media. Spirochetes were counted every 24 hours under a dark field microscope using a Petroff-Hausser cell counter for assessment of cell growth kinetics, whereas cell morphology was examined by transmission electron microscopy. The results revealed that unlike the *bb0323* deletion mutant, both Δ LysM and Δ C mutants display growth kinetics (Fig. 5A), OM

organization, and cell fission (Fig. 5B) similar to the wild-type isolate. This suggests that the N-terminal region of BB0323 (encompassing the first 202 amino acids) is sufficient for supporting normal spirochete growth and fission. Thus, although the C-terminal LysM domain binds isolated borrelial peptidoglycan, our data indicate that this region plays a redundant role in spirochete cell fission and the normal growth process.

LysM domain is required for spirochete persistence in the murine host

We finally assessed whether the C-terminal LysM domain is important for infectivity or whether it too plays redundant roles in cell growth or fission (Fig. 5). Separate groups of C3H/HeN mice were inoculated with 10^5 cells of wild-type, *bb0323* Δ LysM, or *bb0323*-complemented *B. burgdorferi*. Infection was assessed by qRT-PCR analysis of *B. burgdorferi* *flaB* transcript levels in several murine tissues and by re-isolation of spirochetes from infected tissues using culture analysis. The qRT-PCR analysis of pathogen levels in the skin, heart, joint, and bladder indicated that while Δ LysM mutants remained undetectable in all tissues, both wild-type and *bb0323*-complemented spirochetes persisted during infection (Fig. 6). Similarly, wild-type and *bb0323*-complemented *B. burgdorferi* were recovered by culture analysis of murine tissues, whereas *bb0323* Δ LysM mutants could not be isolated. Whereas individual ear samples isolated from a total of six mice infected with either wild-type or *bb0323*-complemented spirochetes tested positive for spirochetes, ear samples from each of the six mice inoculated with Δ LysM mutants remained culture negative (Table S2). Together, these results suggest that the BB0323 LysM domain is required for *B. burgdorferi* infection of the murine host.

Discussion

While BB0323 is required for normal OM organization, cell fission, and infectivity (Stewart et al., 2004, Zhang et al., 2009), precisely how this protein contributes to spirochete biology remains unknown. Here we present strong evidence that BbHtrA (BB0104), a periplasmic serine protease, is potentially involved in the proteolytic processing of BB0323 into distinct polypeptides including a longer N-terminal fragment, as well as a shorter, poorly-immunogenic C-terminal polypeptide. The latter polypeptide harbors a LysM domain (Buist et al., 2008), which binds borrelial peptidoglycan and is potentially essential for mammalian infection but plays a redundant role in supporting cell fission. On the other hand, the N-terminal polypeptide, which can independently support normal borrelial morphology and cell fission, interacts with the C-terminal polypeptide, although the biological significance of BB0323 heteromer formation remains unknown. As BB0323 is one of the few known OM proteins essential for *B. burgdorferi* transmission and persistence throughout the tick-rodent infection cycle (Zhang et al., 2009), further understanding BB0323 processing and biological function may help to determine possible therapeutic methods to combat Lyme disease.

Although the precise sequence of BB0323 maturation events in borrelial cells remains unknown, several lines of evidence suggest that BbHtrA, an ortholog of the bacterial periplasmic serine protease and heat shock protein termed HtrA (also known as DegP, or protease Do) (Merdanovic et al., 2011, Clausen et al., 2011, Meltzer et al., 2009), is responsible for the primary cleavage of BB0323. First, even though CtpA, a C-terminal protease, has been shown to be involved in BB0323 processing (Ostberg et al., 2004), the occurrence of low molecular weight BB0323 in the *ctpA* mutant (30 kDa compared to the predicted or recombinant version in *E. coli* of 42 kDa) suggests that BB0323 likely undergoes additional unidentified proteolytic event(s) prior to the processing by CtpA. Second, based on our studies using recombinant protein, we speculate that an autocatalytic event for BB0323 proteolysis seems unlikely, although there is a possibility that the recombinant protein lacks the activity of the native protein. Finally, we show that BbHtrA

interacts with BB0323 and specifically cleaves the protein into a larger N-terminal and a shorter C-terminal polypeptide. Although HtrA is shown to have serine protease activity that degrades unfolded or misfolded proteins at higher temperatures (Clausen et al., 2011), BbHtrA cleaved BB0323 specifically to a 30 kDa N-terminal and an ~15 kDa C-terminal polypeptide. Notably, the size of BB0323 in the *ctpA* mutant is also 30 kDa, which corresponds to the N-terminal polypeptide. Despite our concerted attempts, we were unable to isolate a *BbHtrA* deletion mutant, which suggests a housekeeping function of the protein. Based on our current data, we speculate that BbHtrA produces a 30 kDa BB0323 N-terminal polypeptide, which is then further processed by CtpA (Ostberg et al., 2004) to eventually produce a 27 kDa mature polypeptide. Such multi-step post-translational maturation of BB0323, particularly involving a potential chaperone-protease like BbHtrA, is intriguing and possibly relevant to the tight quality control of important proteins like BB0323, which could have pleiotropic roles in pathogen biology and infection. HtrA is a highly conserved protein with a serine protease domain and one or two carboxy-terminal peptide-binding domains and uses its antagonizing functions as both a protease and chaperone to control protein quality in an ATP-independent manner (Clausen et al., 2011). Originally identified as a thermal stress-induced protein, HtrA is implicated in many cellular processes, including cell fission and bacterial virulence and can trigger or modulate various signaling pathways by cleaving or sequestering regulatory proteins (Clausen et al., 2011). The function of the *E. coli* homolog of HtrA is essential even under optimal laboratory conditions (Clausen et al., 2002). Therefore, owing to its versatile roles as a protease and yet-to-be characterized chaperone, BbHtrA is an ideal candidate for the maturation and/or regulation of proteins such as BB0323 that are important for outer membrane integrity and borrelial cell fission.

Our data suggest specific interaction between BB0323 polypeptides and in addition, confirms that amino acid region 226-242 is important for the formation of a complex of BB0323 N- and C-terminal polypeptides. Although BB0323 is primarily a subcellular antigen and localizes to the periplasm, the protein is also detectable in the OM (Zhang et al., 2009). As the N-terminus of BB0323 contains a lipidation motif and partitions into the Triton X-114 detergent phase, it is possible that the lipidated N-terminal polypeptide inserts into the OM, while the C-terminal polypeptide binds the peptidoglycan layer via the LysM domain. Thus, we speculate that the processed BB0323 polypeptides interact with each other and/or with other proteins involving regions N-terminal to the LysM domain to form a structural complex that may function similarly to the bacterial Tol-Pal complex (Bernadac et al., 1998, Cascales et al., 2002, Llamas et al., 2000, Yeh et al., 2012) that help in tethering the OM to the protoplasmic cylinder. However, if such an analogue exists in *B. burgdorferi* and allows for the fluidity of the outer membrane needed for spirochetal motility with the subsurface flagellar apparatus, it is conceivable that the complex would need to be restricted to the poles of the cell.

Our data suggest that BB0323 polypeptides individually, or as a complex, could contribute to multiple cellular function(s). Unlike other LysM domain-containing bacterial enzymes involved in peptidoglycan turnover during cell fission (Buist et al., 2008), the recombinant form of full-length BB0323 lacks an identifiable hydrolytic domain and fails to display recognizable degrading ability against the *Micrococcus* cell wall, suggesting a minor role, if any, in peptidoglycan degradation. However, as recombinant protein as well as cell wall from Gram-positive bacteria have been used in our assays, we cannot rule out the role of native BB0323 in peptidoglycan turnover in *B. burgdorferi*, which also features a chemically distinct peptidoglycan likely with the diaminopimelate residue replaced by ornithine (Schleifer & Kandler, 1972). It is also possible that BB0323 is involved in additional cellular functions related to OM biogenesis and/or stability and that its role in peptidoglycan turnover is redundant due to the presence of other LysM domain-containing proteins; for example, BB0262 and BB0761 that also possess potential LysM domains. The latter

speculation is supported by the fact that the BB0323 C-terminal polypeptide binds purified *B. burgdorferi* peptidoglycan via its LysM domain but lacks an essential role in the cell fission process. Additionally, we show that the BB0323 N-terminal polypeptide can independently support cell fission (despite having no recognizable hydrolytic or LysM domain). Interestingly, the N-terminal polypeptide, which supports borrelial growth, morphology, and fission like full-length BB0323 in wild-type cells, failed to support the establishment of spirochete infection, suggesting that a precisely processed N-terminal protein, the LysM domain, or the interaction of mature BB0323 polypeptides is indispensable for infectivity. This reinforces our speculation that processed BB0323 products or specifically the C-terminal polypeptide containing the LysM domain is involved in additional physiological functions beyond cellular organization or fission including those required for host infectivity that remain subjects of future investigation. Nevertheless, based on the fact that *bb0323* is expressed throughout the spirochete infection cycle (Zhang et al., 2009) and that *bb0323* deletion impairs pathogen survival in both the murine host and the arthropod vector (Zhang et al., 2009), the function of BB0323 and/or the LysM domain is likely to be important for *B. burgdorferi* survival in ticks as well.

Experimental Procedures

B. burgdorferi and mice

B. burgdorferi strain B31 clone A3 is an infectious, clonal derivative of the type B31-M1 (Elias *et al.*, 2002) used for all genetic manipulations reported in this study. The generation of *bb0323* mutant and complemented isolates, as well as the use of additional *B. burgdorferi* sensu lato isolates were described previously (Zhang et al., 2009). Four-to-six-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 recombinant proteins, site-directed mutagenesis, and generation of antisera

The list of oligonucleotide primers used for the production of various recombinant proteins representing the full-length or truncated versions of *B. burgdorferi* proteins is presented in Table S1. All recombinant proteins were produced in *E. coli* using pGEX-6P-1 (GE Healthcare, Piscataway, NJ), as detailed earlier (Zhang et al., 2009). Mutated versions of proteins were generated by site-directed mutagenesis using oligonucleotide primers (Table S1) and a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Isolation of the purified recombinant proteins, either fused to GST or without any fusion after removal of the GST tag, was performed according to the manufacturer's instructions (GE Healthcare), also previously described (Promnare et al., 2009, Zhang et al., 2009). Generation of additional polyclonal antisera against full-length BB0323 and all other recombinant proteins in mice and assessment of the titer and specificity of the antisera using ELISA and immunoblotting were performed as described (Promnare et al., 2009, Zhang et al., 2009).

Polymerase chain reaction

The oligonucleotide sequences for each of the primers used in specific PCR reactions are indicated in Table S1. Total RNA was isolated from *in vitro*-grown *B. burgdorferi* at various growth phases or from infected mouse tissues using TRIzol reagent (Invitrogen), further treated with DNase I (NEB, Ipswich, MA) to reduce DNA contamination, and reverse transcribed to cDNA (AffinityScript, Santa Clara, CA). The cDNA samples were used in RT-PCR reactions and were analyzed by agarose gels or were assessed by quantitative RT-

PCR (qRT-PCR) analyses using iQ SYBR Green Supermix (BioRad, Hercules, CA), as detailed earlier (Promnares et al., 2009, Zhang et al., 2009).

Generation of *B. burgdorferi* mutants

Additional genetically manipulated isolates of *B. burgdorferi* were generated by reintroduction of truncated versions of *bb0323* into the *bb0323* deletion strain (Zhang et al., 2009). To accomplish this, we sought to clone portions of the *bb0323* open reading frame, its native promoter, and a streptomycin-resistance cassette into pXLF14301, which carries both up and downstream recombination arms for insertion of the DNA construct via allelic exchange into the *B. burgdorferi* chromosome between *bb0445* and *bb0446* as detailed (Promnares et al., 2009, Yang et al., 2009, Zhang et al., 2009). The *bb0323* open reading frame with a truncation of the C-terminal amino acids 203-377 (Δ C) or 328-377 (Δ LysM) was amplified using primers (as indicated in Table S1) and was cloned into the *Pst*I and *Sac*II sites of pKFSS1 (Frank et al., 2003). A DNA fragment containing truncated *bb0323* and the *aadA* cassette was further digested from pKFSS1-*bb0323* with *Sac*II and *Xma*I and was inserted into pXLF14301. The *bb0323* mutants were transformed with the recombinant plasmids, pXLF14301-p Δ LysM or Δ C. Transformants were selected based on their ability to grow in the presence of antibiotics, retention of the same plasmids as in the parental isolates (data not shown), and *bb0323* expression. Plasmid content was determined by PCR to ensure that strains were comparable and that plasmid loss did not contribute to phenotypic differences observed between strains. Clone Δ C had lost the lp28-1 plasmid, which was reinserted into the isolate using a wild-type copy carrying a gentamicin-resistance cassette, as described previously (Grimm et al., 2004). The *bb0323* mutants and the wild-type strains were processed for growth analysis and transmission electron microscopy, as described (Zhang et al., 2009).

Assays for peptidoglycan binding, cell wall lysis, and autolysis of BB0323

Peptidoglycan was purified from cultured *B. burgdorferi*, as previously described (Beck et al., 1990). Analysis of the interaction of peptidoglycan with GST-fused BB0323, BB0323 Δ LysM, or the LysM domain was performed using a published procedure (Eckert et al., 2006) with the following minor modifications. Briefly, one μ g of each GST-fused protein was incubated with aliquots of purified peptidoglycan in phosphate-buffered saline pH 7.2 with 0.05% Tween 20 (PBST) for 10 minutes at room temperature. Unbound protein in the supernatant was separated from peptidoglycan by centrifugation at $17,000 \times g$ for 20 minutes. After washing with PBST three times, bound protein was eluted using SDS-PAGE sample buffer and was analyzed by immunoblotting using anti-GST antibodies (GE Healthcare) and HRP-conjugated anti-goat secondary antibodies. Peptidoglycan interaction with BB0323 or the LysM domain was also examined using a microtiter plate assay, as detailed (Pal et al., 2004). Equal amounts of purified peptidoglycan were immobilized in wells of a microtiter plate and were incubated with full-length or truncated recombinant BB0323 protein fused to GST. Levels of bound protein were determined by incubation with an anti-GST antibody followed by HRP-conjugated anti-goat secondary antibodies and were measured by absorbance at OD₄₅₀. Cell wall lytic activity was assessed in a standard zymographic assay (Buist et al., 1995). The wild-type and *bb0323* mutant lysates were separately resolved using SDS-PAGE gels that contained autoclaved *Micrococcus lysodeikticus* cell lysates, a substrate for lytic enzymes. After electrophoresis, enzymatic activity of resolved borrelial proteins was visualized as clear zones on the opaque SDS-PAGE gel after an overnight incubation in 0.5% Triton X-100 in 50 mM Tris-HCl (pH 7.5) at 37°C.

Autolysis of BB0323 was performed as described (Ferris et al., 2005), with the following modifications. Briefly, various amounts of full-length recombinant BB0323 were

incubated either in 50 mM Glycine-HCl or Glycine-NaOH buffer in various pH (2-10) or temperature (25-34°C) conditions overnight. Protein cleavage was assessed by immunoblot using BB0323 antibodies.

Triton X-114 phase partitioning assay

Triton X-114 phase partitioning assays were performed as previously described (Yang *et al.*, 2010). Briefly, 10⁹ spirochetes were resuspended in 200 µl of PBS (pH 7.4), sonicated, mixed with 200 µl of 10% Triton X-114, and incubated overnight. The lysates were centrifuged to remove Triton X-114-insoluble material, and the supernatant was incubated at 37°C for 15 min followed by separation of the aqueous and detergent phases by centrifugation at 15,000 × *g* for 15 min at room temperature. The separated phases were washed with ice-cold acetone to precipitate the proteins, centrifuged at 13,000 × *g* for 15 min at 4°C, and finally assessed by immunoblotting using antibodies against known hydrophilic (BBA74) or amphiphilic (OspA) proteins as detailed (Yang *et al.*, 2010).

Western blotting, far-Western blotting, GST pull-down, and immunoprecipitation assays

Immunoblotting assays were performed in accordance with standard procedures using *B. burgdorferi* lysates and recombinant proteins resolved using SDS-PAGE or Tricine SDS-PAGE, employing specific antibodies, as detailed in our publications (Promnare et al., 2009, Yang *et al.*, 2009, Zhang *et al.*, 2009). For localization of periplasmic proteins by Western blot analysis, periplasmic or spheroplasmic proteins were extracted using a commercial kit (PeriPreps™ Periplasting Kit, Epicentre) following manufacturer's instructions. Proteins for far-Western analyses were fractionated by electrophoresis in a 12% SDS-PAGE or a 16% Tricine-SDS-PAGE gel before being transferred to nitrocellulose membranes. After transfer, membranes were blocked in Tris-buffered saline pH 7.5 with 0.05% Tween 20 (TBST) and 2% skim milk and were incubated with GST-fused proteins in the blocking buffer. After washing with TBST, bound proteins were identified using anti-GST antibodies and HRP-conjugated anti-goat secondary antibodies. A GST pull-down assay was carried out as detailed (Wei *et al.*, 2001) with modifications as follows: GST or GST-fused recombinant proteins were incubated with glutathione-sepharose beads (GE Healthcare) in TBST at 4°C for 2 h. The beads were washed several times with TBST by centrifugation at 1,000 × *g* for 5 minutes to remove unbound proteins, followed by the addition of SDS-PAGE sample buffer. The eluates were fractionated by SDS-PAGE, and recombinant proteins were detected by immunoblotting. For affinity purification of BB0323-interacting proteins, recombinant BB0323 was conjugated to NHS-activated Sepharose 4 Fast Flow (GE Healthcare). Borrelial lysates solubilized with TBST were applied onto the BB0323-immobilized Sepharose column at 4°C and washed extensively with TBST, and finally bound proteins were eluted using SDS-PAGE sample buffer.

Immunoprecipitation assays and LC-MS/MS-based protein identification analysis were performed as detailed earlier (Yang *et al.*, 2011).

Proteolytic activity of BB0104 (BbHtrA)

About 300 nanograms of recombinant wild-type or mutated BbHtrA with a critical replacement of a serine residue with an alanine at the putative catalytic site (BB0104.S226A) was incubated in 20 µl buffer (50 mM Tris-HCl pH 7.0, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol) at 37°C in the presence of five micrograms of various substrate proteins. The reactions were then terminated by adding SDS-PAGE sample buffer at the indicated times between zero and four hours. Digests were analyzed by Western blotting using specific antisera (Yang *et al.*, 2009) and far-Western blotting using N-terminal BB0323.

Animal infection studies

Groups of C3H/HeN (3 animals/group) were intradermally inoculated with equal numbers of wild-type, *bb0323*-complemented, or Δ LysM *B. burgdorferi* (10^5 spirochetes/mouse), as detailed (Yang et al., 2009). Infection was assessed at 21 days of pathogen challenge by culturing infected ear and heart tissues in the spirochete growth medium or by qRT-PCR analysis, as described (Zhang et al., 2009). The entire animal infection experiment was independently repeated in a similar fashion two additional times.

Statistical analysis

Results are expressed as the mean $\pm$ standard error (SEM). The significance of the difference between the mean values of the groups was evaluated by two-tailed Student's *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. BB0323 is highly conserved and expressed in *B. burgdorferi sensu lato* strains including during early growth phases

(A) Production of BB0323 in major infectious strains of *B. burgdorferi sensu lato*. Equal amounts of lysates prepared from *B. burgdorferi* isolates B31, 297, or N40, *B. garinii* isolate PBi, and *B. afzelii* isolate PKo were immunoblotted with BB0323 antisera generated against the *B. burgdorferi* B31 recombinant protein. Arrow denotes position of BB0323. Protein molecular weight markers are shown on the left. (B) BB0323 production in early and late growth phases of spirochetes in culture. Left panel denotes *bb0323* transcripts that were measured using qRT-PCR and presented as copies of *bb0323* transcripts per copy of *flaB* transcript. The bars represent the mean values, and the error bars represent the SEM values from three independent experiments. Differences between *bb0323* transcript levels in early and late growth phases are significant ($*P < 0.001$). Right panels show protein levels in spirochetes at the indicated growth phases assessed using Ponceau S staining or by immunoblotting with BB0323 antiserum. Arrow denotes position of BB0323. Protein molecular weight markers are shown on the left. (C) Transmission electron microscopic analysis of spirochetes collected at different phases of cell growth. BB0323-associated cell fission defects (arrows) were evident at early and late growth phases of spirochetes *in vitro*.

Figure 2. BB0104 (BbHtrA) is a putative BB0323-processing protease

(A) Interaction of BbHtrA and BB0323 in a GST pull-down assay. GST or GST-fused BB0323 were incubated with glutathione-sepharose beads, washed, and incubated with BbHtrA. Bound proteins were detected by immunoblotting using anti-BbHtrA. (B) Proteolytic activity of BbHtrA produces N- and C-terminal polypeptides of BB0323. Recombinant BbHtrA, as wild-type protein or mutated version with the critical replacement of a serine residue with an alanine (BB0104.S226A), was incubated with full-length BB0323 (top panel) or a control protein, BBA52 (bottom panel), at 37 °C for 0 to 4 h. Immunodetection of BbHtrA, BBA52, and N-terminal polypeptide of BB0323 was accomplished by Western blotting using polyclonal antibodies against the respective proteins. The C-terminal polypeptide of BB0323 is difficult to detect by Western blot analysis, likely due to poor immunogenicity but can be detectable by far-Western blotting, as detailed in Fig. 3C. (C) Detection of BbHtrA and BB0323 in the periplasmic fraction of *B. burgdorferi* cells. Periplasmic (Peri) or spheroplasmic (Sphero) proteins are extracted from cultured spirochetes (Bb) and immunoblotted using BbHtrA and BB0323 antibodies. The higher-molecular-weight band (arrow) could represent either cross-reacting antigens absent in the purified periplasmic fraction (Peri) from lower proteome diversity or a cell cylinder-specific HtrA precursor.

Figure 3. Specific interaction of N- and C-terminal polypeptides of BB0323

(A) Schematic representation of various truncated recombinant BB0323 proteins representing N- and C-terminal polypeptides. (B) Direct interaction of truncated recombinant BB0323 proteins representing N- and C-terminal polypeptides. N-terminal polypeptides were produced without GST tags (N1, N2, and N3). Recombinant glutathione S-transferase (GST) or GST-fused C-terminal polypeptide (GST-C) was immobilized on glutathione beads and incubated with various N-terminal polypeptides, as described in panel A. After thorough washing, bound proteins were recovered and assessed by immunoblotting using anti-BB0323 antiserum. For size comparison, N-terminal polypeptides N1, N2, and N3 were separated in the same gel. The arrowhead denotes a potential cross-reactive antigen. (C) Recombinant N- and C-terminal polypeptides recognize native version of C- and N-terminal BB0323 polypeptides in *B. burgdorferi*, as revealed by far-Western blot analyses. *B. burgdorferi* lysates from equal numbers of WT and *bb0323* mutant spirochetes were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and incubated with GST, GST-fused N2 (GST-N2), or GST-fused C-terminal polypeptide (GST-C). Bound proteins were detected by immunoblotting using anti-GST antibodies. Note that the GST-fused C-terminal polypeptide that encompasses only the LysM domain (LysM) failed to exhibit detectable interaction in the assay. Arrows denote positions of native BB0323 N- and C-terminal polypeptides.

Figure 4. BB0323 LysM domain binds to *B. burgdorferi* peptidoglycan

(A) Schematic representation of full-length BB0323 (BB0323), recombinant truncated BB0323 proteins with either a deletion of the LysM domain (BB0323ΔLysM) or with only the LysM motif (LysM). The proteins were purified as GST-fusion proteins in *E. coli*. (B) Immobilized peptidoglycan purified from *B. burgdorferi* binds recombinant LysM domain protein. Recombinant GST-tagged full-length BB0323, BB0323ΔLysM, LysM, or GST proteins were incubated in microtiter wells coated with peptidoglycan purified from *B. burgdorferi*. The wells were washed, and protein binding was quantified using anti-GST antibodies followed by HRP-conjugated secondary antibodies. The OD₄₅₀ value of BB0323 was expressed as 1.0. The bars represent the mean values, and the error bars represent the SEM values from three independent experiments. The asterisk represents a significant decrease ($P < 0.05$) between BB0323ΔLysM binding to peptidoglycan compared to that of either LysM or BB0323. (C) Interaction of LysM domain with borrelial peptidoglycan in solution. Peptidoglycan extracted from *B. burgdorferi* cells was mixed with BB0323, BB0323ΔLysM, or LysM, centrifuged to separate pellet or supernatant (Sup) fractions, and analyzed by immunoblotting using anti-GST antibodies. Arrow denotes position of BB0323 proteins.

Figure 5. BB0323 LysM domain is not essential for normal *B. burgdorferi* growth *in vitro*
(A) Growth analysis of cultured spirochetes. Wild-type cells (WT), *bb0323* deletion mutants (*bb0323*⁻), and isolates with deletion of the LysM domain (Δ LysM) or C-terminus of BB0323 (Δ C) were assessed for growth *in vitro*. Data represent results from three independent experiments. Differences in spirochete numbers between WT and Δ LysM or Δ C groups were not significant at any time point. Arrow indicates a significant difference ($P < 0.05$) between numbers of *bb0323* deletion mutants compared to all other isolates. **(B)** BB0323 LysM domain is not essential for normal morphology and the fission process in *B. burgdorferi* cells. Complementation of *bb0323* mutants with truncated *bb0323* genes restored normal cell morphology. Arrows denote abnormal OM organization as well as cell fission (represented by non-separated cells) in cases of *bb0323* mutants. WT, *bb0323*⁻, Δ LysM, and Δ C isolates were grown in culture and subjected to transmission electron microscopy.

Figure 6. BB0323 LysM domain is required for spirochete infection of murine hosts

Mice (3 animals/group) were infected with wild type (WT), *bb0323* Δ LysM (Δ LysM), or a *bb0323* deletion mutant complemented with *bb0323* (*bb0323* Com). RNA samples isolated from each group of three mice were pooled together according to the tissue type, and spirochete levels were assessed by qRT-PCR analysis at day 21 by measuring copies of *flaB* transcripts normalized against mouse β -actin levels. The entire animal infection experiment was independently repeated three times. The bars represent the mean values, and the error bars represent the SEM values from the three independent experiments. Asterisks indicate undetectable levels of *bb0323* Δ LysM (Δ LysM) mutants.