BB0259 Encompasses a Peptidoglycan Lytic Enzyme Function for Proper Assembly of Periplasmic Flagella in *Borrelia burgdorferi*

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Assembly of the bacterial flagellar rod, hook, and filament requires penetration through the peptidoglycan (PG) sacculus and outer membrane. In most β- and γ-proteobacteria, the protein FlgJ has two functional domains that enable PG hydrolyzing activity to create pores, facilitating proper assembly of the flagellar rod. However, two distinct proteins performing the same functions as the dual-domain FlgJ are proposed in δ- and ε-proteobacteria as well as spirochetes. The Lyme disease spirochete *Borrelia burgdorferi* genome possesses a FlgJ and a PG lytic SLT enzyme protein homolog (BB0259). FlgJ in *B. burgdorferi* is crucial for flagellar hook and filament assembly but not for the proper rod assembly reported in other bacteria. However, BB0259 has never been characterized. Here, we use cryo-electron tomography to visualize periplasmic flagella in different *bb0259* mutant strains and provide evidence that the E580 residue of BB0259 is essential for PG-hydrolyzing activity. Without the enzyme activity, the flagellar hook fails to penetrate through the pores in the cell wall to complete assembly of an intact periplasmic flagellum. Given that FlgJ and BB0259 interact with each other, they likely coordinate the penetration through the PG sacculus and assembly of a functional flagellum in *B. burgdorferi* and other spirochetes. Because of its role, we renamed BB0259 as flagellar-specific lytic transglycosylase or LTase<sup>bb</sup>.

**Keywords:** Lyme disease, flagella, peptidoglycan hydrolases, cryo-electron tomography, spirochete, *Borrelia*

**INTRODUCTION**

In most bacterial species, the flagellum is a rotary nanomachine framed in a supramolecular structure that initiates assembly from the cell envelope and extends into the extracellular space. However, in motile spirochetes, this migratory organelle resides in the periplasmic space and is therefore called the periplasmic flagellum (Wolgemuth et al., 2006; Charon et al., 2009, 2012). Despite differences in location, the external flagella found in the model organisms *Escherichia coli* and *Salmonella enterica* serovar Typhimurium share a remarkably high degree of similarity with respect to overall structure, as the key components are conserved among the external flagella and the periplasmic flagella of spirochetes (Liu et al., 2009; Chen et al., ...
The supramolecular structure is composed of more than 25 distinct protein subunits and self-assembles to final lengths of up to 20 μm in the model organisms. The individual flagellum consists of three major substructures: a basal body, hook, and filament (reviewed in Nakamura and Minamoto, 2019; Carroll and Liu, 2020). Synthesis of the flagellum begins with the assembly of a basal body composed of an MS ring embedded in the cell membrane and a rod. The MS ring is comprised of multiple copies of FlhG and acts as a platform for assembly of the rod, C-ring, torque-generating stator, and flagellum-specific type III secretion system (T3SS). The rod is composed of five proteins, FlhE, FlhB, FlgC, FlhO/FlgF, and FlgG. These rod proteins are transported from the cytoplasm to the periplasmic space via the T3SS (Saijo-Hamano et al., 2019) and then assemble onto the MS ring—in an ordered fashion from the proximal rod to the distal rod (Zhao et al., 2013; Terashima et al., 2017; Carroll and Liu, 2020). The distal rod serves as the template for subsequent assembly of the P-ring and hook (B. burgdorferi lacks the L-ring; Zhao et al., 2013). The filaments are subsequently polymerized onto the hook to complete assembly of a flagellum. Hook and filament assembly is mediated by the hook cap FlgD and filament cap FlID protein, respectively (Hommia and Iino, 1985; Ohnishi et al., 1994; Yonekura et al., 2000).

In order for the assembly of the rod with 30 nm × 4 nm to penetrate the outer membrane in E. coli or S. enterica, a hole must be created in the peptidoglycan sacculus, as its mesh diameter is narrower than 2 nm (Demchick and Koch, 1996; Herlihy et al., 2014). This PG hydrolytic activity is accomplished by a dual-domain protein FlgJ of S. enterica and E. coli. FlgJ is composed of an N-terminal scaffolding domain required for polymerization of the distal rod and a C-terminal domain required to form a hole in the PG sacculus. The C-terminal domain of FlgJ proteins belongs to the glycosyl hydrolase family in which these β-N-acetyl-glucosaminidases cleave the β,1,4 glycosidic linkage between N-acetylmuramyl and N-acetylglicosaminyl residues of bacterial cell wall peptidoglycan. Unlike lysozymes, however, SLTs are not hydrolases but cleave the β-glycosidic linkages, with simultaneous production of 1,6-anhydromuramyl residues (Holtje et al., 1975; van Asselt et al., 1999; Blackburn and Clarke, 2001; Koraimann, 2003).

As B. burgdorferi FlgJ is reported to be a single-domain protein that lacks a PG-hydrolyzing enzymatic domain, we sought to investigate the genome to identify a protein that harbors this domain (Zhang et al., 2012). BB0531 and BB0259 identified in this communication are both predicted to possess PG-hydrolyzing enzyme activities. BB0531 possesses a glucosaminidase domain; however, subsequent analysis indicates that this protein is not involved in flagellar pore-forming activities. BB0259 possesses an SLT homolog at its C-terminal domain, and further analyses indicate that FlgJ specifically interacts with BB0259. Because of its apparent flagellar specific peptidoglycan lytic enzyme function in the spirochete, BB0259 is renamed as LTase<sup>th</sup>. FlgJ and LTase<sup>th</sup> appear to function distinctively compared to their counterparts from other bacteria. Our model describes how B. burgdorferi FlgJ and LTase<sup>th</sup> work synchronously to form a functional periplasmic flagellum.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

*Borrelia burgdorferi* B31-A is a high-passage noninfectious clone used as the wild-type (WT) strain (Bono et al., 2000; Tilly et al., 2001). The isogenic *bb0259* mutant and complemented strains were constructed as described below. All *B. burgdorferi* strains were grown in Barbour–Stoenner–Kelly II (BSK-II) liquid medium or on soft agarose plates at 35°C in the presence of 2.5% CO<sub>2</sub> (Motalet et al., 2007). Antibiotics, when needed, were supplied in BSK-II medium at proper concentrations: 200 μg/ml kanamycin and/or 40 μg/ml gentamycin. All *Escherichia coli* strains were cultured in Luria-Bertani (LB) liquid medium or on LB agar plates (Bertani, 1951, 2004). When required, 100 μg/ml ampicillin or 4 μg/ml gentamycin was supplemented into the LB medium.

**Bioinformatics**

Basic local alignment search tool (BLAST) was used to determine protein homologs from the sequence database (Altschul et al., 1990, 1997). For the BLAST analysis, a significant homolog is determined based on their E-value [the lower an E-value (the closer it is to zero), the more significant the score is]. Signal peptide was predicted using SignalP 5.0 (Almagro Armenteros et al., 2019) and Phobius
Analysis of bacterial lipoprotein was performed using DOLOP (Madan Babu and Sankaran, 2002; Babu et al., 2006). Clustal Omega was utilized to align multiple amino acid sequences (Sievers et al., 2011). FFAS server was used as described elsewhere.

**Overexpression and Purification of Recombinant Proteins in Escherichia coli**

Multiple attempts to express soluble *B. burgdorferi* BB0259 with variable lengths using short-affinity tags (His6 or Strep) failed, as all these recombinant BB0259 proteins with short affinity tags formed inclusion bodies in the *E. coli* host. In order to produce soluble recombinant BB0259 protein, a DNA fragment harboring the BB0259 open reading frame (ORF) without signal peptide (1-49 aa) was polymerase chain reaction (PCR)-amplified from chromosomal DNA of *B. burgdorferi* B31-A using the primers PF rBB0259-BamHI (GGATCC TGGTTATGGAATTTGAGATTA) and PR rBB0259-PstI (CTGCAGTTAATTTTGGGAATTCGCCC; restriction sites are underlined) and cloned into the pMAL c5x (NEB Inc.) vector. Similarly, the 1xFLAG (DYKDDDDK) tagged FlgJ with gene locus number (BB0771a, also named BB0858) was constructed for far-western or affinity blotting. A DNA fragment of full-length *flgJ* fused with 1xFLAG tag coding sequence (GACTACAAAGACGATGACGACAAG) at C-terminus was amplified by PCR with primers PF MBPFlgJ BamHI (GCTGGAGATCCGAAACACAAATATATCACAATG) and PR MBPFlgJPstI (ATGCATGGAAATTTTGAAATTCGCCC; restriction sites are underlined) and cloned into the pMAL c5x (NEB Inc.) vector. Similarly, the 1xFLAG (DYKDDDDK) tagged FlgJ with gene locus number (BB0771a, also named BB0858) was constructed for far-western or affinity blotting. A DNA fragment of full-length *flgJ* fused with 1xFLAG tag coding sequence (GACTACAAAGACGATGACGACAAG) at C-terminus was amplified by PCR with primers PF MBPFlgJ BamHI (GCTGGAGATCCGAAACACAAATATATCACAATG) and PR MBPFlgJPstI (ATGCATGGAAATTTTGAAATTCGCCC; restriction sites are underlined) and cloned into the pMAL c5x (NEB Inc.) vector.

**SDS-PAGE, Immunoblot, and Affinity Blotting**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as mentioned previously (Motaleb et al., 2000). Exponentially growing *B. burgdorferi* cells were harvested and washed with phosphate-buffered saline (PBS) and resuspended in the same buffer to process the preparation of cell lysate for SDS-PAGE. Immunoblotting was performed with *B. burgdorferi* FlaB, FlgE, MotB, FlhL, and DnaK-specific antibodies (Motaleb et al., 2000, 2011; Sal et al., 2008; Sultan et al., 2015) and Pierce™ ECL or ECL 2 substrate (Thermo Fisher Scientific Inc.). Concentration of the proteins was determined using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. Unless specified, approximately 10 μg of cell lysates was subjected to SDS-PAGE. Far-western or affinity blotting assay with recombinant proteins was performed as described previously (Toker and Macnab, 1997; Kariu et al., 2015; Moon et al., 2016, 2018; Xu et al., 2019). Briefly, 1 μg purified recombinant proteins was subjected to SDS-PAGE and Coomassie blue staining or transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked in the blocking solution (5% BSA, 150 mM NaCl and 0.3% Tween 20, pH 7.4) with gentle shaking for 4 to 6h at room temperature, and then the membranes were incubated with or without the purified FLAG-FlgJ protein at the concentration 2 μg/ml in blocking solution overnight. The membranes were washed 3 times with the washing buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween 20, pH 7.4) and then immunoblotted with monoclonal anti-FLAG® M2 antibody (Sigma-Aldrich Co. LLC) followed by ECL 2 detection, as mentioned above. The X-ray films were exposed to the membranes for less than a minute.

**Construction of the Borrelia burgdorferi Mutants and Complemented Strains**

Construction of the *bb0259* and *bb0531* inactivation plasmids, electroporation, and plating of *B. burgdorferi* were as described earlier (Motaleb et al., 2000; Sultan et al., 2010, 2013; Moon et al., 2016; Xu et al., 2017). *bb0259* was inactivated by *P*<sup>agr</sup>-kanamycin resistance cassette (*P*<sup>agr-aph</sup>, which possessed the *flgb* promoter driving kanamycin resistance gene (Ge et al., 1997; Bono et al., 2000; Li et al., 2010; Zhang et al., 2012; Moon et al., 2016; Xu et al., 2017, 2019). In short, DNA fragment including upstream 394-bp region of *bb0259*, *bb0259* containing five *HindIII* sites, and downstream 324-bp region of *bb0259* was amplified by PCR from chromosomal DNA of wild-type *B. burgdorferi* strain B31-A through Expand™ High Fidelity PCR System (Sigma-Aldrich Inc.) using primers BB0259-KO-F (GGATCCATATGGAATTTGATTATATA) and BB0259-KO-R (GCGGCCGCCTGCAGTTAATTTTGGGAATTCGCCC) respectively, and cloned into pGem-T Easy (Promega Inc.), yielding pGEM-T Easy::*bb0259*. *P*<sup>agr</sup>-Kan was introduced into pGEM-T Easy::*bb0259*.
through the first and last HindIII sites located within bb0259 gene, creating pGEM-T Easy::bb0259KO-P_{bb0259}-Kan, of which the direction of transcription of P_{bb0259}-Kan was confirmed to be the same as that of bb0259. Plasmid pGEM-T Easy::bb0259KO-P_{bb0259}-Kan was linearized by NotI restriction digestion and electroporated into competent B. burgdorferi B31-A cells to create Δbb0259 mutant cells. bb0531 mutant was similarly prepared using a promoter-less kanamycin cassette (Pl-Kan), as described (Sultan et al., 2010). Kanamycin-resistant transformants were screened by PCR to confirm the genotypes of the mutants.

The bb0259 mutant was complemented in trans using the B. burgdorferi E. coli shuttle vector pBSV2G (Elias et al., 2003). In short, bb0259 was PCR amplified using primers BB0259 Comp F (GATCCGATATTTAATAGGTTCGCTTT) and ligated into pGEM-T Easy, yielding plasmid pbb259ORF-Easy. DNA fragment containing bb0259 ORF was then released using NotI restriction digestion from pbb259ORF-Easy, and further ligated into NotI-treated P_{bb0259}-motB-Easy vector (Sultan et al., 2015), creating plasmid P_{bb0259}-bb0259-Easy. Finally, this plasmid was digested with NotI and cloned into the same restriction site of the shuttle vector pBSV2G, generating pBSV2G::P_{bb0259}-bb0259. The newly constructed shuttle vector pBSV2G::P_{bb0259}-bb0259 was electroporated into the Δbb0259 mutant cells, followed by selection with both kanamycin and gentamycin. The resistant clones, namely bb0259Δmutant, were analyzed by PCR for confirmation of the presence of plasmid pBSV2G::P_{bb0259}-bb0259.

* Borrelia burgdorferi* bb0259-E580Q (E glutamate to Q glutamine) and bb0259-D606N (D aspartate to N asparagine) point-mutated strains were constructed through allelic exchange mutagenesis (Yang et al., 2003). Simply, using B31-A chromosomal DNA as the template, the left arm (882 bp, partial bb0259 gene), promoter-less kanamycin cassette (Pl-Kan); 846 bp, and right arm (564 bp, including 88 bp leftover from bb0259 gene for potential ribosome binding and 476 bp of partial bb0259 gene) were PCR amplified, respectively, using primer pairs P1F BB0259 E580Q and P2F BB0259 D606N. The PCR products were cloned into the same restriction site of the shuttle vector pBSV2G::P_{bb0259}-Easy. Finally, this plasmid was digested with NotI and cloned into the same restriction site of the shuttle vector pBSV2G, generating pBSV2G::P_{bb0259}-bb0259. The newly constructed shuttle vector pBSV2G::P_{bb0259}-bb0259 was electroporated into the Δbb0259 mutant cells, followed by selection with both kanamycin and gentamycin. The resistant clones, namely bb0259Δmutant, were analyzed by PCR for confirmation of the presence of plasmid pBSV2G::P_{bb0259}-bb0259.

Dark-Field Microscopy and Swarm Plate Motility Assays

Exponentially growing *B. burgdorferi* cells were observed using a Zeiss Axio Imager M1 dark-field microscope to determine bacterial morphology and motility. To evaluate the motility of *B. burgdorferi* cells, swarm plate assays were performed following our well-established protocol (Motaleb et al., 2000, 2007, 2011).

Cryo-ET Analysis of bb0259 and bb0531 Mutant Cells

Frozen-hydrated *B. burgdorferi* specimens were prepared as described previously (Zhao et al., 2013). In short, the bacterial culture was centrifuged at 5,000 × g for 5 min, and the resulting pellets were suspended in PBS to achieve a cell concentration ~1 × 10^9/ml. After adding 10-nm gold marker solution, 5 μl of the cell suspension was placed on freshly glow-discharged holey carbon grid (Quantifoil Cu R2/2, 200 mesh) for 25 s. The grids were blotted with filter paper for 3 to 5 s and rapidly frozen in liquid ethane, using a homemade plunger apparatus as described previously (Zhao et al., 2013).

The Δbb0531, Δbb0259, and complemented bb0259Δmutant strains were imaged at ~170°C using a Polara G2 electron microscope (FEI Company) equipped with a field emission gun and a 4,096-by-4,096 charge-coupled device (16-megapixel) camera (TVIPS Gmbh, Germany). The microscope was operated at 300 kV with a magnification of ×31,000, resulting in an effective pixel size of 5.7 Å. Using the FEI batch tomography program, low-dose single-axis tilt series were collected from each bacterium at ~8 μm defocus with a cumulative dose of ~100 e/Å^2 distributed over 65 images. In total, 80 tomographic reconstructions of Δbb0259 cell tips and 10 tomographic reconstructions of complemented bb0259 cells were generated.

The bb0259 point mutants (bb0259E580Q and bb0259D606N) were imaged with Krios electron microscope (Thermo Fisher) with a field emission gun, a volta phase plate, and a direct electron detector (Gatan K3 Summit). SerialEM was used to collect tilt series at focus. A total dose of 55 e/Å^2 is distributed among 35 tilt images covering angles from −51° to +51° at tilt steps of 3°. For every single tilt series collection, dose-fractionated mode was used to generate 11 frames per projection image. Collected dose-fractionated data were first subjected to the MotionCor2 to generate drift-corrected files (Zheng et al., 2017). IMOD software was used to align the tilt series and to generate tomograms (Kremer et al., 1996).
In total, 240 tomographic reconstructions of \textit{bb0259}-E580Q cells and 10 tomographic reconstructions of \textit{bb0259}-D606N cells were generated.

**Subtomogram Averaging**

A total of 646 flagellar motor subtomograms were picked from \textit{Δbb0259} tomograms. The initial orientation of each particle was manually estimated by the C-ring and hook. The subtomograms were used to generate the averaged structure as described previously (Zhao et al., 2013).

**Three-Dimensional Visualization**

The software package EMAN2 was used for 3D segmentation (Chen et al., 2017). Segmentation of tomograms was performed using supervised machine learning to segment features of interest. The outer membrane, inner membrane, peptidoglycan layer, and flagella were segmented in this manner and manually cleaned using ChimeraX (Goddard et al., 2018). An existing motor model derived from subtomogram averaging was manually inserted into the segmented model. ChimeraX was used for visualization.

**RESULTS**

**An Open Reading Frame Encompasses a Peptidoglycan-Hydrolyzing Enzymatic Domain**

The single open FlgJ of \textit{B. burgdorferi} plays roles in hook and filament assembly but not in rod assembly in \textit{S. enterica} (Nambu et al., 1999; Cohen and Hughes, 2014; Zaloba et al., 2016), as \textit{flgJ} mutant cells possess an intact rod and P-ring but form partial hook and filament structures (Zhang et al., 2012). Given that an enzymatic activity is required for pore formation in the PG layer for assembly of the flagellar hook and filament, and that \textit{B. burgdorferi} FlgJ lacks a peptidoglycan-hydrolyzing enzyme domain, we sought to determine an ORF containing an enzymatic domain in the Lyme disease spirochetal genome. Using the \textit{S. enterica} FlgJ with GenBank accession number NP_460153 as a query in BLAST search, we found no significant homologs in \textit{B. burgdorferi} (not shown; Nambu et al., 1999; Hirano et al., 2001; Cohen and Hughes, 2014). However, using the FFAS server,\(^1\) BB0531 was identified as the only significant homolog of FlgJ (homology score $-41.70$; a lower FFAS score indicates higher confidence of the prediction; Rychlewski et al., 2000; Jaroszewski et al., 2005, 2011). BB0531 is annotated as a hypothetical protein with unknown function in the \textit{B. burgdorferi} genome; however, it is predicted to possess a mannosyl-glycoprotein endo-$β$-N-acetylglucosaminidase domain responsible for peptidoglycan remodeling or hydrolyzing activity (Pfam: PF01832). BB0531 shares only 15% amino acid sequence identity with \textit{S. enterica} FlgJ and alignment of amino acid sequences of \textit{S. enterica} FlgJ and BB0531 from FFAS shows that the catalytic residues of FlgJ are not well conserved in BB0531 leading us to speculate that BB0531 is not involved in peptidoglycan hydrolyzing activity for flagellar penetration (not shown).

Because \textit{B. burgdorferi} lacks a significant homolog of \textit{S. enterica} FlgJ, we used the model organism's SLT domain as a query in the BLAST search and identified BB0259 as a homolog in \textit{B. burgdorferi}. BB0259 shares 35–41% amino acid sequence identity with significant E-value at the SLT domain (Figure 1; Pfam: PF01464; Thunnissen et al., 1994; van Asselt et al., 1999; Koraimann, 2003). Importantly, the motifs and amino acid residues required for peptidoglycan lytic enzymatic activity are highly conserved in BB0259 (Figure 1). BB0259 possesses a signal sequence (1-24 aa) with lipobox consensus residues LVSC, an unknown domain with no homologs (25-558 aa), and an SLT domain at the C-terminal region (559-717 aa; Figure 1). The presence of these features led us to hypothesize that BB0259 is secreted into the periplasmic space using its signal sequence to create pores in the PG layer, thereby completing assembly of the flagellar hook–filament.

**Mutants in \textit{bb0259}, but Not \textit{bb0531}, Exhibit Defective Motility and Altered Morphology**

Completion of the appropriately assembled flagellar rod, hook, and filament is critical for the spirochete motility and morphology (Motaleb et al., 2000, 2015; Sal et al., 2008; Sultan et al., 2013; Zhao et al., 2013). If the SLT domain of BB0259 or the glucosaminidase domain of BB0531 displays PG-hydrolyzing activities, which are specific for flagellar assembly, then a deletion mutant in \textit{bb0259} or \textit{bb0531} is expected to be unable to create pores in the PG layer, resulting in defective rod/ hook/filament assembly and a motility-deficient phenotype. To test this proposition, we created deletion mutants \textit{Δbb0259} and \textit{Δbb0531} (Supplementary Figures S1, S2, and S5). While the \textit{Δbb0531} cells lack any observable phenotype with respect to motility and morphology, the \textit{Δbb0259} mutant cells observed under a dark-field microscope are completely non-motile (Figure 2A and not shown). Swarm plate motility assays also indicated that \textit{Δbb0259} mutant cells are deficient in swarming out from the initial site of inoculation in soft agarose plates (Figure 2B). Furthermore, while wild-type cells show characteristic flat-wave morphology, \textit{Δbb0259} mutant cells are rod-shaped (Figure 2A). To demonstrate that the phenotypes described here are devoid of a polar effect on downstream gene expression or a secondary mutation elsewhere in the genome, we complemented the mutant \textit{in trans} using a shuttle vector (Supplementary Figures S1, S2). Complemented \textit{bb0259} (\textit{bb0259}com) cells observed under a microscope as well as in swarm plate motility assays indicated morphology and motility phenotypes restored to wild-type level (Figures 2A,B). Furthermore, SLT proteins important for hydrolyzing the PG sacculus for flagellar insertion and subsequent assembly of an intact flagellum are reported to be secreted in the periplasm using their Sec-signal peptide (Garcia-Ramos et al., 2018). Consistent with this observation, a recent study reported that BB0259 is associated with the inner membrane (Toledo et al., 2018). To determine whether the Sec-signal sequence is required

\(^{1}\)https://ffas.godziklab.org/ffas-cgi/cgi/ffas.pl
for BB0259 proteins to be secreted in the periplasm, where they create pores in the PG sacculus for flagellar assembly and thereby provide motility to the cell, we complemented the non-motile Δbb0259 mutant without the signal sequence (bb0259Δsec). As described above, while the full-length bb0259 was able to complement the Δbb0259 mutant and therefore restored the morphology and motility phenotypes (Figures 2A, B), the Sec-signal sequence-deleted variant bb0259Δsec was unable to restore any of the phenotypes (data not shown). These results suggest that the signal sequence is essential for BB0259 proteins to be secreted in the periplasm.

The altered motility and morphology phenotypes exhibited by the deletion mutant suggest that the rod/hook/filament structure was not properly assembled due to a lack of the SLT enzymatic activity of BB0259 that would hydrolyze the PG layer for flagellar structures to assemble through the PG sacculus and provide motility to the bacterial cell. B. burgdorferi cells lacking the gene-encoding filament FlaB or hook FlgE were reported to be non-motile and rod-shaped (Motaleb et al., 2000; Sal et al., 2008; Sultan et al., 2013). We therefore tested whether these gene products were synthesized in the Δbb0259 cells. Western blotting results (Supplementary Figure S3) indicate that the mutant cells synthesized very few FlaB proteins, with no effect on synthesis of the hook protein FlgE. Lack of the filaments in the Δbb0259 cells may thus have resulted in the motility-deficient and rod-shaped morphology (Figures 2A, B).

**Glutamate E580 of BB0259 Is the Catalytic Site Residue**

The SLT enzyme domain proteins possess a conserved ES-GLMQ-AYNAG motif in which the glutamate [E] residue is reported to be the catalytic residue (Figure 1; Thunnissen et al., 1994; van Asselt et al., 1999; Koraimann, 2003). The SLT domain identified in the C-terminal region of BB0259 possesses an E residue at position 580. To determine whether the E residue is the catalytic site, we created a point mutant in E580 of BB0259, resulting in bb0259-E580Q. B. burgdorferi (Supplementary Figures S1, S2). Furthermore, the E223 and D248 residues of the S. enterica FlgJ enzymatic domain were reported to be important for PG-hydrolyzing activities (Nambu et al., 1999), and we noticed that the BB0259 SLT domain also encompasses similarly distanced E580 and D606 residues.
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Even though BB0259 shares limited homology with *S. enterica* FlgJ, and E580 and D606 do not align with E223 and D248 residues of FlgJ (not shown), we created a *bb0259*-D606N *B. burgdorferi* point mutant to determine whether this non-conserved D606 residue codes for any enzyme function (*Supplementary Figures S1, S2*). The *bb0259*-E580Q point mutant exhibited phenotypes identical to those of the deletion mutant Δ*bb0259* with respect to morphology and motility, whereas the *bb0259*-D606N cells were devoid of any detectable phenotype (*Figures 2A,B*). These results indicate that E580 of BB0259 is the catalytic site residue required for BB0259 function, while the D606 residue is not important for PG-hydrolyzing activities.

**Rod/Hook Structures of bb0259 Mutant Cells Lie Underneath the PG Layer**

The morphology and motility phenotypes of the mutant cells led us to speculate that the periplasmic flagellar rod/hook/filament is not able to assemble properly due to the lack of PG-hydrolyzing activity of the Δ*bb0259* and *bb0259*-E580Q cells. Despite using the best possible assays to determine whether BB0259 is able to hydrolyze PG, as this protein possesses an SLT domain, our numerous attempts to demonstrate such activity resulted in little success. Attempts to determine the PG-hydrolyzing enzyme assays using various published assay conditions were unsuccessful even when utilizing the purified *B. burgdorferi* PG as a substrate (Jutras et al., 2016). As an alternative to enzyme assays, we used cryo-electron tomography (cryo-ET) to directly visualize the mutant’s flagella in situ to determine whether the rod/hook/filament assembles or whether the flagella are able to penetrate the PG-layer in the mutant cells. As shown in cryo-ET images (*Supplementary Figure S4*), the Δ*bb0259* mutant cells appear able to synthesize or assemble the rod, P-ring, and hook structures but lack the filament structure (*Supplementary Figures S4A,B*). The hook length of the Δ*bb0259* mutant cells was measured to be the same as that of wild-type cells (~51 nm; *n* = 20), and the hooks of the mutant cells look to be underneath the peptidoglycan layer (*Figures 3E–H, 4D*). However, similar cryo-ET data from the wild-type and complemented *bb0259* cell tips show the intact rod, P-ring, and hook as well as filament (*Supplementary Figures S4D,E,G*). The averaged motor structure from the mutant cells appears to be identical to the wild-type motor structure, without any visible defect in the rod, P-ring, stator, C-ring, export apparatus, or collar structure (*Supplementary Figures S4C,F*). Importantly, the hooks from the *bb0259*-E580Q or Δ*bb0259* cells were found to be underneath the peptidoglycan layer (*Figures 3E–H, 4D*), whereas an intact rod-hook-filament structure was clearly assembled in the wild-type, *bb0259*-D606N point mutant, and complemented *bb0259* cells (*Figures 3A–D, 4C*). The Δ*bb0259* or *bb0259*-E580Q mutant completed assembly of all the components of the flagella except the filament, and its hooks were not able to penetrate...
through the PG (Figures 3E–H, 4D). These results suggest that BB0259 possesses PG-lytic activity; that residue E580, but not D606, is essential for the catalytic activity of the enzyme; and that hydrolyzing the PG sacculus to allow the hook to penetrate through the cell wall is essential for completion of the assembly of an intact flagellum. We therefore renamed the BB0259 protein flagellar-specific LTase\textsubscript{Bb}. On the other hand, cryo-ET images of the Δbb0531 mutant cells show that the periplasmic flagella are completely assembled like the wild-type cells without any detectable defect in penetrating the PG layer or assembly of the flagella indicating that BB0531 is not involved in PG hydrolytic activity for flagellar penetration (Supplementary Figure S6).

**BB0259 or LTase\textsubscript{Bb} Interacts With the Single-Domain Protein FlgJ**

The requirement of PG-hydrolyzing activity for proper assembly of periplasmic flagella and consequently for motility suggests that LTase\textsubscript{Bb} is essential for creating space within the peptidoglycan sacculus for insertion of cell-envelope spanning structures such as flagella. Given that the single-domain FlgJ protein lacks a PG-hydrolyzing enzyme domain, LTase\textsubscript{Bb} is postulated to interact with FlgJ as the enzyme creates pores in the PG sacculus for the hook to assemble through the layer, subsequently allowing the completion of hook–filament structures for cells to be mobile. To test this proposition or to determine which proteins FlgJ interacts with, we performed far-western or affinity blotting. Recombinant FlgJ protein was used as a probe to incubate a PVDF membrane transferred with the distal rod protein FlgG, hook protein FlgE, hook cap protein FlgD, hook–filament junction protein FlgL, filament protein FlaB, and LTase\textsubscript{Bb}. As shown in Figure 5, FlgJ specifically interacts with LTase\textsubscript{Bb} and the hook cap protein FlgD.

**DISCUSSION**

Soluble lytic transglycosylases cleave the glycosidic linkage between N-acetyl-l-muramoyl and N-acetyl-l-glucosaminyl residues with the simultaneous production of a 1,6-anhydromuramoyl product (Holtje et al., 1975; Herlihey et al., 2014). These enzymes, transglycosylases, are abundant in bacteria, found in different forms, and crucial for generating pores by
hydrolyzing the PG sacculus for biosynthesis and recycling of PG, bacterial cell division, and the insertion of cell membrane-spanning structures such as flagella (Herlihey et al., 2014). *B. burgdorferi* LTaseBb appears similar to the *S. enterica* and *E. coli* SLT proteins as it possesses the conserved ES-GLMQ-AYNAG motif, with E580 being the catalytic residue (Figure 1).

Furthermore, the C-terminal domain with the enzymatic motif encompassing the amino acid residues 559-717 is also similar in size to the PG-hydrolytic domains of other bacteria (de la Mora et al., 2012). While a confirmatory enzymatic assay is required to state whether BB0259 in fact possesses any enzymatic activity, our numerous attempts at such an assay...
were unsuccessful. It is noteworthy that measuring the peptidoglycan-degrading or lytic activity of FlgJ/SLT proteins poses significant technical drawbacks. The lack of a defined substrate, the heterogeneity of both the substrate and resulting hydrolyzing products in terms of peptide chain composition and extent of PG sacculus cross-linking, and any modification to the glycan chains contribute to the complexity of analysis of the peptidoglycan. However, genetic studies concomitant with direct visualization of the flagella in situ by cryo-ET enabled us to propose that BB0259 possesses peptidoglycan pore-forming activities and that E580 is the catalytic residue for the peptidoglycan lytic activities, as the bb0259-E580Q mutant’s hooks were unable to penetrate the peptidoglycan sacculus (Figure 3).

LTase$^Bb$ possesses an N-terminal peptide with unknown function (25-558 aa). As the C-terminal domain possesses the enzymatic motif, we speculate that the N-terminal domain is involved in binding to FlgJ, as LTase$^Bb$ secretes into the periplasm using its Sec-signaling sequence. Since LTase$^Bb$ appears to possess peptidoglycan lytic activities and FlgG and FlgJ are required for proper rod/hook/filament assembly (Zhang et al., 2012; Zhao et al., 2013), we propose that these proteins physically interact to facilitate the insertion of the flagella through the PG layer as the enzyme creates pores in the sacculus (Figure 6).

Our direct visualization of the mutant’s flagella in situ by cryo-ET shows that the hooks are unable to pass through the PG sacculus, and therefore, these structures were detected just underneath the PG layer (Figures 3E–H, 4D). Consequently, the flagella did not assemble properly, leading to the non-motile and rod-shaped morphology (Figures 2A,B).

*Borrelia burgdorferi* FlgJ and the SLT domain-containing LTase$^Bb$ are distinct from their counterparts in *S. enterica*. For example, FlgJ from *S. enterica* is involved in flagellar rod formation or required for the transition from rod to hook formation (Cohen and Hughes, 2014). However, cryo-ET data from the *B. burgdorferi* ΔflgJ mutant show that the mutant completed assembly of the rod and P-ring but assembled a partial hook and filament structures (Zhang et al., 2012). It is noteworthy that, unlike the model organisms, the rod and P-ring in this spirochete are not able to contact the PG layer, as the rod is significantly smaller than in the model organisms (17 nm vs. 30 nm in *E. coli*; Zhao et al., 2013; Saijo-Hamano et al., 2019). It is the spirochete-specific collar that contacts the PG sacculus, while the hook penetrates the peptidoglycan layer to complete assembly and formation of the filament in *B. burgdorferi* (Zhao et al., 2013). FlgJ in the spirochete is therefore likely important for assembly of the hook/filament during the hook-to-filament transition (Zhang et al., 2012). The mutants of bb0259 (or LTase$^Bb$) also assemble the rod, P-ring, and hook but are unable to synthesize/assemble the filament (Figures 3, 4, Supplementary Figure S3). The hooks are not able to penetrate the PG layer due to the lack of PG lytic activity in Δbb0259 or bb0259-E580Q mutants (ΔLTase$^Bb$ or LTase$^Bb$-E580Q, respectively). This result also enforces the

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**FIGURE 6**: A working model of periplasmic flagellar hook–filament assembly in the spirochete *B. burgdorferi*. At the onset of rod assembly, many flagellar components assemble, such as the MS ring, C ring, export apparatus, stators, and collar. Subsequently, the rod components (FliE, FlgB, FlgC, FlhO/FlgF, and FlgG) are sequentially secreted into the periplasmic space through flagellar T3SS and assemble (Zhao et al., 2013). The hook cap protein FlgD and its chaperone or interacting protein FlgJ form at the distal end of the rod and initiate hook assembly. Binding of FlgJ with the pre-localized SLT enzyme BB0259 or L Tate$^Bb$ in the periplasmic leaflet of the cell wall promotes hydrolysis of the sacculus as the hook completes assembly through the cell wall. Assembly of the filament (FlaA and FlaB) is promoted by the filament cap.
notion that FlgJ and LTase \(^{ab}\) are crucial for hook/filament assembly and/or for hook-to-filament transition (Figure 6).

\(\Delta bb0259/\Delta LTase^{ab}\) or \(\Delta bb0259/E580Q/\Delta LTase^{ab}/E580Q\) mutant phenotypes appear to be very similar to that of the \(\Delta flaB\) mutant (Zhao et al., 2013). In both cases, the mutants complete assembly and formation of the motor, rod, P-ring, and hook but lack the filament (compare Figures 3F,G with Supplementary Figure S4H) as the PG enzyme activity of BB0259 is believed to be retained in \(\Delta flaB\) cells, again reinforcing that BB0259 or LTase \(^{bb}\) is a PG hydrolyzing enzyme. Moreover, the hook length of the \(\Delta LTase^{ab}, \Delta flaB,\) and wild-type cells is the same (\(~51 \text{ nm; } n = 20\) and consistent with the previously reported hook length (Zhao et al., 2013), suggesting that LTase \(^{ab}\) is not involved in hook assembly. All these data suggest that FlgJ and LTase \(^{ab}\) are distinct from their counterparts in other flagellated bacteria reported to date. Importantly, in \(S. enterica\), FlgJ interacts with the rod proteins including the distal rod FlgG as FlgJ is considered a rod-capping or scaffolding protein (Hirano et al., 2001; Cohen and Hughes, 2014). However, that is not the case in \(B. burgdorferi\). FlgJ interaction with FlgG is undetectable (Figure 5), and the \(\Delta flgJ\) mutant’s rod structures are intact in \(B. burgdorferi\) (Zhang et al., 2012).

Based on reports of FlgJ and this communication, we propose a model for how LTase \(^{bb}\) and FlgJ work synergistically with the hook cap protein FlgD to complete assembly of the hook–filament in \(B. burgdorferi\) (Figure 6). In this model, LTase \(^{ab}\) uses Sec-dependent pathway to be secreted into the periplasm. Using the flagellar type III secretion system, FlgJ and hook-capping protein FlgD compile onto the rod as the rod completes assembly onto the MS-ring. Interactions of FlgJ with BB0259 or LTase \(^{bb}\) and FlgD (Figure 5) enable the hook to initiate and complete its assembly through the PG holes created by LTase \(^{ab}\) wherein FlgJ chaperones or interacts with FlgD for the hook to complete its assembly in the periplasm (Figure 6). Without the FlgJ, the hook FlgE synthesis or assembly is therefore incomplete (Zhang et al., 2012) due to a lack of interaction between FlgD and FlgJ or lack of chaperone functions of FlgJ for FlgD. We speculate that the hook-to-filament assembly occurs as in other bacteria, as we have reported (Zhao et al., 2013). Additional in-depth analysis is warranted to better understand the spatiotemporal synthesis of the FlgJ, FlgD, and LTase \(^{ab}\) proteins and hook–filament assemblies in \(B. burgdorferi\).

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

HX, BH, and DAF conducted the experiments. JL and MM designed the experiments. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.692707/full#supplementary-material

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