Molecular Characterization of the Flagellar Hook in *Bacillus subtilis*

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The structure of the Gram-positive flagellum is poorly understood, and *Bacillus subtilis* encodes three proteins homologous to the flagellar hook protein from *Salmonella enterica*. Here we generated a modified *B. subtilis* hook protein that could be fluorescently stained using a cysteine-reactive dye. We used the fluorescently labeled hook to demonstrate that FlgE is the hook structural protein and that FliK regulated hook length. We further demonstrate that two proteins of unknown function, FlhO and FlhP, and the putative hook cap, FlgD, were required for hook assembly, such that when *flhO*, *flhP*, or *flgD* was mutated, hook protein was secreted into the supernatant. All mutants defective in hook completion resulted in homogeneously reduced $\sigma^{D}$-dependent gene expression due to the action of the anti-sigma factor FlgM.

Many bacteria are motile by rotating extracellular appendages called flagella, but flagellar assembly and structure are best understood in the Gram-negative bacteria *Escherichia coli* and *Salmonella enterica* (45). Thrust is generated by rotating the filament, a long, hollow, helical structure polymerized from approximately 20,000 subunits of a single protein called flagellin (also called FliC or Hag) (44). The filament is assembled by secreting each flagellin subunit through the duct of the nascent structure such that polymerization occurs at the distal tip (31, 73). Flagellin secretion is driven by a type III protein secretion system housed within the flagellar basal body that is anchored to the cell envelope (16, 48). The basal body also interacts with proton channels to rotate a rod that ultimately turns the filament. Between the rotating rod and the helical filament is a short, curved, hollow linker domain called the hook.

The hook is a flexible universal joint that transmits torque from the rod to the filament and changes the angle of rotation (6, 64). In *E. coli* and *S. enterica*, the hook is assembled from a single repeating monomer unit called FlgE (32, 35, 40, 66). The flgE gene (also called flaK or flaFV), which encodes the FlgE protein, was identified from partial-function alleles that altered the electrophoretic mobility of the hook structural subunit (1, 38). To build the hook, the hook cap protein FlgD must first be assembled on the end of the completed rod structure (59). Then, like flagellin, FlgE subunits are secreted through the basal body rod complex, inserted underneath FlgD, and polymerized into a curved hollow cylinder (56, 59). The hook has an average length of 55 nm, and hook length is controlled by the regulatory protein FliK and the flagellar secretion component FlhB (27). Loss of FliK and particular alleles of FlhB result in elongated hook structures called polyhooks, and the cells fail to produce a flagellar filament (27, 65, 67).

FliK is thought to act as a molecular ruler in which the length of the FliK primary sequence is proportional to the length of the hook (17, 34, 55). Prior to hook completion, FliK is secreted intermittently through the basal body rod and the nascent hook structure (17, 48, 49). When the hook–basal body is the length of the extended FliK protein, the FliK N terminus interacts with the hook cap, the middle section occupies the secretion channel, and the FliK C terminus interacts with FlhB in the cytoplasm (34, 51, 52, 55). Interaction with FliK activates autoproteolysis of FlhB to cause a switch in the substrate specificity of the secretion apparatus (19, 20, 52, 71). After specificity has been switched, the secretion apparatus becomes proficient for the secretion of late-class flagellar proteins.

One of the earliest proteins secreted after the substrate specificity switch is the negative regulator FlgM (23). FlgM is an anti-sigma factor that directly binds to and antagonizes the activity of the alternative sigma factor $\sigma^{D}$ (also called $\sigma^{D}$ or FliA) (33, 58). Prior to completion of the hook–basal body complex, FlgM accumulates in the cytoplasm and inhibits $\sigma^{D}$ activity. Once the hook is complete, FlgM is secreted and $\sigma^{D}$ is released to direct RNA polymerase to express the late-class flagellar genes, including fliC, encoding flagellin (30, 36, 41). Thus, not only is the length of the completed flagellar hook regulated, but hook completion has profound regulatory effects on downstream gene expression and subsequent flagellar assembly.

The Gram-positive bacterium *Bacillus subtilis* is motile and encodes many flagellar structural proteins in the 31-gene fla-che operon, but their putative functions are largely inferred by homology to their *E. coli* and *S. enterica* counterparts (2, 3, 11, 60, 74). *B. subtilis* also encodes FlgM, which binds to and inhibits the $\sigma^{D}$ homolog $\sigma^{D}$ (SigD), which directs flagellin expression (7, 10, 21, 54). In addition, $\sigma^{D}$ activity is inhibited in the absence of completed basal bodies in an FlgM-dependent manner (5, 14, 29). FlgM has never been reported to be secreted in *B. subtilis*, and how FlgM activity is coordinated with flagellar assembly is unknown. Finally, the *B. subtilis* genome contains a gene annotated as *flkI* that is predicted to encode an FliK homolog, but the role of the *B. subtilis* FliK protein in hook length regulation has not been explored.

*B. subtilis* encodes three homologs of the hook structural protein: FlgE, FlhO, and FlhP. The FlgE protein was predicted to constitute the primary structural subunit of the hook due to its...
high abundance in biochemical preparations of purified *B. subtilis* hook-based body complexes (39). In contrast, FlhO and FlhP were present in much lower abundance, and their functions are unknown (39). Whereas the *flgE* gene is coexpressed with other hook-based body proteins in the *fla-che* operon, *flhO* and *flhp* are expressed as a separate and remote putative dicistron. Here we mutated each of the three genes encoding hook homologs and found that each is required for motility. Using genetic, biochemical, and cytological approaches, we conclude that FlgE is the primary structural subunit of the hook and demonstrate that FlhO and FlhP are required for hook assembly (39). We demonstrate that cells with mutated *flik* produce polyhook structures and that cells with a mutated hook reduce σ52-dependent gene expression in a FlgM-dependent manner.

**MATERIALS AND METHODS**

**Strains and growth conditions.** All strains used in this study are listed in Table 1. *B. subtilis* strains were grown in Luria-Bertani (LB) (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) broth or on LB plates fortified with 1.5% Bacto agar at 37°C. When appropriate, antibiotics were included at the following concentrations: 10 μg/mL tetracycline, 100 μg/mL spectinomycin, 5 μg/mL chloramphenicol, 5 μg/mL kanamycin, and 1 μg/mL erythromycin plus 25 μg/mL lincomycin (macrolides-lincosamides-streptogramin B [MLS]).

**Swarm expansion assay.** Cells were grown to mid-log phase at 37°C in LB broth and resuspended to an optical density at 600 nm (OD600) of 10 in pH 8.0 phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4) containing 0.5% India ink (Higgins). Freshly prepared LB containing 0.7% Bacto agar (25 mL plate) was dried for 20 min in a laminar flow hood, centrally inoculated with 10 μL of the cell suspension, dried for another 10 min, and incubated at 37°C. The India ink demarks the origin of the colony, and the swarm radius was measured relative to the origin. For consistency, an axis was drawn on the back of the plate and swarm radius measurements were taken along this transect.

**Microscopy.** For cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) microscopy, cells were grown at 37°C to an OD600 of 0.6 to 1.0, and 1 mL was washed once in pH 8.0 PBS, pelleted, and resuspended in 50 μL PBS containing 5 μg/mL membrane stain FM4-64 (Molecular Probes). For fluorescence microscopy of flagella, 0.5 mL of broth containing 5 μg/mL Alexa Fluor 488 C3 maleimide (Molecular Probes), and incubated for 5 min at room temperature (8). Cells were then washed twice with 50 μL PBS, and membranes were stained by resuspension in 50 μL of PBS containing 5 μg/mL FM4-64. Three microliters of suspension was placed on a microscope slide and immobilized with a poly-L-lysine-treated coverslip.

Fluorescence microscopy was performed with a Nikon 80i microscope with a Nikon Plan Apo 100× phase-contrast objective and an Excite 120 metal halide lamp. FM4-64 was visualized with a C-FL HYQ Texas Red filter cube (excitation filter, 532 to 587 nm; barrier filter, >590 nm). CFP fluorescence signals were viewed using a C-FL HYQ CFP filter cube (excitation filter, 426 to 446 nm; barrier filter, 460 to 500 nm). YFP was visualized using a C-FL HYQ YFP filter cube (excitation filter, 490 to 510 nm; barrier filter, 520 to 550 nm). Images were captured with a Photometrics Coolsnap HQ2 camera in black and white, false colored, and superimposed using Metamorph image software.

**Strain construction.** All PCR products were amplified from purified chromosomal DNA from *B. subtilis* strain 3610. All constructs were first introduced into the domesticated strain PY79 by natural competence and then transferred to the 3610 background using SPP1 phage-mediated generalized phage transduction (72). All primers used in this study are listed in Table S1 in the supplemental material. All plasmids used in this study are listed in Table S2 in the supplemental material.

(i) In-frame deletions. To generate the ΔflgE in-frame markerless deletion construct pDP306, the region upstream of *flgP* was PCR amplified using the primer pair 1483/1484 and digested with EcoRI and XhoI, and the region downstream of *flgE* was PCR amplified using the primer pair 1485/1486 and ligated into the EcoRI and BamHI sites of pMiniMAD, which carries a temperature-sensitive origin of replication and an erythromycin resistance cassette (62). The plasmid pDP306 was introduced into PY79 by single-crossover

![Image](http://jb.asm.org/downloaded_from/4620/017520306.jpg)
integration by transformation at the restrictive temperature for plasmid replication (37°C) using MLS resistance as a selection. The integrated plasmid was then transduced into 3610. To evict the plasmid, the strain was incubated in 3 ml LB broth at a permissive temperature for plasmid replication (22°C) for 14 h, diluted 30-fold in fresh LB broth, and incubated at 22°C for another 8 h. Dilution and outgrowth were repeated 2 more times. Cells were then serially diluted and plated on LB agar at 37°C. Individual colonies were patched on LB plates and LB plates containing MLS to identify MLS-sensitive colonies that had evicted the plasmid. Chromosomal DNA from colonies that had excised the plasmid was purified and screened by PCR using primers 1483/1486 to determine which isolate had retained the ΔflhH allele.

To generate the ΔflhH in-frame markerless deletion construct pKB114, the region upstream of flhH was PCR amplified using the primer pair 1850/1524 and digested with EcoRI and Sall, and the region downstream of flhH was PCR amplified using the primer pair 1525/1526 and digested with Sall and BamHI. The two fragments were then simultaneously ligated into the EcoRI and BamHI sites of pMiniMAD. pKB114 was integrated into the B. subtilis PV79 genome, transduced to strain 3610, and evicted as described above. Colonies were screened by PCR using primers 1850/1524 to determine which isolate had retained the ΔflhH allele.

To generate the ΔflhP in-frame markerless deletion construct pCC12, the region upstream of flhP was PCR amplified using the primer pair 2347/2348 and digested with EcoRI and BamHI, and the region downstream of flhP was PCR amplified using the primer pair 2349/2350 and digested with BamHI and Sall. The two fragments were then simultaneously ligated into the EcoRI and Sall sites of pMiniMAD. pCC12 was integrated into the B. subtilis PV79 genome, transduced into strain 3610, and evicted as described above. Colonies were screened by PCR using primers 2347/2350 to determine which isolate had retained the ΔflhP allele.

To generate the Δflk in-frame markerless deletion construct pKB93, the region upstream of flk was PCR amplified using the primer pair 1387/1388 and digested with EcoRI and Xhol, and the region downstream of flk was PCR amplified using the primer pair 1389/1390 and digested with Sall and BamHI. The two fragments were then simultaneously ligated into the EcoRI and Sall sites of pMiniMAD. pCC12 was integrated into the B. subtilis PV79 genome, transduced into strain 3610, and evicted as described above. Colonies were screened by PCR using primers 1387/1390 to determine which isolate had retained the Δflk allele.

To generate the ΔflgD in-frame markerless deletion construct pDP328, the region upstream of flgD was PCR amplified using the primer pair 2035/2036 and digested with EcoRI and Xhol, and the region downstream of flgD was PCR amplified using the primer pair 2035/2036 and digested with Xhol and BamHI. The two fragments were then simultaneously ligated into the EcoRI and BamHI sites of pMiniMAD. pDP328 was integrated into the B. subtilis PV79 genome, transduced into strain 3610, and evicted as described above. Colonies were screened by PCR using primers 2035/2036 to determine which isolate had retained the ΔflgD allele.

(i) ΔflgD::kan. The ΔflgD::kan insertion-deletion allele was generated by long flanking homology PCR (using primers 624 and 625 and primers 626 and 627), and DNA containing a kanamycin drug resistance gene (pDG780) was used as a template for marker replacement (24, 69).

(ii) Implementation constructs. To generate the ΔflgD::flgE complementation construct pDP324, the ΔflgD::flgE promoter was amplified using primer pair 2015/2016 and was digested with EcoRI and Xhol. The flgE region was amplified primer pair 2017/2018 and was digested with Xhol and BamHI. The two fragments were ligated simultaneously into the EcoRI and BamHI sites of pDG364 containing the polylinker and the erythromycin resistance cassette between the arms of the thrC gene (25).

To generate the ΔflgD::flhP complementation construct pCC13, the ΔflgD promoter was amplified using primer pair 1251/2344, and the flhP gene was amplified using primer pair 2344/2345. The ΔflgD-containing PCR product was digested with EcoRI and Xhol, the flhP-containing product was digested with Xhol and BamHI, and the two digested fragments were cloned simultaneously into the EcoRI and BamHI sites of pDG364.

To generate the ΔflgD::flgD complementation construct pDP403, the ΔflgD::flgD promoter was amplified using primer pair 2460/2461 and was digested with EcoRI and Xhol. The flgD region was amplified using primer pair 3163/3164 and was digested with Xhol and BamHI. The two fragments were ligated simultaneously into the EcoRI and BamHI sites of pDG364.

(iv) LacZ reporter constructs. To generate the β-galactosidase (lacZ) reporter construct pCC1, the lacZ promoter region was PCR amplified using 3610 DNA as a template and primer pair 1251/1252. The PCR product was digested with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of plasmid pDG268, which carries a chloramphenicol resistance marker and a polylinker upstream of the lacZ gene between two arms of the amyE gene (4).

To generate the native-site lacZ integrant flgH::lacZ cat pDP405, the flhP gene was PCR amplified using 3610 DNA as a template and primer pair 3210/3211. The PCR product was digested with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of plasmid pEX44, which carries chloramphenicol resistance marker and a polylinker upstream of the lacZ gene.

(v) MBP-FlgE protein expression construct. To generate the maltose binding protein (MBP)-FlgE fusion expression construct, the flgE gene was PCR amplified using primer pair 1992/1993, digested with EcoRI and Xhol, and cloned into the EcoRI and BamHI sites of plasmid pMal-p2X (New England BioLabs).

SPPI phage transduction. To 0.2 ml of dense culture grown in TY broth (LB broth supplemented after autoclaving with 10 mM MgSO4 and 100 μM MnSO4), serial dilutions of SPPI phage stock were added and statically incubated for 15 min at 37°C. To each mixture, 3 ml TYS (molten TY supplemented with 0.5% agar) was added, poured atop fresh TY plates, and incubated at 37°C overnight. Top agar from the plate containing near-confluent plaques was harvested by scraping into a 50-ml flat-bottomed tube, vortexed, and centrifuged at 5,000 × g for 10 min. The supernatant was treated with 25 μg/ml (final concentration) DNase before being passed through a 0.45-μm syringe filter and stored at 4°C.

Recipient cells were grown to stationary phase in 2 ml TY broth at 37°C. Cells (0.9 ml) were mixed with 5 μl of SPPI donor phage stock, and then 9 ml of TY broth was added to the mixture and allowed to stand at 37°C for 30 min. The transduction mixture was then centrifuged at 5,000 × g for 10 min, the supernatant was discarded, and the pellet was resuspended in the remaining volume. One hundred microliters of cell suspension was then plated on TY fortified with 1.5% agar, the appropriate antibiotic, and 10 mM sodium citrate.
β-Galactosidase assay. Cells were harvested from cultures growing at 37°C in LB broth. Cells were collected in 1-ml aliquots and suspended in equal volume of Z buffer (40 mM NaH₂PO₄, 60 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM KCl, and 38 mM 2-mercaptoethanol). Lysozyme was added to each sample to a final concentration of 0.2 mg ml⁻¹ and incubated at 30°C for 15 min. Each sample was diluted in Z buffer to a final volume of 500 μl, and the reaction was started with 100 μl of 4 mg ml⁻¹ 2-nitrophenyl β-D-galactopyranoside in Z buffer and stopped with 250 μl of 1 M Na₂CO₃. The OD₄₂₀ of the reaction mixture was measured, and the β-galactosidase-specific activity was calculated according to the equation 

\[ \text{OD}_{420}(\text{time} \times \text{OD}_{600}) \times \text{dilution factor} \times 1,000. \]

**FlgE protein purification.** The MBP-FlgE fusion protein expression vector pCC10 was transformed into Rosetta-gami and incubated for 30 min at 37°C. Ten microliters of lysate was mixed or anti-Hag (1:80,000 dilution) primary antibody and a 1:10,000 diluted with anti-FlgE (1:20,000 dilution), anti-SigA (1:40,000 dilution), and protein was sent to Cocalico Biologicals Inc. for serial injection into a affinity, high-specificity detection of FlgE protein in Western blot analysis.

Western blotting. A Milligram of purified MBP-FlgE protein was sent to Cocalico Biologicals Inc. for serial injection into a rabbit host for antibody generation. Crude serum was sufficient for high-affinity, high-specificity detection of FlgE protein in Western blot analysis.

RESULTS

**Mutants defective in FlgE, FlhO, and FlhP fail to assemble the flagellar filament and have reduced flagellin gene expression.** The molecular composition of the flagellar hook in **B. subtilis** is unknown. The **B. subtilis** genome encodes three proteins, FlgE, FlhO, and FlhP, homologous to the single flagellar hook protein, FlgE, in **S. enterica** (Fig. 1A). To determine the contribution of each protein to flagellum-based motility, the gene encoding each homolog, flgE, flhO, and flhP, respectively, was separately mutated by in-frame markerless deletion (Fig. 1B). Mutation of any of the three genes abolished swimming in wet mounts as observed by phase-contrast microscopy and abolished swimming motility, defined as the ability to spread rapidly atop the surface of a 0.7% agar petri plate (Fig. 1C) (37). We conclude that FlgE, FlhO, and FlhP are required for motility.

To confirm that the loss-of-motility phenotypes resulted from the absence of the putative hook proteins, each individual gene was restored by complementation (Fig. 1B). To complement the flgE mutation, the flgE gene was fused to the P₃₄₆-P₃₄₆- fla-che promoter of the fla-che operon and inserted at the ectopic amyE site (amyE:: P₃₄₆-P₃₄₆- fla-che- flgE) (18, 70). To complement the flhO mutation, the flhO gene and 500 bp immediately upstream (P₃₄₆- flhO) was inserted at the amyE site (amyE:: P₃₄₆- fla-che- flhO). The flhP gene appeared to be part of an operon with flhO, and to complement the flhP mutation, the flhP gene was fused to the P₃₄₆ promoter region and inserted at the amyE site (amyE:: P₃₄₆- fla-che- flhP). In each case, introduction of the complementation construct restored wild-type swarming motility to the strain with the corresponding gene mutated (Fig. 1C). We conclude that each mutation resulted in a loss of motility due to the disruption of the indicated gene and that the phenotype was not due to polar effects on downstream gene expression. We further infer that the region upstream of the flhO gene contains a promoter sufficient for expressing the flhO and flhP genes.

In **E. coli** and **S. enterica**, completion of the flagellar hook is required to assemble the flagellar filament. To determine whether the loss-of-motility phenotypes resulted from filament assembly, flagellar filaments were stained with a cysteine-reactive dye in a strain expressing the flagellar filament protein Hag with a cysteine residue introduced on an exposed surface (amyE::P₃₄₆-P₃₄₆- hag(208°C)) (8). Wild-type cells expressing the modified filament produced many flagella on the cell surface (Fig. 2A). In contrast, cells with flgE, flhO, or flhP mutated failed to synthesize detectable flagellins (Fig. 2A). We conclude that FlgE, FlhO, and FlhP are each required for filament assembly, as mutation of the gene that encodes each protein abolished filament assembly.

The failure to synthesize flagellar filaments may have been either due to a defect in flagellin protein synthesis or due to a defect in flagellin polymerization such that flagellin subunits were released into the supernatant. To measure cell-associated flagellin protein levels, cell pellets of the wild type and various mutants were harvested, resolved by SDS-PAGE, electroblotted, and probed with an anti-Hag antibody in Western blot analysis. Each lysate was also probed with an anti-SigA antibody to detect the constitutive vegetative housekeeping sigma factor σ^A as a loading control. Whereas flagellin levels were high in the wild type, flagellin was severely reduced in each of the flgE, flhO, and flhP backgrounds (Fig. 3A). To measure the amount of secreted flagellin, cell supernatants were concentrated by trichloroacetic acid (TCA) precipitation, resolved by SDS-PAGE, electroblotted, and probed with an anti-Hag antibody in Western blot analysis. Small amounts of flagellin were found in the supernatant of wild-type cells, presumably the result of secretion of unpolymerized flagellin and/or filament shearing (Fig. 3A). The flagellin found in the supernatant was unlikely to be the result of premature cell lysis, as the cytoplasmic σ^A protein was undetectable (Fig. 3B). Finally, no flagellin was detected in the supernatants of the flgE, flhO, or flhP mutants suggesting that the reduction of flagellin in cell pellets was not due to secretion of flagellin into the supernatant (Fig. 3A). We conclude that mutation of any of the putative hook components results in a dramatic reduction in total flagellin protein level.

Flagellin protein levels may be reduced due to a reduction in flagellin gene expression. To measure flagellin gene expression, the promoter of flagellin (P₃₄₆- hag) was fused to the gene encoding yellow fluorescent protein and inserted at an ectopic site (amyE::P₃₄₆- hag-YFP). Cells with flgE, flhO, or flhP mutated appeared to express YFP at a level lower than the wild type, and the cells grew in
short chains (Fig. 4). Chaining may result from a decreased expression of the LytF cell-separating autolysin, which, like flagellin, is expressed by the alternative sigma factor $\sigma^H$ (12, 46). To measure LytF expression, the promoter of LytF (P$_{lytF}$) was fused to the gene encoding cyan fluorescent protein and inserted at an ectopic site (thrC::P$_{flhO}$-CFP). Cells with $\text{flgE}$, $\text{flhO}$, and $\text{flhP}$ mutated appeared to express CFP from P$_{lytF}$ at an undetectable level (Fig. 4). We conclude that mutation of the putative hook proteins reduces the expression of both the flagellin and LytF promoters uniformly in the population and likely acts at the level of the $\sigma^H$ sigma factor.

FIG 1 $B. subtilis$ encodes three proteins homologous to the hook structural protein, and each is required for motility. (A) Multiple-sequence alignment of FlgE from $Salmonella enterica$ serovar Typhimurium (Sen) and FlgE, FlhO, and FlhP from $Bacillus subtilis$ (Bsu). (B) Genetic map of regions carrying the $\text{flgE}$, $\text{flhO}$, $\text{flhP}$, and $\text{fliK}$ genes, including the locations of mutations used in the study and the organization of ectopic complementation constructs. Note that $\text{flgE}$ and $\text{fliK}$ are encoded within the long, 25-kb $\text{fla-che}$ operon, whereas $\text{flhO}$ and $\text{flhP}$ are expressed as a separate dicistron. (C) Quantitative swarm expansion assays. Each graph contains wild-type strain 3610 (solid circles), a strain containing a mutation in the gene indicated in the upper left corner (open circles), and a strain containing the indicated mutation and ectopic complementation construct of the gene indicated in the upper left corner (gray circles). In the leftmost graph, the gray triangles indicate complementation with the $\text{flgET123C}$ allele. The following strains were used to generate the swarm expansion assays: 3610 (wild type), DS4681 ($\Delta\text{flgE}$), DS5161 ($\Delta\text{flhO}$), DS5944 ($\Delta\text{flhO}$ $\text{thrC}$::P$_{flhO}$-$\text{flhO}$), DS7351 ($\Delta\text{flhP}$), DS7360 ($\Delta\text{flhP}$ $\text{amyE}$::P$_{flhO}$-$\text{flhP}$), DS4536 ($\Delta\text{fliK}$), DS7673 ($\Delta\text{flgE}$ $\text{amyE}$::P$_{fla-che}$-$\text{flgE}$ T123C), DS8037 ($\Delta\text{flgE}$ $\text{amyE}$::P$_{fla-che}$-$\text{flgE}$ T123C), and DS9920 ($\Delta\text{flgD}$ $\text{amyE}$::P$_{fla-che}$-$\text{flgD}$).
FlgE is the primary structural subunit of the hook. (A) Fluorescence micrographs in which membranes have been stained with FM4-64 (false colored in red) overlaid with the HagT209C allele of flagellin that was stained with a maleimide dye (false colored in green). Scale bar is 2 μm.

(B) Fluorescence micrographs in which membranes have been stained with FM4-64 (false colored red) overlaid with the FlgET123C allele of the hook that was stained with a maleimide dye (false colored in green). Scale bar is 2 μm. The following strains were used to generate this panel: DS7671 (wild type), DS4681 (fliK), DS5161 (flhO), DS8808 (fliK flgE), DS8839 (flhP), DS8840 (fliK flgE), and DS9892 (sigD). See Fig. S1 in the supplemental material for an enlarged panel of polyhooks in the ∆fliK strain.

FIG 2

The sigma factor σ^72 is known to be antagonized by binding directly to the anti-sigma factor FlgM (7). To determine whether FlgM was antagonizing σ^72 in the fliE, flhO, and flhP mutants, σ^72-dependent gene expression was measured quantitatively by generating transcriptional fusions of the gene encoding β-galactosidase fused to either the Phag or PlytF promoter. Mutation of flgE, flhO, or flhP reduced the expression of both Phag and PlytF 10-fold relative to that in the wild type (Fig. 5). Furthermore, expression was restored to levels above that in the wild type by the deletion of the gene encoding FlgM (Fig. 5). We conclude that σ^72-dependent gene expression (including flagellin and LytF expression) is low in the putative hook mutants due to the activity of the anti-sigma factor FlgM.

The FlgE protein is the structural subunit of the hook, and FlhO, FlhP, and FlgD are required for hook assembly. To dissect the roles of the FlgE, FlhO, and FlhP proteins, we generated a strain to visualize the flagellar hook by introducing a cysteine residue on an exposed surface of a hook protein such that the hook could be labeled with fluorescent cysteine-reactive dye. We chose the FlgE protein for modification because previous work suggested that FlgE was substantially more abundant than either FlhO or FlhP in flagellar basal body-hook preparations (39). To identify candidate residues, the primary sequences of FlgE from B. subtilis and S. enterica were aligned, and surface-exposed serine and threonine residues were predicted by mapping onto the S. enterica FlgE three-dimensional structure (22, 64). Threonine at position 123 was predicted to be surface exposed, and site-directed mutagenesis was used to replace the residue with a cysteine (FlgET123C) in the flgE complementation construct (amyE::P_{amyE}^{E. coli}-flgET123C) (Fig. 1A). The ectopically integrated flgET123C complementation construct was functional and restored swarming motility to a ∆flgE deletion mutation (Fig. 1C). Furthermore, fluorescence microscopy of the flgET123C allele introduced as a merodiploid into wild-type cells resulted in a faint punctate staining pattern when the cysteine-reactive dye was added (Fig. 2B). Stronger fluorescence was detected in a ∆flgE
mutant complemented by the ectopic $\text{FlgET}_{123C}$, presumably due to the lack of competition between the stainable and wild-type alleles. We conclude that $\text{FlgET}_{123C}$ was functional for supporting motility and could be fluorescently stained.

The puncta observed when staining the $\text{FlgET}_{123C}$ allele may represent flagellar hooks, but hooks are short and the curved shape is below the limit of resolution for fluorescence microscopy. To increase the size of flagellar hooks, we mutated the putative hook length regulator $\text{FliK}$ because $S$. enterica $\text{FliK}$ mutants fail to limit hook length, resulting in elongated “polyhooks” (27). An in-frame markerless deletion of $B$. subtilis $\text{FliK}$ (Fig. 1B) abolished swimming and swarming motility (Fig. 1C), abolished flagellar filament assembly (Fig. 2A), reduced flagellin protein levels (Fig. 3A), and reduced $\sigma^{70}$-dependent gene expression in a $\text{FlgM}$-dependent manner (Fig. 4 and 5). Thus, mutation of $\text{FliK}$ appeared to phenocopy mutation of $\text{FlgE}$, $\text{FlhO}$, or $\text{FlhP}$. When the $\text{FlgET}_{123C}$ allele was expressed and fluorescently labeled in a $\Delta\text{flgE}$ double mutant background, elongated structures consistent with polyhooks were observed (Fig. 2B; see Fig. S1 in the supplemental material). We conclude that $\text{FlgET}_{123C}$ puncta represent flagellar hooks, that $\text{FlgE}$ is a primary component of the hook structure, and that $\text{FliK}$ in $B$. subtilis is a hook length regulator.

To determine the roles of $\text{FlhO}$ and $\text{FlhP}$ in hook assembly, the $\text{FlgET}_{123C}$ allele was introduced into $\Delta\text{flhO}$ or $\Delta\text{flhP}$ $\text{FlgE}$ double mutant backgrounds. Cells defective in hook completion grow in short chains. Fluorescence micrographs of cells of the indicated genotype were membrane stained with FM4-64 (false colored in red) and contained a reporter for flagellin ($P_{\text{tag}}\text{-YFP}$, false colored in green) and autolysin ($P_{\text{pho}}\text{-CFP}$, false colored in blue) expression. Merge indicates an overlay of the three channels of fluorescence. Scale bar, 2 μm. The following strains were used to generate this figure: DS7006 (wild type), DS8705 ($\Delta\text{flgE}$), DS8706 ($\Delta\text{flhO}$), DS8707 ($\Delta\text{flhP}$) DS8704 ($\Delta\text{flhK}$), and DS9894 ($\Delta\text{flgD}$).

**FIG 4** Cells defective in hook completion grow in short chains. Fluorescence micrographs of cells of the indicated genotype were membrane stained with FM4-64 (false colored in red) and contained a reporter for flagellin ($P_{\text{tag}}\text{-YFP}$, false colored in green) and autolysin ($P_{\text{pho}}\text{-CFP}$, false colored in blue) expression. Merge indicates an overlay of the three channels of fluorescence. Scale bar, 2 μm. The following strains were used to generate this figure: DS7006 (wild type), DS8705 ($\Delta\text{flgE}$), DS8706 ($\Delta\text{flhO}$), DS8707 ($\Delta\text{flhP}$) DS8704 ($\Delta\text{flhK}$), and DS9894 ($\Delta\text{flgD}$).
double mutants. No puncta were observed when either strain was fluorescently labeled, suggesting that both FlhO and FlhP were required for assembly of the hook (Fig. 2B). A cell way in which assembly of the hook could be abrogated is by a reduction in the amount of FlgE protein synthesized. To measure FlgE protein levels, cell pellets of the wild type and various mutants were harvested. Lysates from each pellet were resolved by SDS-PAGE, electrobotted, and probed with an anti-FlgE antibody in Western blot analysis. Whereas FlgE levels were high in the wild-type and flhK mutant pellets, FlgE was severely reduced in the flhO and flhP mutant backgrounds (Fig. 3C). Instead, FlgE was found at high levels secreted into the supernatant in the mutant backgrounds (Fig. 3C). We conclude that FlgD is required for hook completion. The FlhO and FlhP are expressed from multiple promoters. To characterize the regulation of flhO and flhP, the PflhO promoter region was cloned upstream of the lacZ gene encoding β-galactosidase and inserted at an ectopic locus (amyE::PflhO-lacZ). The PflhO promoter region was proficient for driving lacZ gene expression, supporting the presence of a functional promoter (Table 2). Some genes required for flagellar assembly are expressed under the control of σD, the alternative sigma factor that also expresses flagellin. The PflhO promoter appeared to be σD dependent, as mutation of the sigD gene encoding σD abolished expression of the PflhO-lacZ reporter (Table 2). Furthermore, PflhO expression increased when the gene encoding FlgM, the anti-sigma factor that antagonizes σD activity, was mutated (Table 2). Finally, within the 500-bp region upstream of flhO there was a candidate promoter sequence, TTTA[15-bp spacer]TCCTATAT, that differed at four positions (underlined) from the predicted σD consensus binding site TAAA[14 to 16-bp spacer]GCCGATAT (26). We conclude that upstream of flhO and flhP is a promoter, PflhO, that is σD dependent.

If the expression of flhO and flhP is under the control of a σD-dependent promoter and FlhO and FlhP are required for hook assembly, then σD might also be required for hook assembly. A cell mutant in the gene encoding σD, sigD, grew in long chains due to the failure to express σD-dependent autolysins and was defective for filament biosynthesis due to the fact that hag is under exclusive σD control (Fig. 2A) (12, 53). When a sigD mutant encoding the FlgET123C allele was stained with a maleimide dye and observed by fluorescence microscopy, faint puncta could be detected (Fig. 2B). Likewise, near-wild-type levels of FlgE protein were found in the cell pellet of a sigD mutant (Fig. 3C). We conclude that σD is not essential for hook assembly.

If σD is not required for hook assembly, then σD must not be absolutely required for the expression of FlhO and FlhP. We hypothesized that flhO and flhP must be expressed from one or more additional promoters besides PflhO. To detect additional promoter activity, the lacZ gene was integrated downstream of flhP at the native site in wild-type cells and in cells with either sigD or flgM mutated. Wild-type cells expressed the native-site flhP::lacZ inte-
grant at roughly the same level as the ectopically integrated \(P_{flhO-lacZ}\) fusion (Table 2). Likewise, expression of \(flhP\P_{O-lacZ}\) decreased in the \(sigD\) mutant and increased in the \(flgM\) mutant (Table 2). Unlike that of the \(P_{flhO-lacZ}\) fusion, however, expression of \(flhP\P_{O-lacZ}\) was not abolished in the absence of \(\sigma^\beta\), and a low 4 Miller units of activity was detected. We conclude that a \(\sigma^\beta\)-independent promoter is present upstream of \(flhP\), and we infer that the low level of expression from this promoter is sufficient to support hook assembly in a \(sigD\) mutant. We infer that it is important for cells to be able to complete hook assembly by a \(\sigma^\beta\)-independent mechanism because hook completion is necessary to activate \(\sigma^\beta\) by antagonizing FlgM (Fig. 5).

**DISCUSSION**

The hook is a critical component of the flagellum. The hook (i) connects the filament to the basal body, (ii) acts as a universal joint to change the angle of rotation that is transmitted to the filament, (iii) serves as a secretion conduit and polymerization platform for filament proteins, (iv) instructs a change in specificity of the secretion apparatus when completed, and (v) indirectly controls late-class flagellar gene transcription. Finally, in *B. subtilis*, hook completion has recently been shown to be required to relieve translation inhibition on the flagellin transcript (57). Despite its importance, the hook has been relatively poorly studied in *B. subtilis*, and three different proteins that are homologous to the hook structural subunit of *S. enterica* are encoded in the *B. subtilis* genome. Here we introduced a unique cysteine residue into the FlgE primary sequence such that FlgE could be fluorescently labeled, an approach that has proved successful for labeling the filament (8, 68). We found that FlgE formed hooks that appeared as puncta when stained for fluorescence microscopy, and we support the inference that FlgE is the primary hook structural subunit (39). Furthermore, we used hook staining to demonstrate that FliK was the *B. subtilis* hook length regulator and that the FlgD hook cap and the FlgE homologs of unknown function, FlhO and FlhP, were required for hook assembly.

The gene predicted to encode FliK in *B. subtilis* was shown to control flagellar hook length, because when fliK was mutated, long FlgE-containing structures resembling polyhooks were observed (Fig. 2B; see Fig S1 in the supplemental material). FliK is thought to act like a molecular ruler during secretion, where the length of the FliK primary sequence is proportional to the length of the hook (34, 65). The hook of *S. enterica* is approximately 55 nm long, and the FliK primary sequence is 405 amino acids (15, 27). The hook of *B. subtilis* is approximately 71 nm long, and the FliK primary sequence is 487 amino acids; i.e., they are 129% and 120% longer, respectively, than their *S. enterica* counterparts (15, 39). Although the conservation between FliK homologs is poor, the two proteins nonetheless seem to function similarly, with lengths correlated to that of the hook they control.

FliD, FlhO, and FlhP, are required for hook polymerization such that in their absence, FlgE is secreted into the supernatant. The role of FlgD is likely similar to that in *S. enterica*, where FlgD is loaded on the end of the rod and ushers FlgE subunits into the extending hook complex. Two models could account for the requirement of FlhO and FlhP (Fig. 6). In the first model, FlhO and FlhP form the distal rod (Fig. 6A). The flagellar rod of *S. enterica* is complex and consists of four proteins, FlgB, FlgC, FlgF, and FlgG (28). FlgB and FlgC form the proximal rod, with FlgB likely adjacent to the FliF/FliE basal body complex (50). FlgE and FlgG form the distal rod, with FlgG likely being the most distal subunit involved in outer membrane penetration (13). In *B. subtilis*, the genes encoding the FlgB and FlgC putative proximal rod proteins are encoded at the 5′ end of the long *fla-che* operon directly adjacent to the genes encoding FliF and FliE (Fig. 1B) (74). FlhO and FlhP could be the distal rod proteins, because the hook and rod classes are evolutionarily related and difficult to distinguish at the sequence level (43, 61, 74). Further, FlhO and FlhP mutants may secrete FlgE into the supernatant like a FlgD mutant because in their absence, FlgD might be unable to load (Fig. 6). We note that mutations in the distal rod of *S. enterica* do not cause FlgE to be accumulated in the supernatant and instead cause secretion of FlgE to the periplasm, where FlgE is proteolytically degraded (9, 42, 59). Thus, in *B. subtilis*, mutants defective in the rod and hook cap may resemble one another with respect to FlgE secretion due to the lack of an outer membrane.

In the second model, FlhO and FlhP could function with FlgD to form an extended cap (Fig. 6B). FlgD would first load on to the end of the rod as described for *S. enterica*. We note that FlhO and FlhP could still serve as the distal rod in this model and play a role in FlgD cap loading. If FlhO and FlhP are not part of the distal rod, however, they would presumably be the first two proteins ushered underneath FlgD. In either case, once the extended cap is assembled, FlgE subunits would be polymerized, not underneath the FlgD protein but underneath the entire FlgD/FlhO/FlhP complex. The distinction between the distal rod and extended cap models therefore lies in where FlgE is being inserted, either distal or proximal to FlhO and FlhP, respectively. We tend to favor a model in which FlhO and FlhP form the distal rod but do not serve as an extended cap, as this model is more parsimonious, requires fewer changes in insertion points, is supported by the homology of the proteins involved, and is more consistent with the *S. enterica* paradigm. We note that if FlhO and FlhP do not form the distal rod, then no other rod-hook homologs are encoded in the chromosome and therefore the *B. subtilis* rod may be simpler than the rod assembled by *S. enterica*.

Although cells with *flhO* and *flhP* mutated resembled cells with *flgD* mutated in nearly all respects, the *flgD* mutant had one additional phenotype. A cell-associated fluorescent haze with rare puncta was observed when cells encoding the FlgE<sup>122R</sup>-GFP allele at an
ectopic site simultaneously had mutations in flgD and flgE and were labeled with a maleimide stain. The fluorescent puncta in this case may either represent nonspecific aggregation of FlgE protein on the cell surface or represent partially assembled hooks. If the puncta represent hooks, the hooks presumably remain incomplete due to the failure of the flgD mutant to assemble flagellar filaments (Fig. 2A). The haze of fluorescence suggests that in the absence of FlgD, FlgE was being sequestered at a high level and retained nonspecifically on the cell surface, similar to the surface retention of Yop proteins that occurs during artificial high-level secretion in Yersinia (47). Why the flgD mutant would result in expression or secretion of FlgE at higher levels than either the flhO or flhP mutant is unknown, but this may suggest that FlgD has an additional regulatory effect on FlgE expression in B. subtilis. We note that a regulatory connection between the two proteins has been previously reported, inasmuch as the translation of FlgE protein was impaired by deletions in the flgD gene of S. enterica (42).

The structural completion of the hook has regulatory consequences. Hook completion is required to antagonize FlgM and thereby activate σ^D^-dependent gene expression. Although P_{fiber} promoter that expresses FlhO and FlhP, is a σ^D^-dependent and FlhO and FlhP are required for hook synthesis, we found that a sigD mutant was nonetheless proficient for forming flagellar hooks (Table 2 and Fig. 2B). The ability to form hooks in the absence of σ^D^-is likely due to a low level of flhO and flhP transcription originating from an upstream promoter or incomplete termination of transcript from the upstream gene, mbl (Table 2). Suboptimal expression of FlhO and FlhP may account for the faint FlgE^T123C puncta and the enhanced secretion of FlgE into the supernatant of the fla-che operon (14).

The hook is a critical structural intermediate in flagellar assembly; its length is regulated, and its completion governs subsequent events via autocleavage mechanism. J. Bacteriol. 190:5775–5784.


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