

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 σ^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, 63, 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 secre-

tion 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 σ^{28} (also called σ^F or FliA) (33, 58). Prior to completion of the hook-basal body complex, FlgM accumulates in the cytoplasm and inhibits σ^{28} activity. Once the hook is complete, FlgM is secreted and σ^{28} 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 σ^{28} homolog σ^D (SigD), which directs flagellin expression (7, 10, 21, 54). In addition, σ^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 *fliK* 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

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high abundance in biochemical preparations of purified *B. subtilis* hook-basal 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-basal 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 σ^D -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 culture was harvested at an OD₆₀₀ of 0.5 to 2.0 and washed once in 1.0 ml PBS. The suspension was pelleted, resuspended in 50 µl of PBS containing 5 µg/ml Alexa Fluor 488 C₅ maleimide (Molecular Probes), and incubated for 5 min at room temperature (8). Cells were then washed twice with 500 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 fluorescent 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

TABLE 1 Strains

Strain	Genotype
3610	Wild type
DK17	$\Delta flgDE$ <i>amyE::P_{fla-che}-flgE^{T123C} cat</i>
DK22	<i>flhP</i> Ω <i>lacZ cat</i>
DK23	<i>sigD::tet flhP</i> Ω <i>lacZ cat</i>
DK24	<i>flgM::tet flhP</i> Ω <i>lacZ cat</i>
DS793	<i>amyE::P_{hag}-lacZ cat</i>
DS1916	<i>amyE::P_{hag}-hag^{T209C} spec (8)</i>
DS3159	<i>sigD::tet amyE::P_{hag}-hag^{T209C} spec</i>
DS4536	$\Delta fliK$
DS4681	$\Delta flgE$
DS5161	$\Delta flhO$
DS5775	<i>amyE::P_{lytF}-lacZ cat</i>
DS5776	<i>amyE::P_{flhO}-lacZ cat</i>
DS5806	<i>flhP::kan amyE::P_{lytF}-lacZ cat</i>
DS5807	$\Delta flhO$ <i>amyE::P_{lytF}-lacZ cat</i>
DS5808	$\Delta flgE$ <i>amyE::P_{lytF}-lacZ cat</i>
DS5809	$\Delta fliK$ <i>amyE::P_{lytF}-lacZ cat</i>
DS5821	<i>flhP::kan amyE::P_{hag}-lacZ cat</i>
DS5822	$\Delta fliK$ <i>amyE::P_{hag}-lacZ cat</i>
DS5823	$\Delta flgE$ <i>amyE::P_{hag}-lacZ cat</i>
DS5824	$\Delta flhO$ <i>amyE::P_{hag}-lacZ cat</i>
DS5894	$\Delta flhP$ <i>amyE::P_{hag}-hag^{T209C} spec</i>
DS5895	$\Delta fliK$ <i>amyE::P_{hag}-hag^{T209C} spec</i>
DS5896	$\Delta flgE$ <i>amyE::P_{hag}-hag^{T209C} spec</i>
DS5897	$\Delta flhO$ <i>amyE::P_{hag}-hag^{T209C} spec</i>
DS5944	$\Delta flhO$ <i>thrC::P_{flhO}-flhO mls</i>
DS6388	$\Delta flgE$ <i>amyE::P_{D-3^P} fla-che-flgE cat</i>
DS6555	$\Delta flgD$
DS7006	<i>thrC::P_{lytF}-CFP mls amyE::P_{hag}-YFP cat</i>
DS7351	$\Delta flhP$
DS7360	$\Delta flhP$ <i>amyE::P_{flhO}-flhP cat</i>
DS7671	<i>amyE::P_{D-3^P} fla-che-flgE^{T123C} cat</i>
DS7673	$\Delta flgE$ <i>amyE::P_{D-3^P} fla-che-flgE^{T123C} cat</i>
DS7770	<i>flgM::tet amyE::P_{hag}-lacZ cat</i>
DS8037	$\Delta fliK$ <i>amyE::P_{D-3^P} fla-che-fliK cat</i>
DS8704	$\Delta fliK$ <i>thrC::P_{lytF}-CFP mls amyE::P_{hag}-YFP cat</i>
DS8705	$\Delta flgE$ <i>thrC::P_{lytF}-CFP mls amyE::P_{hag}-YFP cat</i>
DS8706	$\Delta flhO$ <i>thrC::P_{lytF}-CFP mls amyE::P_{hag}-YFP cat</i>
DS8707	$\Delta flhP$ <i>thrC::P_{lytF}-CFP mls amyE::P_{hag}-YFP cat</i>
DS8795	<i>flgM::tet amyE::P_{lytF}-lacZ cat</i>
DS8800	<i>flgM::tet flhP::kan amyE::P_{lytF}-lacZ cat</i>
DS8801	<i>flgM::tet flhO amyE::P_{lytF}-lacZ cat</i>
DS8802	<i>flgM::tet flgE amyE::P_{lytF}-lacZ cat</i>
DS8803	<i>flgM::tet flhP::kan amyE::P_{hag}-lacZ cat</i>
DS8804	<i>flgM::tet flhP::kan amyE::P_{hag}-lacZ cat</i>
DS8805	<i>flgM::tet flhP::kan amyE::P_{hag}-lacZ cat</i>
DS8806	<i>flgM::tet flgE amyE::P_{hag}-lacZ cat</i>
DS8807	<i>flgM::tet flhO amyE::P_{hag}-lacZ cat</i>
DS8808	$\Delta fliK$ $\Delta flgE$ <i>amyE::P_{D-3^P} fla-che-flgE^{T123C} cat</i>
DS8839	$\Delta flhO$ $\Delta flgE$ <i>amyE::P_{D-3^P} fla-che-flgE^{T123C} cat</i>
DS8840	$\Delta flhP$ $\Delta flgE$ <i>amyE::P_{D-3^P} fla-che-flgE^{T123C} cat</i>
DS9840	$\Delta flgD$ <i>amyE::P_{hag}-lacZ cat</i>
DS9841	$\Delta flgD$ <i>amyE::P_{lytF}-lacZ cat</i>
DS9852	<i>flgM::tet flgD amyE::P_{hag}-lacZ cat</i>
DS9853	<i>flgM::tet flgD amyE::P_{lytF}-lacZ cat</i>
DS9892	<i>sigD::tet flgE amyE::P_{D-3^P} fla-che-flgE^{T123C} cat</i>
DS9893	$\Delta flgD$ <i>amyE::P_{hag}-hag^{T209C} spec</i>
DS9894	$\Delta flgD$ <i>amyE::P_{hag}-YFP cat thrC::P_{lytF}-CFP mls</i>
DS9915	<i>sigD::tet amyE::P_{flhO}-lacZ cat</i>
DS9916	<i>flgM::tet amyE::P_{flhO}-lacZ cat</i>
DS9920	$\Delta flgD$ <i>amyE::P_{D-3^P} fla-che-flgD cat</i>

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 $\Delta flgE$ in-frame markerless deletion construct pDP306, the region upstream of *flhP* 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 digested with XhoI and BamHI. The two fragments were then simultaneously 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

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 $\Delta flgE$ allele.

To generate the $\Delta flhO$ in-frame markerless deletion construct pKB114, the region upstream of *flhO* was PCR amplified using the primer pair 1850/1524 and digested with EcoRI and SalI, and the region downstream of *flhO* was PCR amplified using the primer pair 1525/1526 and digested with SalI and BamHI. The two fragments were then simultaneously ligated into the EcoRI and BamHI sites of pMiniMAD. pKB114 was integrated into the *B. subtilis* PY79 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 $\Delta flhO$ allele.

To generate the $\Delta flhP$ 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 SalI. The two fragments were then simultaneously ligated into the EcoRI and SalI sites of pMiniMAD. pCC12 was integrated into the *B. subtilis* PY79 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 $\Delta flhP$ allele.

To generate the $\Delta fliK$ in-frame markerless deletion construct pKB93, the region upstream of *fliK* was PCR amplified using the primer pair 1387/1388 and digested with EcoRI and XhoI, and the region downstream of *fliK* was PCR amplified using the primer pair 1389/1390 and digested with SalI and BamHI. The two fragments were then simultaneously ligated into the EcoRI and BamHI sites of pMiniMAD (XhoI and SalI have compatible ends). pKB93 was integrated into *B. subtilis* PY79 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 $\Delta fliK$ allele.

To generate the $\Delta flgD$ in-frame markerless deletion construct pDP328, the region upstream of *flgD* was PCR amplified using the primer pair 2033/2034 and digested with EcoRI and XhoI, and the region downstream of *flgD* was PCR amplified using the primer pair 2035/2036 and digested with XhoI and BamHI. The two fragments were then simultaneously ligated into the EcoRI and BamHI sites of pMiniMAD. pDP328 was integrated into the *B. subtilis* PY79 genome, transduced into strain 3610, and evicted as described above. Colonies were screened by PCR using primers 2033/2036 to determine which isolate had retained the $\Delta flgD$ allele.

(ii) $\Delta flhP::kan$. The $\Delta flhP::kan$ 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).

(iii) **Complementation constructs.** To generate the $P_{D3P_{fla-che}}flgE$ complementation construct pDP324, the $P_{D3P_{fla-che}}$ promoter was amplified using primer pair 2015/2016 and was digested with EcoRI and XhoI. The *flgE* region was amplified primer pair 2017/2018 and was digested with XhoI and BamHI. The two fragments were ligated simultaneously into the EcoRI and BamHI sites of pDG364 containing the polylinker and the chloramphenicol resistance cassette between the arms of the *amyE* gene (25). To generate the $P_{D3P_{fla-che}}flgE^{T123C}$ allele complementation construct pCC16, site-directed mutagenesis was conducted using the QuikChange II kit (Stratagene) on pDP324 in two sequential steps. First, primer pair 2246/2247 was used to change codon 123 of *flgE* from ACT

(threonine) to TCT. Next, primer pair 2378/2379 was used to change the TCT codon to TGT (cysteine). Sequences were verified by sequencing with primers 1853/1854.

To generate the $P_{D3P_{fla-che}}fliK$ complementation construct pCC14, the $P_{D3P_{fla-che}}$ promoter was amplified using primer pair 2015/2016 and was digested with EcoRI and XhoI. The *fliK* region was amplified using primer pair 2535/2536 and was digested with XhoI and BamHI. The two fragments were ligated simultaneously into the EcoRI and BamHI sites of pDG364.

To generate the $P_{flhO}-flhO$ complementation construct pCC7, the *flhO* gene and DNA immediately upstream were amplified using primer pair 1251/1681. The PCR product was digested with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of pDG1664 containing the polylinker and the erythromycin resistance cassette between the arms of the *thrC* gene (25).

To generate the $P_{flhO}-flhP$ complementation construct pCC13, the P_{flhO} promoter was PCR amplified using primer pair 1251/2344, and the *flhP* gene was amplified using primer pair 2344/2345. The P_{flhO} -containing PCR product was digested with EcoRI and XhoI, the *flhP*-containing product was digested with XhoI and BamHI, and the two digested fragments were cloned simultaneously into the EcoRI and BamHI sites of pDG364.

To generate the $P_{D3P_{fla-che}}flgD$ complementation construct pDP403, the $P_{D3P_{fla-che}}$ promoter was amplified using primer pair 2460/2461 and was digested with EcoRI and XhoI. The *flgD* region was amplified using primer pair 3163/3164 and was digested with XhoI 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 P_{flhO} 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 *flhP* Ω *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 BamHI, and cloned into the EcoRI and BamHI sites of plasmid pMal-p2X (New England BioLabs).

SPP1 phage transduction. To 0.2 ml of dense culture grown in TY broth (LB broth supplemented after autoclaving with 10 mM MgSO₄ and 100 μ M MnSO₄), serial dilutions of SPP1 phage stock were added and statically incubated for 15 min at 37°C. To each mixture, 3 ml TYSA (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 conical tube, vortexed, and centrifuged at 5,000 $\times$ g for 10 min. The supernatant was treated with 25 μ g/ml (final concentration) DNase before being passed through a 0.45- μ m 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 μ l of SPP1 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 $\times$ 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 μ l, and the reaction was started with 100 μ l of 4 mg ml⁻¹ 2-nitrophenyl β -D-galactopyranoside in Z buffer and stopped with 250 μ l of 1 M Na₂CO₃. The OD₄₂₀ of the reaction mixture was measured, and the β -galactosidase-specific activity was calculated according to the equation [OD₄₂₀/(time $\times$ OD₆₀₀)] $\times$ dilution factor $\times$ 1,000.

FlgE protein purification. The MBP-FlgE fusion protein expression vector pCC10 was transformed into Rosetta-gami *E. coli*, grown to an OD₆₀₀ of $\sim$ 0.8 in 750 ml of Luria-Bertani broth supplemented with 0.2% glucose and 100 μ g/ml ampicillin, induced with 0.2 mM IPTG (isopropyl- β -D-thiogalactopyranoside) and grown at 16°C for 12 h. Cells were pelleted, resuspended in column buffer (20 mM Tris-HCl, 400 mM NaCl, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride [PMSF]), disrupted on a French press, and lysed by sonication. Lysed cells were ultracentrifuged at 20,000 $\times$ g for 40 min. Cleared supernatant was combined with amylose resin (New England Biolabs) and incubated overnight with gentle rocking at 4°C. The resin-lysate mixture was poured onto a 1-cm separation column (Bio-Rad), and the resin was allowed to pack and was washed with column buffer. MBP-FlgE fusion protein bound to the resin was then eluted using column buffer supplemented with 10 mM maltose. Elution products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue stained to verify purification of the MBP-FlgE fusion, and pure fractions were dialyzed into dialysis buffer (300 mM NaCl, 0.5 mM EDTA, 10% glycerol, 50 mM Na₃PO₄) and stored at -20°C.

FlgE antibody preparation. One 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.

Western blotting. *B. subtilis* strains were grown in LB to an OD₆₀₀ of $\sim$ 1.0, and 1 ml was harvested by centrifugation, resuspended to an OD₆₀₀ of 10 in lysis buffer (20 mM Tris [pH 7.0], 10 mM EDTA, 1 mg/ml lysozyme, 10 μ g/ml DNase I, 100 μ g/ml RNase I, 1 mM PMSF), and incubated for 30 min at 37°C. Ten microliters of lysate was mixed with 2 μ l 6 $\times$ SDS loading dye. Samples were separated by 12% SDS-PAGE. The proteins were electroblotted onto nitrocellulose and developed with anti-FlgE (1:20,000 dilution), anti-SigA (1:40,000 dilution), or anti-Hag (1:80,000 dilution) primary antibody and a 1:10,000 dilution of secondary antibody (horseradish peroxidase [HRP]-conjugated goat anti-rabbit immunoglobulin G). Immunoblots were developed using the Immun-Star HRP developer kit (Bio-Rad).

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 swarming 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 $P_{D-3}P_{fla-che}$ promoter of the *fla-che* operon and inserted at the ectopic *amyE* site (*amyE::P_{D-3}P_{fla-che}-flgE*) (18, 70). To complement the *flhO* mutation, the *flhO* gene and 500 bp immediately upstream (P_{flhO}) were inserted at the *amyE* site (*amyE::P_{flhO}-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 P_{flhO} promoter region and inserted at the *amyE* site (*amyE::P_{flhO}-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 mutation of the putative hook proteins affected 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::P_{hag}-hag^{T209C}*) (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 filaments (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 (P_{hag}) was fused to the gene encoding yellow fluorescent protein and inserted at an ectopic site (*amyE::P_{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

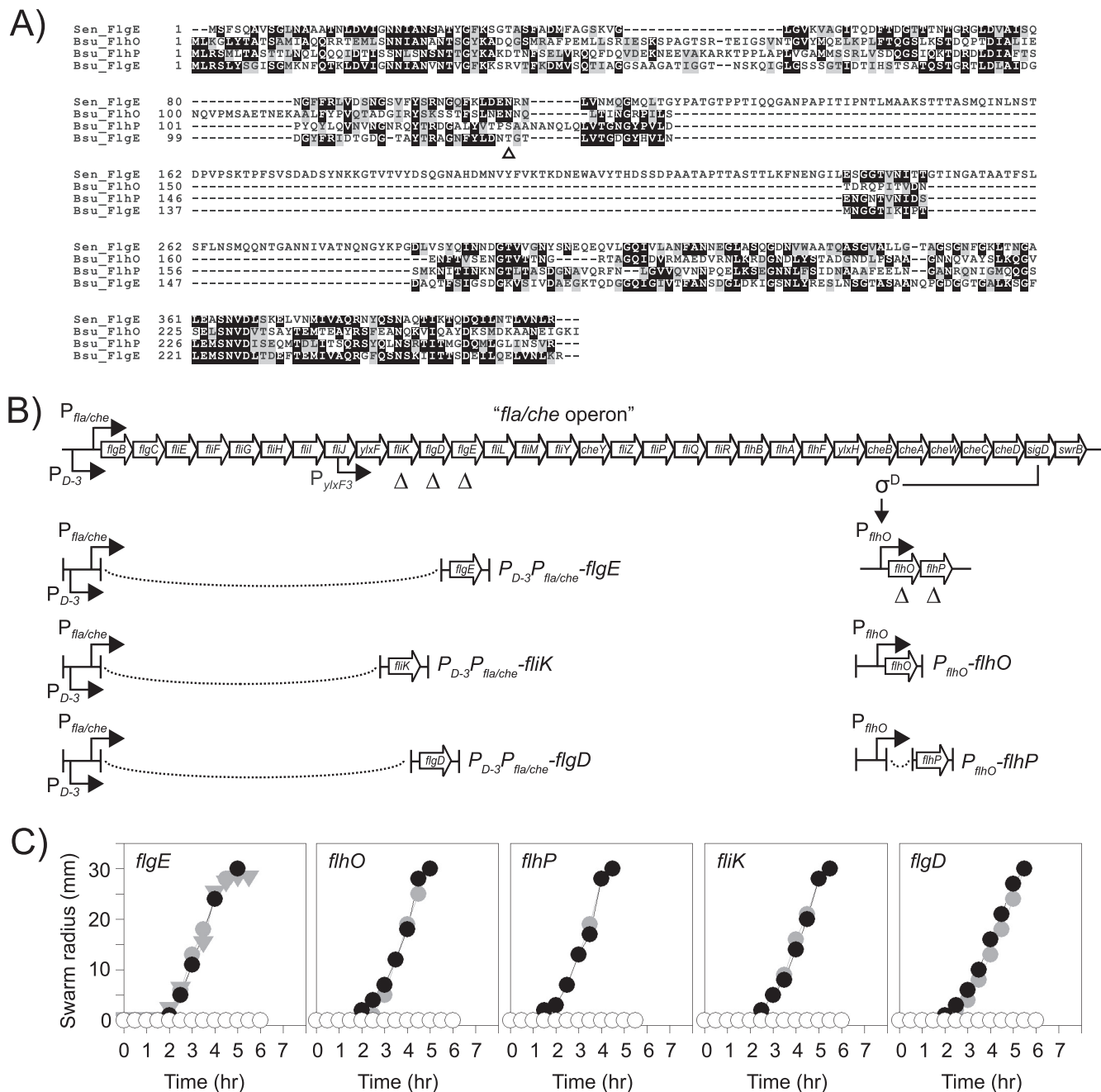


FIG 1 *B. subtilis* encodes three proteins homologous to the hook structural protein, and each is required for motility. (A) Multiple-sequence alignment of FlgE from *Salmonella enterica* serovar Typhimurium (Sen) and FlgE, FlhO, and FlhP from *Bacillus subtilis* (Bsu). (B) Genetic map of regions carrying the *flgE*, *flhO*, *flhP*, and *fliK* genes, including the locations of mutations used in the study and the organization of ectopic complementation constructs. Note that *flgE* and *fliK* are encoded within the long, 25-kb *fla-che* operon, whereas *flhO* and *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 *flgE*^{T123C} allele. The following strains were used to generate the swarm expansion assays: 3610 (wild type), DS4681 (Δ *flgE*), DS6388 (Δ *flgE amyE::P_{D-3}P_{fla-che}-flgE*), DS5161 (Δ *flhO*), DS5944 (Δ *flhO thrC::P_{flhO}-flhO*), DS7351 (Δ *flhP*), DS7360 (Δ *flhP amyE::P_{flhO}-flhP*), DS4536 (Δ *fliK*), DS8037 (Δ *fliK amyE::P_{D-3}P_{fla-che}-fliK*), DS7673 (Δ *flgE amyE::P_{D-3}P_{fla-che}-flgE*^{T123C}), DS6555 (Δ *flgD*), and DS9920 (Δ *flgD amyE::P_{D-3}P_{fla-che}-flgD*).

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 σ^D (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_{lytF}-CFP*). Cells with *flgE*, *flhO*, and *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 σ^D sigma factor.

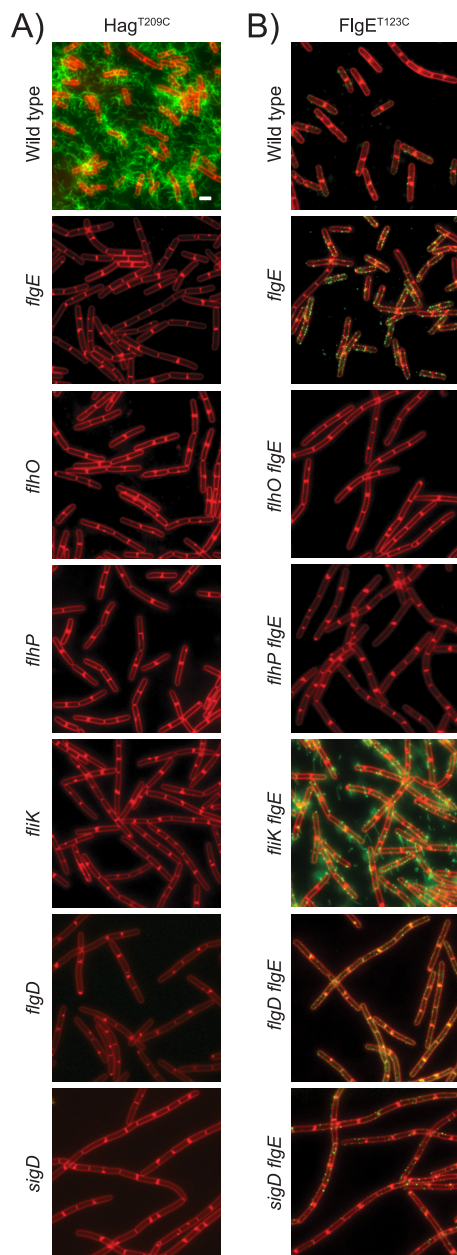


FIG 2 FlgE is the primary structural subunit of the hook. (A) Fluorescence micrographs in which membranes have been stained with FM4-64 (false colored in red) overlaid with the Hag^{T209C} allele of flagellin that was stained with a maleimide dye (false colored in green). Scale bar, 2 μ m. The following strains were used to generate this panel: wild type (DS1916), *flgE* (DS5896), *flhO* (DS5897), *flhP* (DS5894), *fliK* (DS5895), *flgD* (DS9893), and *sigD* (DS3159). (B) Fluorescence micrographs in which membranes have been stained with FM4-64 (false colored red) overlaid with the FlgE^{T123C} allele of the hook that was stained with a maleimide dye (false colored in green). Scale bar is 2 μ m. The following strains were used to generate this panel: DS7671 (wild type), DS7673 (*flgE*), DS8839 (*flhO flgE*), DS8840 (*flhP flgE*), DS8808 (*fliK flgE*), DK17 (*flgD flgE*), and DS9892 (*sigD flgE*). See Fig. S1 in the supplemental material for an enlarged panel of polyhooks in the Δ *fliK* strain.

The sigma factor σ^D is known to be antagonized by binding directly to the anti-sigma factor FlgM (7). To determine whether FlgM was antagonizing σ^D in the *flgE*, *flhO*, and *flhP* mutants, σ^D -dependent gene expression was measured quantitatively by

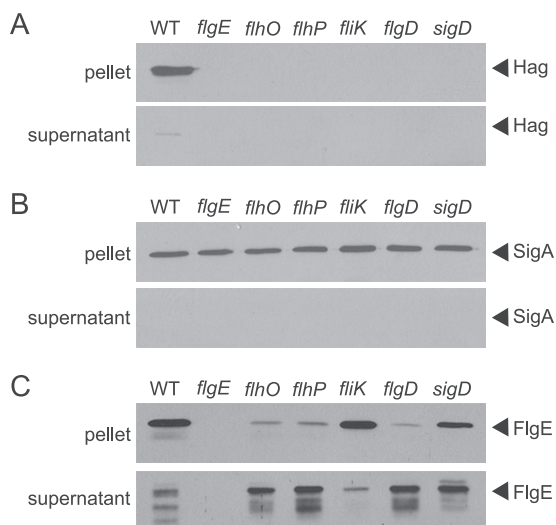


FIG 3 FlgE is secreted into the supernatant of cells with *FlhO*, *FlhP*, and *FlgD* mutated. Cells were grown to mid-log phase, and cell pellets and supernatants were separated. Cell pellets were lysed, and supernatants were concentrated by TCA precipitation of dissolved proteins. Lysates and supernatants were separately resolved by SDS-PAGE, electroblotted, and probed with anti-Hag antibody (A), anti-SigA antibody (B), or anti-FlgE antibody (C). All three panels were generated using the same samples. The following strains were used to generate the samples: 3610 (wild type [WT]), DS4681 (*flgE*), DS5161 (*flhO*), DS7351 (*flhP*), DS4536 (*fliK*), DS6555 (*flgD*), and DS323 (*sigD*).

generating transcriptional fusions of the gene encoding β -galactosidase fused to either the P_{hag} or P_{lytF} promoter. Mutation of *flgE*, *flhO*, or *flhP* reduced the expression of both P_{hag} and P_{lytF} 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^D -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 (FlgE^{T123C}) in the *flgE* complementation construct (*amyE::P_{D-3P_{fla-che}}-flgE^{T123C}*) (Fig. 1A). The ectopically integrated FlgE^{T123C} complementation construct was functional and restored swarming motility to a Δ *flgE* deletion mutation (Fig. 1C). Furthermore, fluorescence microscopy of the *flgE^{T123C}* 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 Δ *flgE*

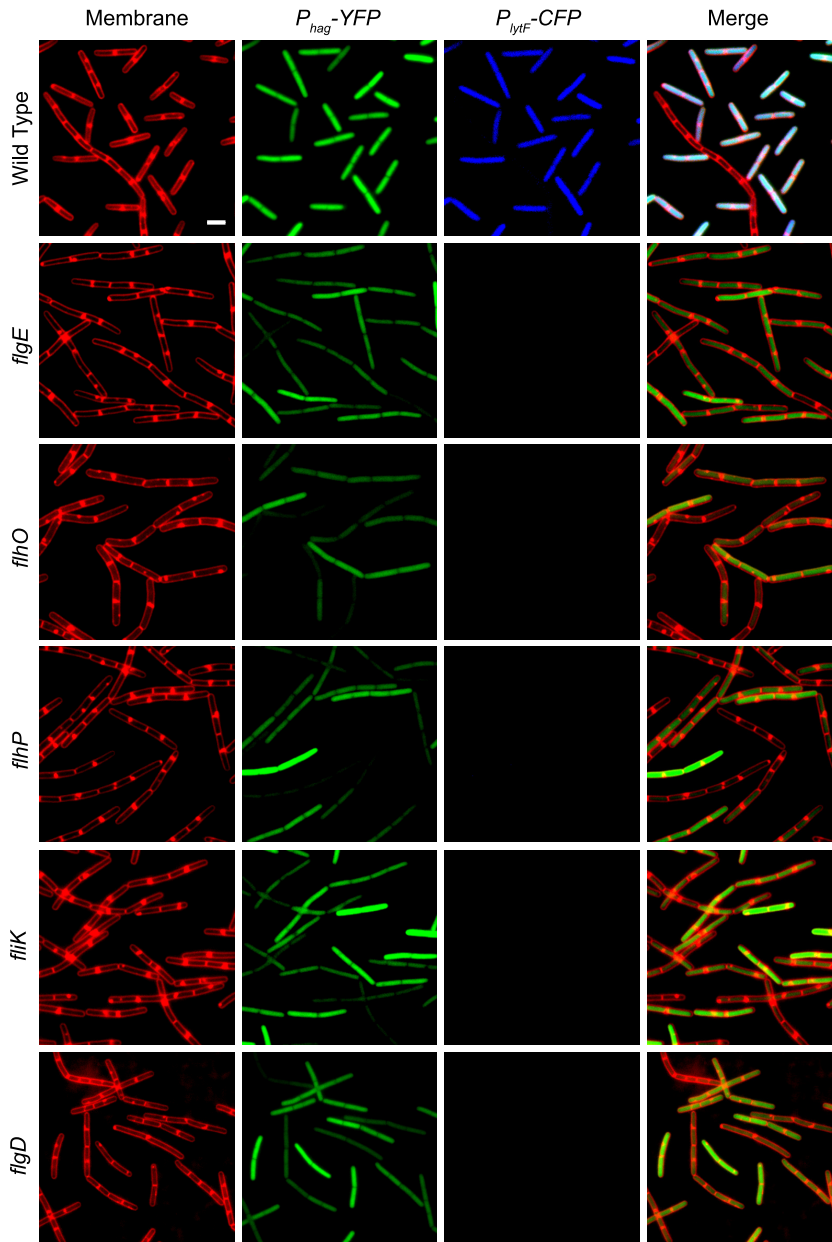


FIG 4 Cells defective in hook completion grow in short chains. Fluorescence micrographs of cells of the indicated genotype were membrane stained with FM4-64 (false colored in red) and contained a reporter for flagellin (P_{hag} -YFP, false colored in green) and autolysin (P_{lytF} -CFP, false colored in blue) expression. Merge indicates an overlay of the three channels of fluorescence. Scale bar, 2 μ m. The following strains were used to generate this figure: DS7006 (wild type), DS8705 ($\Delta flgE$), DS8706 ($\Delta flhO$), DS8707 ($\Delta flhP$), DS8704 ($\Delta fliK$), and DS9894 ($\Delta flgD$).

mutant complemented by the ectopic $flgE^{T123C}$, presumably due to the lack of competition between the stainable and wild-type alleles. We conclude that $FlgE^{T123C}$ was functional for supporting motility and could be fluorescently stained.

The puncta observed when staining the $FlgE^{T123C}$ 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 *FliK* because *S. enterica fliK* mutants fail to limit hook length, resulting in elongated “polyhooks” (27). An in-frame markerless deletion of *B. subtilis fliK* (Fig. 1B) abolished swimming and swarming motility (Fig. 1C), abolished flagellar

filament assembly (Fig. 2A), reduced flagellin protein levels (Fig. 3A), and reduced σ^D -dependent gene expression in a *FlgM*-dependent manner (Fig. 4 and 5). Thus, mutation of *fliK* appeared to phenocopy mutation of *flgE*, *flhO*, or *flhP*. When the $FlgE^{T123C}$ allele was expressed and fluorescently labeled in a $\Delta fliK \Delta flgE$ double mutant background, elongated structures consistent with polyhooks were observed (Fig. 2B; see Fig. S1 in the supplemental material). We conclude that $FlgE^{T123C}$ puncta represent flagellar hooks, that *FlgE* is a primary component of the hook structure, and that *FliK* in *B. subtilis* is a hook length regulator.

To determine the roles of *FlhO* and *FlhP* in hook assembly, the $FlgE^{T123C}$ allele was introduced into $\Delta flhO \Delta flgE$ or $\Delta flhP \Delta flgE$

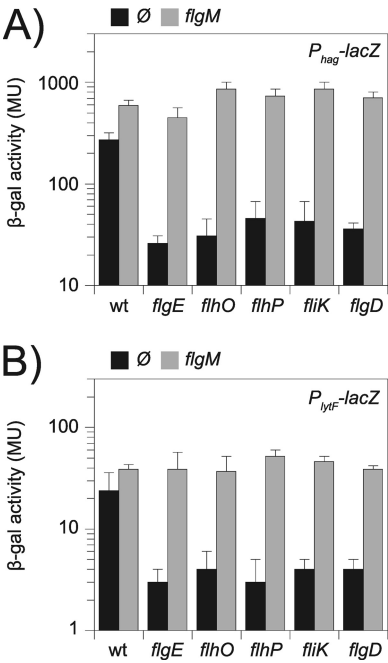


FIG 5 Cells defective in hook completion are reduced for expression of flagellin (P_{hag}) and LytF autolysin (P_{lytF}) in a FlgM-dependent manner. Results of β -galactosidase assays of strains of the indicated genotype that express either a P_{hag} -lacZ reporter (A) or a P_{lytF} -lacZ reporter (B) are shown. In each panel, black bars represent the genotype indicated on the x axis ($\emptyset$) and gray bars represent the genotype indicated on the x axis that also contains a mutation in *flgM* (*flgM*). Error bars are the standard deviations from three replicates. The raw data used to generate this figure are included in Table S3 in the supplemental material. MU, Miller units.

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). One 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, electroblotted, and probed with an anti-FlgE antibody in Western blot analysis. Whereas FlgE levels were high in the wild-type and *fliK* 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 *flhO* and *flhP* mutants relative to the other backgrounds (Fig. 3C). We conclude that FlgE is secreted but not polymerized in the absence of FlhO and FlhP.

Accumulation of FlgE in the supernatant of cells lacking FlhO and FlhP was reminiscent of the phenotype reported for cells lacking the FlgD hook cap in *S. enterica* (59). The *B. subtilis* *flgD* gene (formerly *ylxG*) is predicted to encode a homolog of the hook capping protein FlgD from *S. enterica* and is located immediately upstream of the *flgE* gene in the *B. subtilis* *fla-che* operon (Fig. 1B). An in-frame markerless deletion of *flgD* abolished motility and was ectopically complemented by inserting the *flgD* gene fused to the $P_{D-3}P_{fla-che}$ promoter at the ectopic *amyE* site (*amyE::P_{D-3}P_{fla-che}-flgD*) (Fig. 1B and C). Like other mutants defective in hook completion, the *flgD* mutant grew in short chains (Fig. 4), and showed a 10-fold reduction in σ^D -dependent gene expression that was dependent on FlgM (Fig. 5). Furthermore, the *flgD* mutant

secreted FlgE hook protein abundantly into the supernatant, as had been observed with *S. enterica* *flgD* mutants (Fig. 3) (53). Thus, with respect to FlgE secretion, cells with *flhO* and *flhP* mutated closely resembled cells with *flgD* mutated in *B. subtilis*. When a *flgD flgE* double mutant that encoded an ectopically integrated FlgE^{T123C} allele was stained with a maleimide dye and observed by fluorescence microscopy, fluorescence was observed as a haze over the cell with occasional puncta (Fig. 2B). If the puncta represent hooks, we infer that the hooks fail to be functionally complete, as the *flgD* mutant was defective in assembling flagellar filaments (Fig. 2A). We conclude that FlgD is required for hook completion.

FlhO and FlhP are expressed from multiple promoters. To characterize the regulation of *flhO* and *flhP*, the P_{flhO} promoter region was cloned upstream of the *lacZ* gene encoding β -galactosidase and inserted at an ectopic locus (*amyE::P_{flhO}-lacZ*). The P_{flhO} 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 σ^D , the alternative sigma factor that also expresses flagellin. The P_{flhO} promoter appeared to be σ^D dependent, as mutation of the *sigD* gene encoding σ^D abolished expression of the P_{flhO} -lacZ reporter (Table 2). Furthermore, P_{flhO} expression increased when the gene encoding FlgM, the anti-sigma factor that antagonizes σ^D 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 σ^D consensus binding site TAAA[14- to 16-bp spacer]GCCGATAT (26). We conclude that upstream of *flhO* and *flhP* is a promoter, P_{flhO} , that is σ^D dependent.

If the expression of *flhO* and *flhP* is under the control of a σ^D -dependent promoter and FlhO and FlhP are required for hook assembly, then σ^D might also be required for hook assembly. A cell mutated in the gene encoding σ^D , *sigD*, grew in long chains due to the failure to express σ^D -dependent autolysins and was defective for filament biosynthesis due to the fact that *hag* is under exclusive σ^D control (Fig. 2A) (12, 53). When a *sigD* mutant encoding the FlgE^{T123C} allele was stained for 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. 3). We conclude that σ^D is not essential for hook assembly.

If σ^D is not required for hook assembly, then σ^D 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 P_{flhO} . 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-

TABLE 2 P_{flhO} is a σ^D -dependent promoter

Genotype	lacZ expression (Miller units) in strain carrying ^a :	
	<i>amyE::P_{flhO}-lacZ</i>	<i>flhP</i> lacZ
Wild type	51 $\pm$ 2 (DS5776)	46 $\pm$ 4 (DK22)
<i>sigD</i>	1 $\pm$ 0 (DS9915)	4 $\pm$ 1 (DK23)
<i>flgM</i>	107 $\pm$ 5 (DS9916)	93 $\pm$ 9 (DK24)

^a All cultures were grown to an OD₆₀₀ of 1.0 in LB medium. All values are the averages of three replicates $\pm$ standard deviations. Strains used to generate the data are indicated in parentheses.

grant at roughly the same level as the ectopically integrated $P_{flhO-lacZ}$ fusion (Table 2). Likewise, expression of $flhP\Omega lacZ$ decreased in the $sigD$ mutant and increased in the $flgM$ mutant (Table 2). Unlike that of the $P_{flhO-lacZ}$ fusion, however, expression of $flhP\Omega lacZ$ was not abolished in the absence of σ^D , and a low 4 Miller units of activity was detected. We conclude that a σ^D -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 σ^D -independent mechanism because hook completion is necessary to activate σ^D 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.

FlgD, 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). In 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). FlgF and FlgG form

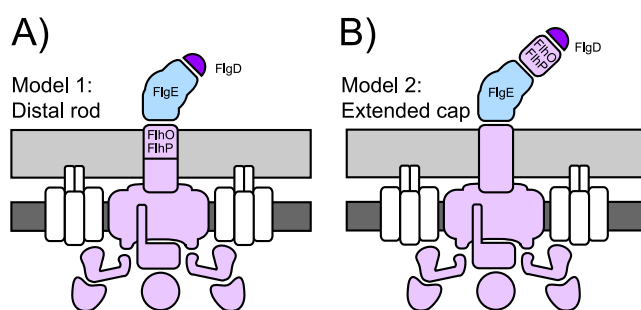


FIG 6 Two models for the function of FlhO and FlhP. (A) FlhO and FlhP may form the distal rod. In this model, FlgD is inserted on the end of FlhO and FlhP, and FlgE subunits are inserted underneath FlgD. (B) FlhO and FlhP may form an extended cap. In this model, FlhO and FlhP either form the distal rod or are the first proteins inserted underneath the FlgD cap. In either case, FlgE subunits are inserted underneath FlhO and FlhP. The basal body structure is shown in pink. The hook (FlgE) is shown in blue. The predicted locations of FlhO and FlhP are indicated. Dark gray indicates membrane. Light gray indicates peptidoglycan.

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^{T123C} 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 secreted 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_{flhO} , the promoter that expresses FlhO and FlhP, is 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, *mbf* (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 *sigD* mutant (Fig. 2A and 3). Furthermore, low levels of FlhO and FlhP may be sufficient for hook completion, as the two proteins were present in purified hook-basal bodies at low abundance (39). The role of σ^D -amplified expression of *flhO* and *flhP* then may be to create a positive feedback loop for the antagonism of FlgM: the more hooks that are completed, the more FlgM is antagonized and the more hooks are made. Similarly, we note that a σ^D -dependent promoter (P_{ybcF3}) was recently identified upstream of the *flhK*, *flgD*, and *flgE* hook genes within the *fla-che* operon (14).

The hook is a critical structural intermediate in flagellar assembly; its length is regulated, and its completion governs subsequent flagellar gene expression. Most studies of flagella have been conducted with Gram-negative bacteria, and we presume that Gram-positive flagella may require different structures and regulatory systems to accommodate the substantial difference in envelope structure. In particular, the Gram-positive basal body has two fewer rings than its *S. enterica* counterpart, as it transits a much thicker layer of peptidoglycan and lacks an outer membrane (15). Although many proteins involved in flagellar assembly are conserved, conservation is poor, and when dealing with such divergent bacteria, it is important to validate sequence-based predictions with structure-function studies. In total, we assign functional roles to five flagellar proteins previously unstudied in *B. subtilis*: the hook (FlgE), the hook length regulator (FlhK), and hook assembly factors FlgD, FlhO, and FlhP. We note that if FlhO and FlhP represent distal rod proteins, they are interesting candidates for the study of flagellar biology specifically in Gram-positive bacteria, as the rod is the structure best situated to accommodate variations in the cell envelope.

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REFERENCES

1. Aizawa S-I, Dean GE, Jones CJ, Macnab RM, Yamaguchi S. 1985. Purification and characterization of the flagellar hook-basal body complex of *Salmonella typhimurium*. J. Bacteriol. 161:836–849.
2. Aizawa S-I, Zhulin IB, Márquez-Magaña L, Ordal GW. 2002. Chemotaxis and motility in *Bacillus subtilis*, p 437–452. In Sonenshein AL, Hoch JA, Losick R (ed), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, DC.
3. Albertini AM, Caramori T, Crabb WD, Scoffone F, Galizzi A. 1991. The *flaA* locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. J. Bacteriol. 173:3573–3579.
4. Antoniewski C, Savelli B, Stragier P. 1990. The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. J. Bacteriol. 172:86–93.
5. Barill D, Caramori T, Galizzi A. 1994. Coupling of flagellin gene transcription to flagellar assembly in *Bacillus subtilis*. J. Bacteriol. 176:4558–4564.
6. Berg HC, Anderson RA. 1973. Bacteria swim by rotating their flagellar filaments. Nature 245:380–382.
7. Bertero MG, Gonzales B, Tarricone C, Cecilian F, Galizzi A. 1999. Overproduction and characterization of the *Bacillus subtilis* anti-sigma factor FlgM. J. Biol. Chem. 274:12103–12107.
8. Blair KM, Turner L, Winkelman JT, Berg HC, Kearns DB. 2008. A molecular clutch disables flagella in the *Bacillus subtilis* biofilm. Science 320:1636–1638.
9. Bonifield HR, Yamaguchi S, Hughes KT. 2000. The flagellar hook protein, FlgE, of *Salmonella enterica* serovar Typhimurium is posttranscriptionally regulated in response to the state of flagellar assembly. J. Bacteriol. 182:4044–4050.
10. Caramori T, Barill D, Nessi C, Sacchi L, Galizzi A. 1996. Role of FlgM in σ^D -dependent gene expression in *Bacillus subtilis*. J. Bacteriol. 178:3113–3118.
11. Carpenter PB, Zuberi AR, Ordal GW. 1993. *Bacillus subtilis* flagellar proteins Flp, FliQ, FliR and FlhB are related to *Shigella flexneri* virulence factors. Gene 137:243–245.
12. Chen R, Guttenplan SB, Blair KM, Kearns DB. 2009. Role of the σ^D -dependent autolysins in *Bacillus subtilis* population heterogeneity. J. Bacteriol. 191:5775–5784.
13. Chevance FFV, et al. 2007. The mechanism of outer membrane penetration by the eubacterial flagellum and implications for spirochete evolution. Genes Dev. 21:2326–2335.
14. Cozy LM, Kearns DB. 2010. Gene position in a long operon governs motility development in *Bacillus subtilis*. Mol. Microbiol. 76:273–285.
15. DePamphilis ML, Adler J. 1971. Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 105:384–395.
16. Erhardt M, Namba K, Hughes KT. 2010. Bacterial nanomachines: the flagellum and type III injectisome. Cold Spring Harbor Perspect. Biol. 2:a000299. doi:10.1101/cshperspect.a000299.
17. Erhardt M, Singer HM, Wee DH, Keener JP, Hughes KT. 2011. An infrequent molecular ruler controls flagellar hook length in *Salmonella enterica*. EMBO J. 30:2948–2961.
18. Estacio W, Santa Anna-Arriola S, Adedipe M, Márquez-Magaña LM. 1998. Dual promoters are responsible for transcription initiation of the *fla-che* operon in *Bacillus subtilis*. J. Bacteriol. 180:3548–3555.
19. Ferris HU, et al. 2005. FlhB regulates ordered export of flagellar components via autocleavage mechanism. J. Biol. Chem. 280:41236–41242.
20. Fraser GM, et al. 2003. Substrate specificity of the type III flagellar protein export in *Salmonella* is controlled by subdomain interactions in FlhB. Mol. Microbiol. 48:1043–1057.
21. Fredrick K, Helmann D. 1996. FlgM is a primary regulator of σ^D activity, and its absence restores motility to a *sinR* mutant. J. Bacteriol. 178:7010–7013.
22. Fujii T, Kato T, Namba K. 2009. Specific arrangement of the α -helical

- coiled coils in the core domain of the bacterial flagellar hook for the universal joint function. *Structure* 17:1485–1493.
23. Gillen KL, Hughes KT. 1991. Molecular characterization of *flgM*, a gene encoding a negative regulator of flagellin synthesis of *Salmonella typhimurium*. *J. Bacteriol.* 173:6453–6459.
 24. Guérout-Fleury AM, Shazand K, Frandsen N, Stragier P. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* 167:335–336.
 25. Guérout-Fleury AM, Frandsen N, Stragier P. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. *Gene* 180:57–61.
 26. Helmann JD, Moran Jr, CP. 2002. RNA polymerase and sigma factors, p 289–312. In Sonenshein AL, Hoch JA, Losick R (ed), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, DC.
 27. Hirano T, Yamaguchi S, Oosawa K, Aizawa S-I. 1994. Roles of FliK and FlhB in determination of flagellar hook length in *Salmonella typhimurium*. *J. Bacteriol.* 176:5439–5449.
 28. Homma M, Kutsukake K, Hasebe M, Iino T, Macnab RM. 1990. FlgB, FlgC, FlgF, and FlgG, a family of structurally related proteins in the flagellar basal body of *Salmonella typhimurium*. *J. Mol. Biol.* 211:465–477.
 29. Hsueh Y-H, et al. 2011. DegU-phosphate activates expression of the anti-sigma factor FlgM in *Bacillus subtilis*. *Mol. Microbiol.* 81:1092–1108.
 30. Hughes KT, Gillen KL, Semon MJ, Karlinsey JE. 1993. Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science* 262:1277–1280.
 31. Iino T. 1969. Polarity of flagellar growth in *Salmonella*. *J. Gen. Microbiol.* 56:227–239.
 32. Iino T, et al. 1988. New unified nomenclature for the flagellar genes of *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* 52:533–535.
 33. Iyoda S, Kutsukake K. 1995. Molecular dissection of the flagellum specific anti-sigma factor, FlgM, of *Salmonella typhimurium*. *Mol. Gen. Genet.* 249:417–424.
 34. Journé L, Agrain C, Broz P, Cornelis GR. 2003. The needle length of bacterial injectisomes is determined by a molecular ruler. *Science* 302:1757–1760.
 35. Kagawa H, Owaribe K, Asakura S, Takahashi N. 1976. Flagellar hook protein from *Salmonella* SJ25. *J. Bacteriol.* 125:68–73.
 36. Karlinsey JE, et al. 2000. Completion of the hook-basal body complex of the *Salmonella typhimurium* flagellum is coupled to FlgM secretion and *fliC* transcription. *Mol. Microbiol.* 37:1220–1231.
 37. Kearns DB, Losick R. 2003. Swarming motility in undomesticated *Bacillus subtilis*. *Mol. Microbiol.* 49:581–590.
 38. Komeda Y, Silverman M, Simon MI. 1978. Identification of the structural gene for the hook subunit protein of *Escherichia coli* flagella. *J. Bacteriol.* 133:364–371.
 39. Kubori T, et al. 1997. Purification and characterization of the flagellar hook-basal body complex of *Bacillus subtilis*. *Mol. Microbiol.* 24:399–410.
 40. Kutsukake K, Iino T, Komeda Y, Yamaguchi S. 1980. Functional homology of *fla* genes between *Salmonella typhimurium* and *Escherichia coli*. *Mol. Gen. Genet.* 178:59–67.
 41. Kutsukake K. 1994. Excretion of the anti-sigma factor through a flagellar substructure couples flagellar gene expression with flagellar assembly in *Salmonella typhimurium*. *Mol. Gen. Genet.* 243:605–612.
 42. Lee HJ, Hughes KT. 2006. Posttranscriptional control of the *Salmonella enterica* flagellar hook protein FlgE. *J. Bacteriol.* 188:3308–3316.
 43. Liu R, Ochman H. 2007. Stepwise formation of the bacterial flagellar system. *Proc. Natl. Acad. Sci. U. S. A.* 104:7116–7121.
 44. Macnab RM. 1992. Genetics and biogenesis of bacterial flagella. *Annu. Rev. Genet.* 26:131–158.
 45. Macnab RM. 2003. How bacteria assemble flagella. *Annu. Rev. Microbiol.* 57:77–100.
 46. Margot P, Pagni M, Karamata D. 1999. *Bacillus subtilis* 168 gene *lytF* encodes a γ -D-glutamate-meso-diaminopimelate murepeptidase expressed by the alternative vegetative sigma factor, σ^D . *Microbiology* 145:57–65.
 47. Michiels T, Wattiau P, Brasseur R, Ruyschaert Cornelis J-MG. 1990. Secretion of Yop proteins by yersiniae. *Infect. Immun.* 58:2840–2849.
 48. Minamino T, Macnab R. 1999. Components of the *Salmonella* flagellar export apparatus and classification of export substrates. *J. Bacteriol.* 181:1388–1394.
 49. Minamino T, González-Pedrajo B, Yamaguchi K, Aizawa SI, Macnab RM. 1999. FliK, the protein responsible for flagellar hook length control in *Salmonella*, is exported during hook assembly. *Mol. Microbiol.* 34:295–304.
 50. Minamino T, Yamaguchi S, Macnab RM. 2000. Interaction between FliE and FlgB, a proximal rod component of the flagellar basal body of *Salmonella*. *J. Bacteriol.* 182:3029–3036.
 51. Minamino T, Ferris HU, Moriya N, Kihara M, Namba K. 2006. Two parts of the T3S4 domain of the hook-length control protein FliK are essential for the substrate specificity switching of the flagellar type III export apparatus. *J. Mol. Biol.* 362:1148–1158.
 52. Minamino T, Moriya N, Hirano T, Hughes KT, Namba K. 2009. Interaction of FliK with the bacterial flagellar hook is required for efficient export specificity switching. *Mol. Microbiol.* 74:239–251.
 53. Mirel DB, Chamberlin MJ. 1989. The *Bacillus subtilis* flagellin gene (*hag*) is transcribed by the σ^{28} form of RNA polymerase. *J. Bacteriol.* 171:3095–3101.
 54. Mirel DB, Lauer P, Chamberlin MJ. 1994. Identification of flagellar synthesis regulatory and structural genes in a σ^D -dependent operon of *Bacillus subtilis*. *J. Bacteriol.* 176:4492–4500.
 55. Moriya N, Minamino T, Hughes KT, Macnab RM, Namba K. 2006. The type III flagellar export specificity switch is dependent on FliK ruler and a molecular clock. *J. Mol. Biol.* 359:466–477.
 56. Moriya N, Minamino T, Imada K, Namba K. 2011. Genetic analysis of the bacterial hook-capping protein FlgD responsible for hook assembly. *Microbiology* 157:1354–1362.
 57. Mukherjee S, et al. 2011. CsrA-FliW interaction governs flagellin homeostasis and a checkpoint on flagellar morphogenesis in *Bacillus subtilis*. *Mol. Microbiol.* 82:447–461.
 58. Ohnishi K, Kutsukake K, Suzuki H, Iino T. 1992. A novel transcriptional regulation mechanism in the flagellar regulon of *Salmonella typhimurium*: an anti-sigma factor inhibits the activity of the flagellum-specific sigma factor, σ^F . *Mol. Microbiol.* 6:3149–3157.
 59. Ohnishi K, Ohto Y, Aizawa Macnab S-IRM, Iino T. 1994. FlgD is a scaffolding protein needed for flagellar hook assembly in *Salmonella typhimurium*. *J. Bacteriol.* 176:2272–2281.
 60. Ordal GW, Márquez-Magaña L, Chamberlin MJ. 1993. Motility and chemotaxis, p 765–784. In Sonenshein AL, Hoch JA, Losick R (ed), *Bacillus subtilis* and other Gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, DC.
 61. Pallen MJ, Penn CW, Chaudhuri RR. 2005. Bacterial flagellar diversity in the post-genomic era. *Trends Microbiol.* 13:143–149.
 62. Patrick JE, Kearns DB. 2008. MinJ (YvjD) is a topological determinant of cell division in *Bacillus subtilis*. *Mol. Microbiol.* 70:1166–1179.
 63. Patterson-Delafield J, Martinez JR, Stocker BAD, Yamaguchi S. 1973. A new *fla* gene in *Salmonella typhimurium*—*flaR*—and its mutant phenotype—superhooks. *Arch. Microbiol.* 90:107–120.
 64. Samatey FA, et al. 2004. Structure of the bacterial flagellar hook and implication for the molecular universal joint mechanism. *Nature* 431:1062–1068.
 65. Shibata S, et al. 2007. FliK regulates flagellar hook length as an internal ruler. *Mol. Microbiol.* 64:1404–1415.
 66. Silverman MR, Simon MI. 1972. Flagellar assembly mutants in *Escherichia coli*. *J. Bacteriol.* 112:986–993.
 67. Suzuki T, Iino T. 1981. Role of the *flaR* gene in flagellar hook formation in *Salmonella* spp. *J. Bacteriol.* 148:973–979.
 68. Turner L, Zhang R, Darnton NC, Berg HC. 2010. Visualization of flagella during bacterial swarming. *J. Bacteriol.* 192:3259–3267.
 69. Wach A. 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* 12:259–295.
 70. West JT, Estacio W, Márquez-Magaña L. 2000. Relative roles of the *fla/che* P_A, P_{D-3}, and P_{sigD} promoters in regulating motility and *sigD* expression in *Bacillus subtilis*. *J. Bacteriol.* 182:4841–4848.
 71. Williams AW, Yamaguchi S, Togashi F, Aizawa SI, Kawagishi I, Macnab RM. 1996. Mutations in *fliK* and *flhB* affecting flagellar hook and filament assembly in *Salmonella typhimurium*. *J. Bacteriol.* 178:2960–2970.
 72. Yasbin RE, Young FE. 1974. Transduction in *Bacillus subtilis* by bacteriophage SPP1. *J. Virol.* 14:1343–1348.
 73. Yonekura K, Maki-Yonekura S, Namba K. 2003. Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. *Nature* 424:643–650.
 74. Zuberi AR, Ying C, Bischoff DS, Ordal GW. 1991. Gene-protein relationships in the flagellar hook-basal body complex of *Bacillus subtilis*: sequences of the *flgB*, *flgC*, *flgG*, *fliE*, and *fliF* genes. *Gene* 101:23–31.