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A Tetratricopeptide Repeat Domain Protein has Profound Effects on Assembly of Periplasmic Flagella, Morphology, and Motility of the Lyme disease spirochete *Borrelia burgdorferi*

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SUMMARY

Spirochetes possess a unique periplasmic flagellar motor component called the collar. However, little is known about the composition or function of the flagellar collar proteins. To identify a collar protein, we have inactivated almost all genes annotated as motility-related in the *Borrelia burgdorferi* genome and identified only FlbB, which comprises the base of the collar. Since the major components of the collar complex remained unidentified, we took advantage of a protein-protein interaction map developed in another spirochete, *Treponema pallidum* to identify proteins of unknown function that could be collar proteins. Subsequently, using various comprehensive approaches, we identified a tetratricopeptide repeat protein BB0236 as a potential candidate for the collar. Biochemical assays indicated that FlbB interacts with BB0236. Furthermore, *bb0236* mutant analyses indicated that BB0236 is crucial for collar structure assembly, cellular morphology, motility, orientation of periplasmic flagella, and assembly of other flagellar structures. Moreover, using comparative motor analyses, we propose how the collar structure is assembled in *B. burgdorferi*. Together, our studies provide new insights into the organization and the complex assembly inherent to the unique spirochetal collar structure.

INTRODUCTION

Borrelia burgdorferi is the causative agent of Lyme disease, which is the most common vector-borne illness in the United States and Europe (Mead, 2015). Flagella-driven motility and chemotaxis are reported to be crucial for the pathogenesis of many bacteria including the spirochetes (Xu *et al.*, 2017, Novak *et al.*, 2016, Moon *et al.*, 2016a, Sultan *et al.*, 2015, Motaleb *et al.*, 2015, Sultan *et al.*, 2013b, Li *et al.*, 2010, Lambert *et al.*, 2012, Wunder *et al.*, 2016, Butler & Camilli, 2005, Lertsethtakarn *et al.*, 2011, Guyard *et al.*, 2013, Sze *et al.*, 2012, Lin *et al.*, 2015). Unlike other externally flagellated bacteria, spirochetes' flagella are

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located between the outer membrane and peptidoglycan layer i.e. in the periplasmic space (Kudryashev *et al.*, 2009, Charon *et al.*, 2009, Charon *et al.*, 2012, Wolgemuth, 2015). *B. burgdorferi* possesses 7–11 periplasmic flagella that are inserted at each pole of the cell and extend toward the other pole as they wrap around the cell cylinder to produce the spirochete's distinctive flat-wave morphology. This normal orientation of the periplasmic flagella toward the other pole of the cell is partly determined by FlhL, which is also essential for the spirochetes morphology and smooth swimming of the bacteria (Motaleb *et al.*, 2011). In motile cells, these periplasmic flagella form a ribbon-like structure. Periplasmic flagella are not only crucial for motility but also for morphology of *B. burgdorferi*, as *flaB* mutant cells are non-motile and exhibit a rod-shaped morphology (Kudryashev *et al.*, 2009, Charon *et al.*, 2009, Charon *et al.*, 2012, Sultan *et al.*, 2015, Motaleb *et al.*, 2015, Sultan *et al.*, 2013a, Motaleb *et al.*, 2000).

The architectural structures and amino acid sequences of the proteins encoded by various components of the periplasmic flagella resemble those of external flagella including the MS-ring, C-ring, P-ring, stator, hook, export apparatus, and the filament (Ge & Charon, 1997, Heinzerling *et al.*, 1997, Motaleb *et al.*, 2000, Zhao *et al.*, 2013, Zhao *et al.*, 2014, Chen *et al.*, 2011, Lin *et al.*, 2015). However, due to the unique location in the periplasmic space and the large torque required for driving the cell body, periplasmic flagella possess a unique spirochete-specific component known as the collar that is absent in all other bacterial flagella reported to date (Chen *et al.*, 2011, Zhao *et al.*, 2014). While the periplasmic collar complex is specific to the spirochetes, we know very little about the proteins contributing to this novel structure or its assembly in any spirochete (Chen *et al.*, 2011, Zhao *et al.*, 2014). FlhB is the first and only protein that was shown to be involved in collar assembly (Moon *et al.*, 2016b). However, FlhB is a small membrane protein, which likely forms the base of the collar. Based on its large structure (~71 nm in diameter and ~24 nm in height) and the fact that other large structures described below are comprised by multiple proteins, we hypothesized that the periplasmic collar complex is also constructed by several proteins (Moon *et al.*, 2016b).

In this communication, we identified BB0236 as a potential candidate for inclusion in the periplasmic collar structure. This protein possesses tetratricopeptide repeat (TPR) domains, but it is currently annotated as a hypothetical protein with unknown function in *B. burgdorferi* genome (Fraser *et al.*, 1997). Using mutational analysis followed by various approaches including cryo-electron tomography, we show that BB0236 is involved in collar assembly and is crucial for cell morphology, motility, orientation of periplasmic flagella and assembly of other important flagellar motor components. Additionally, BB0236 directly interacts with FlhB, supporting our model that the collar is a multi-protein complex. Moreover, using various flagellar mutants for motor analyses, we propose how the flagellar collar structure is assembled in *B. burgdorferi*.

RESULTS

Screening for a periplasmic flagellar collar protein in *B. burgdorferi*

We recently reported that FlhB is important for periplasmic flagellar collar assembly in *B. burgdorferi* (Moon *et al.*, 2016b). FlhB is a small protein (205 amino acids) that is unlikely

to make-up the large collar complex structure. Using green fluorescent protein fusion, we previously demonstrated that FlbB is located at the base of the collar and is embedded in the cytoplasmic membrane using its transmembrane domain (Moon *et al.*, 2016b). We hypothesized that other unidentified collar proteins are assembled onto the FlbB base. Thus, deletion of *flbB* has a dramatic effect on the entire collar complex, as the whole structure is diminished in the *flbB* mutant (Moon *et al.*, 2016b). These data lead us to predict that the collar is comprised of multiple proteins. This proposition is supported by the fact that other relatively smaller complexes such as the flagellar C-ring, flagellar type III export apparatus or type IV secretion structures are composed of multiple proteins (Fronzes *et al.*, 2009, Sowa & Berry, 2008, Minamino, 2014). To identify additional collar proteins, we employed various comprehensive strategies. Since we have analyzed almost all motility-related genes annotated in the *B. burgdorferi* genome, and found no collar proteins other than FlbB, we took advantage of a protein-protein interaction (PPI) map developed in another spirochete, *Treponema pallidum*, which identified 176 PPIs between known motility proteins and proteins of unknown functions using a comprehensive array-based yeast-two-hybrid screen (Rajagopala *et al.*, 2007). We hypothesized that some of the proteins of unknown function could be a periplasmic collar protein in *T. pallidum*. We refined the list of candidates by removing all protein homologs that have been characterized in other non-spirochetal bacteria because the periplasmic collar is a spirochete-specific feature (Chen *et al.*, 2011, Zhao *et al.*, 2014). We found a FlbB homolog in the PPI map (TP0567) that is annotated as a hypothetical protein in *T. pallidum*. TP0567 shares 32% amino acid sequence identity with *B. burgdorferi* FlbB (E-value, 5e-17). Moreover, TP0567 was shown to interact with TP0421, TP0708, and TP0675 in that PPI map (Fig. 1A). TP0675 and TP0708 have no significant homologs in *B. burgdorferi*, whereas TP0421 shares 27% amino acid sequence identity with *B. burgdorferi* BB0236 (E-value, 6e-80; Fig. 1A). Since FlbB was found to be involved in periplasmic flagellar collar assembly and the FlbB homolog TP0567 is proposed to interact with TP0421, we hypothesized that TP0421 protein's homolog BB0236 is a potential candidate for the periplasmic collar structure.

bb0236 is located in an operon consisting of seven genes, *bb0229*—*bb0236* (Fig. S1). None of the genes in this operon is predicted to be involved in motility or chemotaxis. BB0236 is annotated as a hypothetical protein (668 amino acids) that harbors TPR and NHL domains, and an uncharacterized ChID domain known as Mg-chelatase subunit ChID (proposed to be important for coenzyme transport and metabolism) (Marchler-Bauer *et al.*, 2017) (Fig. S1). TPR domain-containing proteins are reported to be important for the assembly of various multi-protein complexes as well as serving as chaperones for type III secretion systems (T3SS) in other bacteria (Cervený *et al.*, 2013). NHL domain was originally identified in NCL-1, HT2A, and Lin-41 proteins and was also proposed to be important for protein-protein interactions (Slack & Ruvkun, 1998). These observations suggest that BB0236 could serve as a chaperone in the multi-protein collar complex in association with FlbB and other proteins.

Protein-protein interaction between FlbB and BB0236

To determine if BB0236 actually interacts with FlbB as suggested by the *T. pallidum* PPI map, we performed pull-down assays with recombinant proteins MBP-BB0236 and 6xHis-

FlbB. MBP-BB0236 was allowed to bind with amylose resin, and this protein-bound resin was subsequently incubated with 6xHis-FlbB. After incubation and extensive washing, we collected the proteins that were bound to the MBP-BB0236 followed by SDS-PAGE and immunoblotting with anti-FlbB. MBP itself and amylose resin without any protein were used as controls. As shown in Fig. 1B, BB0236 was found to bind with FlbB (lane 1) but not with the resin or MBP (lanes 2 and 3). As an alternative, we performed far-western or affinity blotting to determine interactions between MBP-BB0236 and His-FlbB proteins. Our results shown in Fig. 1C indicate that FlbB interacts with MBP-BB0236 but not the control protein MBP-MCP5 or MBP. Together, these results indicate that FlbB specifically interacts with BB0236 and support our bioinformatics analysis and the PPI map developed in *T. pallidum*.

Construction of bb0236 mutant and complementation in trans

To determine the role of BB0236 in *B. burgdorferi*, we used allelic exchange to replace the endogenous *bb0236* gene with a streptomycin resistance cassette (*P_{flgB}-aadA*) (Fig. S1B) (Frank *et al.*, 2003). PCR analysis of the streptomycin-resistant clones indicated that the *bb0236* gene was replaced with the *P_{flgB}-aadA*, resulting in successful construction of the *bb0236* mutant (Fig. S1C).

To demonstrate that the *bb0236* mutant's phenotype described below is due solely to the mutation and not to a polar effect on downstream gene expression or a secondary alteration elsewhere, we complemented the mutant *in trans* using the shuttle vector pBSV2G containing an intact copy of *bb0236* fused to the constitutive *flgB* promoter (*P_{flgB}-bb0236*) (Elias *et al.*, 2003, Sultan *et al.*, 2015). Furthermore, for detection of the BB0236 protein in *B. burgdorferi*, we fused a 6xHistidine tag at the 3'-end of the gene (*P_{flgB}-bb0236-6xHis*) (Fig. S2A). Subsequent analysis of the complemented cell lysates by western blotting using anti-His antibody detected the BB0236-6xHis in the *bb0236* complemented cells (*bb0236^{om}*), but not in wild-type or mutant cells (Fig. S2B). These results indicate the restoration of BB0236 protein synthesis in the *bb0236^{om}* complemented cells.

Furthermore, we determined the expression of *bb0236* and the genes located immediately upstream and downstream of *bb0236* in the wild-type, *bb0236*, and *bb0236^{om}* cells by qRT-PCR using gene-specific primers (Fig. S3). Our real-time PCR detected the *bb0236* transcripts in wild-type and *bb0236^{om}* cells, but not in the *bb0236* mutant cells, indicating the restoration of *bb0236* synthesis in the *bb0236^{om}* cells (Fig. S3 middle panel). However, due to the *in trans* *bb0236* complementation on the shuttle vector with *flgB* promoter, the level of *bb0236* transcripts synthesized in the *bb0236^{om}* cells was approximately 15.4-fold more than the wild-type cells (Fig. S3 middle panel; $P=0.001$). The expression of the genes upstream (*bb0235*) or downstream (*bb0237*) was not altered in the *bb0236* mutant or *bb0236^{om}* cells compared to the wild-type *B. burgdorferi* (Fig. S3 top and bottom panels). These results suggest that the mutant lacks any polar effect.

BB0236 is important for motility and bacterial morphology

To determine the *bb0236* cells morphology and motility phenotypes, the spirochetes were analyzed using dark-field microscopy and swarm plate motility assays. While the wild-type

cells show its characteristic flat-wave morphology and mobility, the *bb0236* mutant cells were completely non-motile and they are mostly rod-shaped with slightly wave-like morphology near the tip of the mutant cells (Fig. 2). The swarm diameters of *bb0236* are similar to those from other non-motile mutants such as *flaB* and *flbB* (Fig. S4) (Moon *et al.*, 2016b, Sultan *et al.*, 2013a, Motaleb *et al.*, 2000). Complementation of the *bb0236* mutant restored the distinctive flat-wave morphology (Fig. 2). Motility was also restored, albeit not completely when compared to the wild-type cells (Fig. S4, panels A, B). These results indicate that BB0236 is crucial for motility and morphology of *B. burgdorferi*.

The lack of BB0236 has an impact on orientation and assembly of periplasmic flagella

We utilized cryo-ET to visualize the periplasmic flagella in *bb0236* mutants. We found that the *bb0236* mutants assembled less flagellar motors per cell than the wild-type or complemented cells (Table 1, Fig. 3). On average, the motor numbers of *bb0236* mutants were approximately 64% and 50% less than the wild-type and *bb0236^{com}* cells, respectively ($p = 0.0001$; Table 1). In addition, 45% of periplasmic flagella in the *bb0236* cells were detected to be abnormally oriented toward the cell pole instead of their normal orientation toward the cell cylinder seen in the wild-type or *bb0236^{com}* cells (Table 1, Fig. 3). Moreover, 69% of periplasmic flagella were also shorter than wild-type flagella (Table 1). Complemented *bb0236^{com}* cells restored these phenotypes albeit not completely. Approximately 11% of periplasmic flagella were found to be abnormally oriented in the *bb0236^{com}* cells compared to only 2% in the wild-type cells (Table 1). These abnormally oriented flagella were also detected in the *flbB* cells (>82%) and *fliL* cells (~26%) (Moon *et al.*, 2016b, Motaleb *et al.*, 2011). Together, these results indicate that BB0236 is important for motility, morphology, and orientation of periplasmic flagella in *B. burgdorferi*.

BB0236 is important for the formation of the intact periplasmic collar structure

Interaction of BB0236 with FlbB and the phenotypes observed with the *flbB* mutant leading to the hypothesis that *bb0236* gene products may be involved in the assembly of the collar structure. To determine if *bb0236* is actually involved in this process, we visualized the in situ motor structures by cryo-ET and sub-tomogram averaging (Fig. 4). The averaged structure of the *bb0236* motors show major features of the periplasmic flagellar motor, such as the export apparatus, the C-ring, the MS-ring, the rod, and the P-ring (Fig. 4B). Interestingly, a bulge-like structure is detected on the periplasmic side of the MS-ring (Fig. 4B, D; indicated by red arrows) that is not detected in *flbB* (Fig. 4A) (Moon *et al.*, 2016b). However, the large structure surrounding the central rod and the P-ring are absent in the mutant, indicating that the *bb0236* motors lack the flagellar collar structure detected in the wild-type motors (compare WT or *bb0236^{com}* and *bb0236* structures in Fig. 4). Moreover, structural densities associated with the stator (comprised of MotA and MotB) and FliL are also diminished in the *bb0236* mutant cells even though the mutant cells synthesized MotB, FliL, as well as FlbB proteins (compare Fig. 4B-D with F-H; Fig. S5). Complementation of *bb0236* restored the collar and all other associated flagellar structures such as FliL and the stator (Fig. 4F). These results suggest that BB0236 is important for the formation of the collar structure and the assembly of the stator and FliL.

The periplasmic collar structure assembles sequentially, in an ordered fashion

We performed comparative motor analysis from our previous and current mutants such as the *flbB*, *fliL*, and *bb0236* that affected the periplasmic flagellar collar structure to understand the assembly of this complex (Moon *et al.*, 2016b, Motaleb *et al.*, 2011). FliL is a membrane protein associated with the collar complex as it directly interacts with FlbB (Moon *et al.*, 2016b). Our previous report also showed that FliL forms a unique density at the interface between the collar and stator (compare Fig. 5E, J with D, I) (Motaleb *et al.*, 2011). Interestingly, our classification of the *fliL* motors indicate that ~80% of the motors possess an intact collar structure (Fig. 5D, I) while ~20% of the *fliL* motors do not contain the top part of the collar (Fig. 5C, H), suggesting that FliL is important for the assembly of the collar complex, and that it interacts with not just FlbB but also with the (unidentified) collar proteins that make-up the top part of the collar structure (indicated by yellow in Fig. 5I). It is important to note that this type of non-homogenous phenotype seen in *fliL* is common to the bacterial flagellar mutants. For example, in the *flgK*, *flgL*, *fliK*, or *motB* mutant, not all hooks or filaments are similar in size or number (Muramoto *et al.*, 1999, Miller *et al.*, 2014, Sultan *et al.*, 2015).

Since BB0236 and FlbB interacts and FlbB forms the base of the collar, we propose that BB0236 assembles on FlbB (Fig. 5C, H) because the *bb0236* cells (Fig. 5B, G) lack a density detected in the *fliL* (Fig. 5C, H). That structural density likely corresponds to BB0236 (indicated by a question mark in 5H). FlbB interacts with FliL (Moon *et al.*, 2016b). FliL, therefore, either assembles simultaneously with FlbB or subsequently after FlbB to provide support for the assembly of BB0236 structure and the proteins that make the top part of the collar. Presumably, other unidentified proteins (indicated by a ? in 5I) then assemble on BB0236/FliL and so on to make an intact periplasmic collar complex (Fig. 5E, J).

DISCUSSION

The periplasmic collar provides a static framework for recruiting sixteen torque-generating units to produce an intact stator structure, which can produce larger torques to rotate the periplasmic flagellum. Consequently, the rotation of the flagella in the periplasm enables the spirochete to bore through viscous and complex environments in the vertebrate/tick hosts (Beeby *et al.*, 2016, Moon *et al.*, 2016b). Indeed, periplasmic flagellar motility is found to be crucial for every juncture of the spirochete's life cycle (Sultan *et al.*, 2015, Motaleb *et al.*, 2015, Sultan *et al.*, 2013a). The periplasmic collar also ensures that the flagella are oriented toward the cell body but not the cell pole (Table 1). This function is critical to the production of *B. burgdorferi*'s characteristic wave-like morphology and smooth swimming (Moon *et al.*, 2016b, Motaleb *et al.*, 2011).

The TPR-domain protein BB0236 identified in this study is essential for the assembly of periplasmic collar structure (Fig. 4). Proteins with TPR motifs are reported to function as chaperones of a T3SS in several species of bacteria such as *Yersinia* spp. (LcrH), *Pseudomonas aeruginosa* (PcrH), and *Shigella* spp. (LpgC) (Cervený *et al.*, 2013). *B. burgdorferi* contains several TPR domain proteins, however, only two of them—BB0238 and BB0324 (BamD)—have so far been investigated. The function of BB0238 is unknown but it is involved in interacting with BB0323 and stabilizing each other. BamD was reported to be

involved in a β -barrel assembly machinery, which is an outer membrane porin protein complex (Thakur *et al.*, 2017, Groshong *et al.*, 2014, Kariu *et al.*, 2015, Lenhart *et al.*, 2012, Dunn *et al.*, 2015).

Based on these observations, we anticipated that BB0236 may serve as a chaperone in the flagellar collar complex assembly. If this proposition is true, then in the absence of the chaperone (BB0236) the collar should not assemble. Indeed, our data indicate that the collar structure is not assembled in the *bb0236* but is restored in the complemented *bb0236^{com}* cells (Fig. 4). The periplasmic collar structure is considerably larger than other complex membrane structures such as the C-ring complex, flagellar type III export apparatus, or type IV secretion core structure, all of which are composed of multiple proteins (Fronzes *et al.*, 2009, Sowa & Berry, 2008, Minamino, 2014, Lin *et al.*, 2015). Moreover, in addition to its large structure, the finding of the TPR motifs in BB0236, and BB0236 interaction with FlbB strongly suggests that the collar is a multiprotein complex (Figs. 1, 5) (Moon *et al.*, 2016b).

The *bb0236* mutant cells are non-motile (Fig. S4), even though the periplasmic flagellar filament comprised of FlaB is synthesized (Fig. S5), but those periplasmic flagella are inactive due to their missing stators (Fig. 4). The number of flagellar motors and the level of FlaB proteins are also reduced in the mutant cells (Table 1; Fig. S5). The *bb0236* gene is not part of a known flagellar or motility-related gene cluster and it is not genetically linked to any predicted flagellar gene, however, the *bb0236* phenotypes are not unusual given the fact that other non-motile *B. burgdorferi* mutants such as *motB* or *flbB* exhibit similar phenotypes (Moon *et al.*, 2016b, Sultan *et al.*, 2015). These data lead us to propose that the stator and/or collar-stator is likely involved in periplasmic flagellar filament assembly in *B. burgdorferi*.

Moreover, the *bb0236* mutant cells lack the cellular densities associated with FliL and stator structures (Fig. 4) even though the FliL or stator (MotB) protein synthesis was not inhibited in this mutant (Fig. S5), suggesting that the collar/BB0236 is important for the assembly of stator and FliL structures. Since the collar structure is intact in the *motB*- or *fliL*-deletion mutant, we propose that the collar provides support for the assembly of the stator and FliL [(Motaleb *et al.*, 2011); our unpublished data]. Similar results were also obtained with the FlbB (Moon *et al.*, 2016b). While this is the first example showing that the TPR protein BB0236 is important for the periplasmic collar, stator, and FliL, chaperones are reported to be involved in the assembly of flagellar components in several species of bacteria (Macnab, 2003, Macnab, 1999). In *Sinorhizobium meliloti*, the periplasmic chaperone MotE is essential for MotC motor assembly (Eggenhofer *et al.*, 2004). In *Salmonella enterica*, several substrate-specific chaperones are involved in various flagellar apparatus assembly, such as the FlgA periplasmic chaperone which is important for the assembly of the P-ring, FlgN for the hook protein, FliT for the filament capping protein, and FliS for the flagellin assembly (Auvray *et al.*, 2001, Bennett *et al.*, 2001, Nambu & Kutsukake, 2000, Yokoseki *et al.*, 1995, Fraser *et al.*, 1999). Taken together, experimental evidence from this and our previous FlbB studies support the hypothesis that the collar structure is assembled before the stator and FliL. In doing so, the collar provides a foundation for the assembly of the stator and FliL. This proposition is supported by the fact that BB0236 interacts with FlbB, FlbB

binds to FliL, and FliL (and unidentified proteins) interact with the stator proteins MotA-MotB (Suaste-Olmos *et al.*, 2010, Zhu *et al.*, 2015, Moon *et al.*, 2016b).

Moreover, *bb0236* cells exhibit abnormally oriented flagellar filaments (Table 1; Fig. 3). 45% periplasmic flagella was found to be abnormally oriented in the *bb0236* mutant, whereas 82% flagella was found to be abnormally tilted in the *flbB* and only 21% of the flagella were tilted in the *fliL* mutant cells (Table 1) (Moon *et al.*, 2016b, Motaleb *et al.*, 2011). In the *bb0236* mutant, we assume the structure for FlbB is intact (see below), but FliL and stators are abolished, and that is likely why we observed fewer filaments pointed abnormally than in the *flbB* mutant (Moon *et al.*, 2016b). These arguments are supported by the fact that FlbB interacts with BB0236 as well as FliL. Based on these results, we propose that the collar and FliL are essential for the normal orientation of periplasmic flagella. We found a bulge appendage (it is a ring in three dimension) in the *bb0236* cells which was not detected in the *flbB* mutant (outlined in red in Fig. 4B, D, 5G). The identity of this appendage is currently unknown, however, we propose that this bulge could be composed of FlbB.

Despite the possession of periplasmic flagella, the *bb0236* mutant cells are mostly rod-shaped but the tip of the cells is relatively wave-like. However, the mutant cells are missing the stator structure (Fig. 4). Diminished stators may explain why these cells are mostly rod-shaped because we observed a similar phenotype in a stator *motB*-deletion mutant (Sultan *et al.*, 2015). While the complemented *bb0236^{com}* cells restored the collar, stator, and FliL structures that are missing in the mutant cells, the motility phenotype was not fully reestablished, likely due to the use of the multi-copy shuttle vector for complementation (Frank *et al.*, 2003).

In summary, this study hints not only the complexity of the collar structure, but also suggests that the *B. burgdorferi* genome possesses additional genes encoding the flagellar collar proteins that remain to be identified. A significant feature of this communication is the utilization of the *T. pallidum* protein-protein interaction map to directly identify the flagellar collar proteins in *B. burgdorferi*. Thus, the knowledge obtained in *B. burgdorferi* may be applicable to understand the structure and function of periplasmic flagellar motors of the syphilis-causing spirochete, which cannot yet be genetically manipulated.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions.

High-passage, avirulent *B. burgdorferi* strain B31-A was used as a wild-type clone throughout the study (Bono *et al.*, 2000, Elias *et al.*, 2002). Constructions of *bb0236* mutants and its complemented strains are described below. *B. burgdorferi* cells were cultured in liquid Barbour-Stoenner-Kelly (BSK-II) medium, and plating BSK was prepared using 0.4% agarose (Sultan *et al.*, 2013a, Motaleb *et al.*, 2007). Cells were grown at 35°C in a 2.5% CO₂ incubator as described previously (Motaleb *et al.*, 2007). Antibiotics, when required, were included in the *B. burgdorferi* culture medium with the following concentrations: 100 µg ml⁻¹ streptomycin, 40 µg ml⁻¹ gentamicin. *Escherichia coli* cells were grown at 37°C in Luria-Bertani (LB) broth or LB agar (Bertani, 1951). Additional supplements, when required, were

included in the *E. coli* culture medium with the following concentrations: 100 µg ml⁻¹ ampicillin, 35 µg ml⁻¹ chloramphenicol, 100 µg ml⁻¹ spectinomycin, 0.2% glucose, 80 µg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.5 mM isopropyl-β-D-thiogalactoside (IPTG).

Bioinformatics.

Basic local alignment search tool (BLAST) (Altschul *et al.*, 1997) was used to identify a homolog of FlbB and its interacting partner in *Treponema pallidum* from a protein-protein interaction (PPI) map generated using a yeast-two hybrid system between known motility-related proteins and unknown proteins (Rajagopala *et al.*, 2007). The lower an E-value (lower than 0), the more significant the score is.

Pull-down assay and Far-western.

BB0236 and FlbB protein-protein interaction was determined by pull-down assays using recombinant proteins. Briefly, *B. burgdorferi* FlbB was cloned in the expression vector pTrcHis-TOPO (Invitrogen) after removing the transmembrane binding domain (a.a. 1–50). *E. coli* codon plus cells harboring pTrcHis-TOPO::flbB was expressed and purified according to the manufacturer's protocol (Invitrogen). The purified 6xHis-FlbB was dialyzed in Maltose binding protein (MBP)-Column buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.4) for subsequent assays. To recombinantly express bb0236 in *E. coli*, the gene was synthesized after codon optimization. Subsequently, the codon optimized bb0236 was cloned in pMAL c5x expression vector (NEB Inc.) after removing the signal sequence (a.a. 1–20). *E. coli* pLysS cells harboring plasmid BB0236-pMAL c5x or the empty vector pMAL c5x were induced with 1 mM of IPTG for four hours followed by harvesting the cells. The cell pellets were resuspended in 10 ml MBP-Column buffer and disrupted by using a French Pressure (SLM AMINCO Inc.) at 18,000 psi. Cell lysates were centrifuged at 22,000 x g at 4°C for 20 min to separate soluble fractions. The soluble fraction was incubated with 150 µl of amylose resin (NEB Inc.) at 4°C for overnight. Protein-bound amylose resins, i.e., MBP-BB0236 (~2.0 µg protein per reaction), or MBP itself were washed twice with 50 ml of MBP-Column buffer and mixed with 10 µg of 6xHis-FlbB. The total volume was adjusted to 500 µl with MBP-Column buffer. The mixture was then incubated for 1 hr at room temperature with constant agitation. After the incubation, the protein-bound amylose resins were washed twice with 50 ml of MBP-Column buffer. 500 µl SDS loading dye containing 10 mM of maltose was added to the protein-bound amylose resins after the washes, and then heated for 10 minutes in boiling water bath. The boiled samples were subjected to SDS-PAGE, transferred to a PVDF membrane, and immunoblotted using *B. burgdorferi* FlbB-specific antibodies. Far-western or affinity blotting was performed as described (Moon *et al.*, 2016a). Construction, expression and purification of recombinant MBP-MCP5 have been previously reported (Moon *et al.*, 2016a). Approximately 2.0 µg protein (codon optimized MBP-BB0236, MBP-MCP5 or MBP) was subjected to SDS-PAGE, transferred to a PVDF membrane, and then incubated with 4 µg His-FlbB in a blocking solution (5% skim milk, 150 mM NaCl, 10 mM Tris, 0.3% Tween 20, pH 7.4). After extensive washing with 150 mM NaCl, 10 mM Tris, 0.3% Tween-20, pH 7.4, the membrane was immunoblotted with the FlbB polyclonal antibodies. Detection was performed using ECL immunoblotting detection kit (GE Healthcare Inc.).

Construction and complementation of the *bb0236* mutant.

Construction of the *bb0236* inactivation plasmids, electroporation, and plating conditions were described previously (Sultan *et al.*, 2015, Motaleb *et al.*, 2011, Sultan *et al.*, 2011). Briefly, the 5' - (1038 bp), and 3'-flanking (1072 bp) DNA of *bb0236* gene were amplified by PCR from chromosomal DNA of *B. burgdorferi* strain B31-A using primers 236.KO-P1F (CCTCACAAGAATAAACTTCTGCTTTAATAAA) and 236.KO-P1R (GTAGTCGGCAAATAAATTTCTTTTGTGATTT), and 236.KO-P2F (TGAAGCTCGGGTAAATCCCCCTTTACT) and 236.KO-P2R (TTTGAGGGAGCCCCAATGG), respectively. Streptomycin resistance cassette with the *B. burgdorferi* *flgB* promoter (*P_{flgB}-aadA*, 1227 bp) was similarly amplified by PCR from the shuttle vector pKFSS1 using primers 236.KO-StrepF (AAATCAAAAAGAAATTTATTTGCCGACTAC) and 236.KO-StrepR (AGTAAAGGGGATTACCCGAGCTTCA) (Frank *et al.*, 2003). These three pieces of DNA fragments were linked by overlapping PCR, yielding *bb0236_KO_P_{flgB}-aadA*, then cloned into the pGEM-T Easy (Promega Inc.), yielding plasmid Teasy::*bb0236_KO_P_{flgB}-aadA*. Competent B31-A cells were electroporated with *bb0236_KO_P_{flgB}-aadA* DNA that was linearized with *NotI* restriction enzyme digestion to remove the ampicillin restriction marker of the vector, preventing it from being introduced into *B. burgdorferi* (Sultan *et al.*, 2015, Motaleb *et al.*, 2011, Sultan *et al.*, 2011). The transformants were selected with streptomycin. The streptomycin-resistant transformants were isolated and confirmed for the replacement of *bb0236* gene with the *P_{flgB}-aadA* by PCR.

To complement the *bb0236* mutant, the *flgB* promoter (*P_{flgB}*) and *bb0236* gene were PCR amplified from chromosomal DNA of *B. burgdorferi* strain B31-A using primers *P_{flgB}-BamHI-F* (GGATCCCGAGCTTCAAGGAAGATTTC) and *P_{flgB}-R* (ACCAAAAATTAACATATGGAAACCTCCCTC), and BB0236-F (GAGGGAGGTTTCCATATGTTAATTTTGGT) and BB0236-His-*PstI-R* (CTGCAGCTAATGATGATGATGATGATTAATAAAATAT), respectively (restriction sites are in underlined, and 6xHis sites are in bold). A 6x histidine amino acid tag was linked at the C-terminal of *bb0236* gene during the PCR. These two pieces of DNA fragments were linked by overlapping PCR, yielding *P_{flgB}-bb0236-6xHis*. The amplified *P_{flgB}-bb0236-6xHis* DNA was cloned into the pGEM-T Easy (Promega Inc.), yielding plasmid Teasy::*P_{flgB}-bb0236-6xHis*. These and the *B. burgdorferi* shuttle vector pBSV2G were digested with *BamHI* and *PstI*, then ligated to yield pBSV2G::*P_{flgB}-bb0236-6xHis* (Elias *et al.*, 2003). Approximately 50 µg of pBSV2G::*P_{flgB}-bb0236-6xHis* plasmid DNA was electroporated into the *bb0236* cells (Sultan *et al.*, 2015, Sultan *et al.*, 2011). Potential transformants were selected with streptomycin and gentamicin. Resistant transformants were analyzed by PCR to confirm the presence of the plasmid in the transformants (complemented *bb0236^{com}* cells). Furthermore, the expression of BB0236-6xHis proteins in complemented *bb0236^{com}* cells were confirmed by western blot with Pierce™ His Antibody-HRP conjugate (Thermo Scientific) as described below. Restoration of *bb0236* expression in the *bb0236^{com}* cells, and polar effect on downstream (*bb0235*, *ychF*) or effect on upstream (*bb0237*, *Int*) gene expressions were verified by quantitative reverse transcriptase-PCR (qRT-PCR) using gene-specific primers (Motaleb *et al.*, 2000; Sultan *et al.*, 2010; Sultan *et al.*, 2013).

Reverse transcription-polymerase chain reaction (RT-PCR).

Exponentially growing *B. burgdorferi* wild-type cells (2×10^7 cells ml⁻¹) were treated with RNAprotect™ followed by total RNA isolation using the RNeasy mini kit (Qiagen Inc.). Contaminating DNA in the RNA samples was removed by RNase-free Turbo® DNase I (Ambion Inc.) digestion for 3 hr. at 37°C followed by RNeasy mini purification. For RT-PCR, cDNA was prepared from 1 µg RNA using the AffinityScript QPCR cDNA synthesis kit according to the manufacturer's protocol (Agilent Technologies Inc.). The iCycler detection system (Bio-Rad Inc.) was used to measure *bb0236*, and its upstream (*ychF*) and downstream (*Int*) genes transcript levels according to the manufacturer's instructions. *B. burgdorferi* enolase was used as a reference gene (Pitzer *et al.*, 2011, Sultan *et al.*, 2011, Sultan *et al.*, 2010, Motaleb *et al.*, 2004). The gene specific primers (5'–3') were RT-enolase-F (TGGAGCGTACAAAGCCAACATT); RT-enolase-R (TGAAAAACCTCTGCTGCCATTC); *ychF*-qRT-F (GCCTTTCATCGGGAATC); *ychF*-qRT-R (CATCTAATGTTGAGATCGCG); *bb0236*-qRT-F (CAAGATTATTAGGATCAAAGCTTAA); *bb0236*-qRT-R (GGGCTCTTAATTCTTATAATAATGG); *Int*-qRT-F (GTTGCTTATGTACCACCTTTTATAGC); and *Int*-qRT-R (GTTGGCTATTATAAAGTAAATACCG). The relative level of expression was calculated using the 2^{-CT} method (Livak; Schmittgen, 2001; Simm *et al.*, 2009; Pitzer *et al.*, 2011).

SDS-PAGE and immunoblot analyses.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with an enhanced chemiluminescent detection method (GE Health Inc.) were carried out as reported previously (Sultan *et al.*, 2013a, Motaleb *et al.*, 2000). The concentration of protein in cell lysates was determined by a Bio-Rad protein assay kit. Unless otherwise noted, 10 µg of lysate protein was subjected to SDS-PAGE and immunoblotting using proper antibodies.

Dark-field microscopy and swarm plate assays.

Growing *B. burgdorferi* clones were imaged using a Zeiss Imager M1 dark-field microscope connected to a digital camera to determine bacterial morphology (and motility). To determine *B. burgdorferi* motility, we performed swarm plate assays (Xu *et al.*, 2017, Moon *et al.*, 2016b, Moon *et al.*, 2016a). Briefly, approximately 1×10^8 cells in a 5 µl volume were spotted into 0.35% agarose plates containing BSK medium diluted in 1:10 in Dulbecco's phosphate-buffered saline. Since *B. burgdorferi* is a slow-growing organism with a 5 to 12 h generation time, swarm plates were incubated for 7 days to measure swarm diameters (Sultan *et al.*, 2015, Sultan *et al.*, 2013a, Rosa *et al.*, 2005).

Cryo-ET sample preparation.

Frozen-hydrated specimens were prepared as described previously (Sultan *et al.*, 2015, Zhao *et al.*, 2014, Zhao *et al.*, 2013, Liu *et al.*, 2009). Briefly, late-exponential phase *B. burgdorferi* wild-type, *bb0236* mutant, and *bb0236*^{com} complemented cells' pellets were harvested, and then resuspended in 40 µl phosphate buffered saline (PBS, pH 7.4) at a final concentration of $\sim 2 \times 10^9$ cells ml⁻¹. Cell suspensions were mixed with 15 nm gold clusters,

then 5 μ l was deposited onto freshly glow-discharged holey carbon grids (Quantifoil Micro Tools GmbH). Grids were blotted with a filter paper to remove excess fluid, followed by rapid freezing in liquid ethane using a homemade gravity-driven plunger apparatus (Sultan *et al.*, 2015, Zhao *et al.*, 2014, Zhao *et al.*, 2013, Liu *et al.*, 2009).

Cryo-electron tomography.

Tilt series acquisitions were conducted as previously described (Lin *et al.*, 2015, Zhao *et al.*, 2014, Zhao *et al.*, 2013). Cryo-samples were imaged at -170°C using a 300 keV Polara G2 electron microscope (FEI Company) equipped with a field emission gun. Tilt series of *B. burgdorferi* cells were collected by SerialEM software, under low dose mode (Mastronarde, 2005) and were recorded by Direct Detection Camera (Gatan K2 Summit). EM images were acquired at X 9,400 magnification (pixel size of ~ 4.5 Å), and at ~ 7 μm defocus. Single axis tilt series were recorded from -60° to $+60^{\circ}$ with 2° angular increments. The cumulative dose was ~ 60 $\text{e}^{-}/\text{\AA}^2$ distributed over 61 images. Dose fractionation mode was used during tilt series acquisition with each tilt image being fractionated into 8 frames. The stack of 8 frames were aligned and drift-corrected using Motioncorr (Li *et al.*, 2013). Tilt series were automatically aligned and reconstructed using a combination of IMOD (Kremer *et al.*, 1996) and TOMO3D packages (Agulleiro & Fernandez, 2011).

Subtomogram average.

Subtomograms containing a flagellar motor were extracted, aligned, and averaged as described previously (Zhao *et al.*, 2014, Zhao *et al.*, 2013, Liu *et al.*, 2009). Briefly, the flagellar motor subtomograms ($256 \times 256 \times 256$ voxels) were visually identified by two coordinates at the C-ring and the hook positions, and then computationally extracted from the tomographic reconstructions. To accelerate image processing, $4 \times 4 \times 4$ binned subtomograms ($64 \times 64 \times 64$ voxels) were used for initial alignment. The alignment and the averaging were then carried out to $2 \times 2 \times 2$ binned subtomograms.

3D visualization.

The averaged maps were visualized by using UCSF Chimera (Pettersen *et al.*, 2004). Two-dimensional tomographic slices were visualized by using the IMOD software (Kremer *et al.*, 1996).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Identification of a collar protein in *B. burgdorferi* from a *T. pallidum* protein-protein interaction network. The PPI map was developed between known motility proteins and proteins of unknown function by a yeast two hybrid screen. *T. pallidum* protein TP0567 shares 32% amino acid sequence identity with *B. burgdorferi* FlbB (E-value, 5e-17). TP0567 is shown to interact with TP0421, TP0675, and TP0708. Only TP0421 shares significant amino acid sequence identity with *B. burgdorferi* BB0236 (27% identity; E-value, 6e-80).

(B) Interaction between BB0236 and FlbB was determined by pull-down assay. Purified His-FlbB protein was incubated with resin-bound MBP-BB0236 followed by extensive washing and elution. The eluted proteins were separated by SDS-PAGE and then

immunoblotted with anti-FlbB antibody. Lane 1, FlbB protein was detected only in the elution from the MBP-BB0236 bound amylose resin but not in the MBP itself (lane 2) or the empty amylose resin (lane 3). FlbB (~24 kDa) is indicated by an arrowhead. **(C) Far-western or affinity blotting showing specific interaction between BB0236 and FlbB proteins.** Approximately 2.0 µg of MBP proteins were subjected to SDS-PAGE and Coomassie staining (left panel) or transferred to a polyvinylidene difluoride membrane (right panel). The membrane was incubated with His-FlbB and then immunoblotted with anti-FlbB polyclonal antibodies.

Figure 2. Comparison of morphology of *B. burgdorferi* clones detected by dark-field microscopy. Wild-type *B. burgdorferi* cells display a flat-wave morphology, whereas the *bb0236* mutant exhibited a rod-shaped morphology with slight wave-like shape in the cell tips. Complemented *bb0236*^{com} cells restored the morphology phenotype. Similar rod-shaped morphology of *flbB* mutant cells was reported previously (Moon *et al.*, 2016b).

Figure 3. Representative tomographic slices of *bb0236* mutant and *bb0236^{com}* cells. (A) A representative tomographic slice of a *bb0236* cell shows three short and cell pole-oriented abnormal periplasmic flagella. The boxed area in (A) is zoomed and shown in (B). (C) A representative tomographic slice of a *bb0236^{com}* cell shows several normal periplasmic flagella that are oriented toward the cell cylinder, characteristic of WT cells (WT, not shown). (D) The zoom-in view shows the motors and flagellar filaments. White arrows indicate motors, yellow arrows show the flagellar filaments.

Figure 4. Three dimensional structures of flagellar motors from wild-type and mutant cells. A central slice of an averaged *flbB* motor structure (A) is shown for comparing with *bb0236* motor (B). A horizontal dashed line in panel B shown by C indicates location of a cross-section shown in panel C. (C) A cross-section of the averaged *bb0236* motor structure showing the P-ring and rod. (D) A 3D surface rendering of the *bb0236* motor structure. (E) A central slice of an averaged structure of the wild type motor. (F) A central slice of an averaged structure of *bb0236^{com}* motor. A horizontal dashed line in panel F indicates location of the slices shown in panel G. (G) A slice of the averaged structure of *bb0236^{com}* motor showing the complexity of the collar structure. (H) A 3D surface rendering of the *bb0236^{com}* motor structure. Collar (yellow), stator (blue), cytoplasmic membrane (CM: light green), MS-ring (dark green), red (unknown/FlbB), P-ring (light blue), C-ring (aqua), export apparatus (gray).

Figure 5. Comparative analysis of the collar structures from different flagellar mutants.

(A, F) A central section of the averaged *flbB* motor structure and corresponding isosurface rendering. (B, G) A central section of the averaged *bb0236* motor structure and corresponding isosurface rendering. (C, H) A central section of one class average of the *fliL* motor and corresponding isosurface rendering. (D, I) A central section of another class average of the *fliL* motor and corresponding isosurface rendering. Compared with the WT motor (E, J), the motor structures from different mutants are distinct from each other. The collar is a multiprotein complex that assembles sequentially and FlbB forms the base of the collar. Other collar proteins are predicted to assemble on top of each other as illustrated. The identity of the red spike structure in (G) is currently unknown but it could be FlbB because this density is missing in the *flbB* mutant. The question marks indicate that the identity of these proteins is yet to be experimentally confirmed.

Table 1.Phenotypes of *bb0236* mutant's periplasmic flagella detected by cryo-ET.

<i>B. burgdorferi</i> clone	No. of cells analyzed	¹ Total no. of flagella detected	No. of irregular flagella (%)	No. of short flagella (%)	² Average no. of motors $\pm$ SD
Wild-type	15	163	1 (0.6%)	3 (2%)	11 $\pm$ 4
<i>bb0236</i>	23	101	46 (45%)	70 (69%)	4 $\pm$ 3
<i>bb0236^{com}</i>	15	118	14 (11%)	25 (21%)	8 $\pm$ 4

¹Total number of periplasmic flagella (normal and abnormal) were detected from the 'number of cells analyzed' shown on the left column. Percentages were determined by dividing the number of irregular or short periplasmic flagella by the total number of periplasmic flagella x 100. A normal flagellum is defined as being oriented toward the cell body. An abnormal or irregular periplasmic flagellum is defined as being tilted toward the cell pole. Short periplasmic flagella are shorter than the wild-type flagella.

²Statistical analysis by Student's *t-test* suggests that the mutant cells assembled considerably fewer periplasmic flagellar motors per cell tip than wild-type ($p = 0.0001$). The *bb0236^{com}* cells synthesized significantly more motors than the mutant cells ($p=0.001$) but less than the wild-type cells.