FlgN is required for flagellum based motility by Bacillus subtilis

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The assembly of the bacterial flagellum is exquisitely controlled. Flagellar biosynthesis is underpinned by a specialized type III secretion system that allows export of proteins from the cytoplasm to the nascent structure. *Bacillus subtilis* regulates flagellar assembly using both conserved and species-specific mechanisms. Here, we show that YvyG is essential for flagellar filament assembly. We define YvyG as an orthologue of the *Salmonella enterica* serovar Typhimurium type III secretion system chaperone, FlgN, which is required for the export of the hook-filament junction proteins, FlgK and FlgL. Deletion of *flgN* (yvyG) results in a nonmotile phenotype that is attributable to a decrease in hag translation and a complete lack of filament polymerization. Analyses indicate that a flgK-flgL double mutant strain phenocopies deletion of *flgN* and that overexpression of FlgK-flgL cannot complement the motility defect of a ΔflgN strain. Furthermore, in contrast to previous work suggesting that phosphorylation of FlgN alters its subcellular localization, we show that mutation of the identified tyrosine and arginine FlgN phosphorylation sites has no effect on motility. These data emphasize that flagellar biosynthesis is differentially regulated in *B. subtilis* from classically studied Gram-negative flagellar systems and questions the biological relevance of some posttranslational modifications identified by global proteomic approaches.

The bacterial flagellum is a complex molecular motor that has been shown to play roles in motility, surface adherence, biofilm structure, and signal transduction (1–4). The flagellum is organized into three main structural components: the basal body, hook, and filament (1). The basal body consists of the flagellar motor, which is required to power rotation of the flagellum, and a type III secretion (T3S) system that permits the export of proteins required for the biosynthesis of the hook and filament. The hook is a flexible joint that permits a change in the angle of rotation of the flagellum, while the filament acts as a propeller to drive movement. Biosynthesis of the flagellum is tightly regulated at the level of transcription. In the Gram-positive bacterium *Bacillus subtilis*, the proteins needed for the hook-basal body (HBB) are transcribed in the 31-gene fla-che operon (5, 6). The penultimate gene of this operon, *sigD*, encodes the sigma factor σ3 (7, 8) that activates transcription of the late flagellar genes: the flagellar filament gene *hag*; the flagellar stator genes *motA* and *motB*; the anti-sigma factor *flgM*; the hook-filament junction genes *flgK* and *flgL* (9); and the autolysins (10). In wild-type *B. subtilis*, while all cells transcribe the fla-che operon, only a subpopulation of the cells synthesizes flagella (11, 12). This is due to heterogeneity in *sigD* transcription such that a threshold level of *sigD* transcription must be reached to allow sufficient σ3* to protein to accumulate and activate σ3*-regulated promoters (11). The net result is that transcription is temporally ordered such that the HBB genes are expressed before the filament genes (1).

As well as being controlled at the level of transcription, flagellar biosynthesis is regulated posttranscriptionally by flagellar type III secretion system (T3S) chaperones. Regulation at this level has reached to allow sufficient soluble export apparatus proteins FliI and FliH (17), while empty chaperones are recycled with the aid of FliJ (18). Following interaction of the chaperone-substrate complex with the C-terminal cytoplasmic domain of the integral membrane protein FlhA, a series of protein-protein interactions facilitates the entry of the substrate protein to the export gate (19–21), and its subsequent secretion is driven by proton motive force (22, 23). In *S. Typhimurium*, FlIS is a specific chaperone for flagellin (24), FlIT is specific for the FlID filament cap protein, and FlgN (ST-FlgN) is specific for the hook-filament junction proteins, FlgK and FlgL (25, 26). Recently, it has been shown that FlIs is required for Hag (flagellin) secretion in *B. subtilis* (13, 14). In addition, *in silico* analysis has suggested that YvyG of *B. subtilis* is an orthologue of the *S. Typhimurium* protein FlgN (27). However, a defined function for YvyG has not yet been determined experimentally.

Several global proteomic screens have been conducted using *B. subtilis* with the goal of examining the extent and diversity of posttranslational modification (28–30). Intriguingly, these experiments identified YvyG as being phosphorylated on tyrosine 49 (29) and arginine 60 (30). Posttranslational modification of proteins can control cell fate in several ways: (i) by altering protein localization and half-life (31), (ii) by controlling protein activity and affinity to ligands (32), and (iii) by the disruption or promotion of protein-protein interactions (33). The *B. subtilis* flagellum

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has recently been shown to be regulated by mechanisms not identified in other bacterial species (34, 35). Therefore, given the potential for YxyG to play a crucial role in the tightly controlled process of flagellar biosynthesis (31, 36), we hypothesized that protein phosphorylation might present an additional route for *B. subtilis* to regulate flagellar assembly and, therefore, motility. Thus, we aimed to define the function of YxyG during motility by *B. subtilis* and to ascertain the in vivo role of YxyG tyrosine and arginine phosphorylation.

Work presented here identifies *B. subtilis* YxyG (here referred to as *B. subtilis* FlgN) as an orthologue of the *S. Typhimurium* T3S chaperone FlgN, as previously suggested by *in silico* analysis (27). Consistent with this, we prove for the first time that FlgN is required for both swimming and swarming motility in *B. subtilis*. The lack of motility in the *B. subtilis* flgN deletion strain is linked to a block in flagellar biosynthesis. This is a consequence of a complete lack of filament assembly. Analysis of a deletion of *flgK* cannot be compensated for by the overexpression of *flgK* and FlgL. In *S. Typhimurium*, overexpression of *flgK* can compensate for the motility defect of a ∆*flgN* strain (16). However, data presented here demonstrates that in *B. subtilis* deletion of *flgK* cannot be compensated for by the overexpression of *flgK* and FlgL. This leads to the conclusion that there is a stricter dependence on the presence of FlgN in *B. subtilis* for motility than there is for FlgN in *S. Typhimurium* (16, 36). Finally, through the use of site-directed mutagenesis, we demonstrate that mutation of the tyrosine and arginine phosphorylation sites of FlgN has no effect on the ability of *B. subtilis* to become motile. In summary, these data emphasize that flagellar biosynthesis is differentially regulated in *B. subtilis* in comparison to the classically studied Gram-negative bacteria and additionally raises questions regarding the biological relevance of some posttranslational modifications identified by global proteomic approaches.

**MATERIALS AND METHODS**

**Growth conditions and strain construction.** *Escherichia coli* and *Bacillus subtilis* strains were routinely grown in Luria-Bertani (LB) medium (10 g liter⁻¹ NaCl, 5 g liter⁻¹ yeast extract, 10 g liter⁻¹ tryptone) or on LB plates supplemented with 1.5% agar at 37°C unless otherwise stated. *E. coli* strain MC1061 [F' lacC1K lacZΔM15 Trn(tet)] was used for the routine construction and maintenance of plasmids. When required, antibiotics were used at the following concentrations: 100 μg ml⁻¹ ampicillin, 100 μg ml⁻¹ spectinomycin, 25 μg ml⁻¹ chloramphenicol, 10 μg ml⁻¹ kanamycin (*B. subtilis*), 50 μg ml⁻¹ kanamycin (*E. coli*), 1 μg ml⁻¹ erythromycin, and 25 μg ml⁻¹ lincomycin. Strains were constructed using standard protocols. Phage transductions were carried out as previously described (37). When appropriate, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at the concentrations indicated in the figures or figure legends. A full list of strains used in this study is provided in Table S1 in the supplemental material.

**Construction of in-frame deletion strains.** To construct the in-frame deletion of *flgN*, an approach similar to that previously described was used (38). The upstream region of *flgN* was amplified from genomic DNA using primers NSW938 and NSW939, purified, and digested with XbaI and Sall using the restriction sites engineered into the primers. The downstream region of *flgN* was amplified using primers NSW936 and NSW937, purified, and digested with BamHI and XbaI using the restriction sites engineered into the primers. The fragments were simultaneously ligated into pUC19 and sequenced, prior to introduction into pMAD (39), to produce plasmid pNW399. Strain NRS3570 (NCIB3610 ∆flgN) was generated by integration and curing of the region contained in pNW399 in strain NCIB3610. Strains NRS4041 (NCIB3610 ∆flhD), NRS4042 (NCIB3610 ∆flgE), and NRS4060 (NCIB3610 ∆flgK-flgL) were constructed in a similar manner using the primers and plasmids detailed in Tables S2 and S3 in the supplemental material, respectively, and in the supplemental methods.

**Introduction of site-specific mutations to the chromosome.** To introduce site-specific mutations to the chromosome of NCIB3610, a similar approach to that previously described was used (39). Plasmids pNW801 and pNW1012 were used to introduce site-specific mutations in codon 49 of *flgN*. Primers NSW936 and NSW939 were used to amplify a 1,725-bp region of DNA containing the complete *flgN* coding region. The PCR fragment was cloned into pUC19 using Sall and SpfI restriction sites engineered into the primers, resulting in plasmid pNW398. Primer sets NSW942 and NSW943 (*YvyG* A60) and NSW1436 and NSW1437 (*YvyG* E60) were used to introduce point mutations to pNW398 using site-directed mutagenesis by the QuikChange method, according to the manufacturer’s instructions (Stratagene). The resulting plasmids were sequenced to ensure that the correct mutations were introduced. The mutated *flgN* coding region was excised and cloned into pMAD for integration into NCIB3610, as described for the construction of in-frame deletion strains. Further plasmids were constructed in an identical manner for *hag* (Hag T776) and *flgN* (with the R-to-A or R-to-E mutation at position 60 encoded by *flgN* [flgN-R60A or flgN-R60E, respectively]). Full details of the plasmids and primers used are provided in Tables S2 and S3, respectively, in the supplemental material.

**Sigma A antibodies.** To overexpress and purify the σ^A^ protein from *B. subtilis* for antibody preparation, the sigA gene was amplified from the chromosome of the strain NCIB3610 using primers NSW860 and NSW861. The Ncol and XhoI sites engineered into the primer sequence are underlined in Table S3 in the supplemental material. The resulting PCR product was digested with Ncol and XhoI and cloned into the expression vector pEHSIGPTVEF (40) to yield a His6-green fluorescent protein (GFP)-σ^A^ fusion construct named pNW642. A tobacco etch virus (TEV) protease recognition site was placed between the gfp and sigA coding regions.

**E. coli BL21(DE3) cells** carrying the pNW642 vector were grown in LB broth containing ampicillin at an optical density (OD₆₀₀) of 0.3 at 37°C. The cells were chilled to 20°C, and protein expression was induced with 50 μM IPTG overnight. Cells were collected by centrifugation and resuspended in lysis buffer (25 mM Tris [pH 7.5], 250 mM NaCl, 5 mM dithiothreitol [DTT], 30 mM imidazole, lysozyme, DNase I, and complete EDTA-free protease inhibitor cocktail [Roche]). Cells were lysed on a French press using pressure at 15,000 lb/in², and the cellular debris was removed by centrifugation. The supernatant was filtered through a 0.45-μm-pore-size syringe filter before being loaded onto a 1-ml HiTrap HF immobilized metal affinity chromatography (IMAC) column (GE Healthcare) using loading buffer, and the column was then washed with 10 ml of loading buffer (25 mM Tris [pH 7.5], 250 mM NaCl, 5 mM DTT, 30 mM imidazole). The recombinant His₆-GFP-σ^A^ fusion protein was eluted from the column using a gradient of elution buffer (25 mM Tris [pH 7.5], 250 mM NaCl, 5 mM DTT, 500 mM imidazole). The fractions containing the fusion protein were pooled and dialyzed into TEV buffer (50 mM Tris [pH 7.5], 20 mM NaCl, 0.5 mM EDTA, 10% glycerol) using spin concentrators. The dialyzed protein was diluted to 1 mg ml⁻¹ in the TEV buffer, and 1.5 mg of TEV protease was added. The reaction solution was incubated overnight at 4°C with agitation. The resulting σ^A^ and GFP proteins were separated using negative IMAC with the loading and elution buffers described above. The unbound fraction, containing σ^A^, was additionally purified using size exclusion chromatography with a Superdex75 resin (GE Healthcare) and buffer containing 25 mM Tris (pH7.5) and 250 mM NaCl. The purified protein was concentrated to 1 mg ml⁻¹ and sent for rabbit immunization to Dundee Cell Products (Dundee, United Kingdom). The obtained σ^A^ antiserum was affinity pu-
rified against purified recombinant α4 according to a previously described protocol (41).

Secondary-structure prediction. Primary protein sequences of S. Typhimurium FlgN and B. subtilis FlgN were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (42). The secondary structures were predicted using PsiPred (http://bioinf.cs.ucl.ac.uk/psipred/) (43, 44) and aligned against the primary sequence of the proteins.

RNA extraction and RT-PCR. RNA isolation was carried out as described previously (38) using a RiboPure Bacteria RNA Isolation Kit (Ambion), according to the manufacturer’s instructions, and treated with DNase I. To confirm cotranscription of the gene frame was filled with molten 1.5% agarose and covered with a standard microscope slide to flatten the agarose surface. Following solidification of the agarose, the slide was carefully removed, and the cell pellet was resuspended in 90 ml of TET buffer and analyzed by mass spectrometry (FingerPrints Proteomics and Mass Spectrometry Facility, University of Dundee).

Western blot analysis. Cellular proteins were extracted as described previously (37) using low-salt LB medium (5 g liter⁻¹ NaCl, 5 g liter⁻¹ yeast extract, 10 g liter⁻¹ tryptone) supplemented with 0.4% and 0.7% Bacto agar, respectively. Plates were incubated at 37°C, and the extent of swimming or swarming was noted at defined time intervals.

Staining of flagella and fluorescence microscopy. Cells carrying the Hag T200y mutation (45) were grown to mid-exponential phase, and 0.5 ml of cells was harvested by centrifugation at 4,000 × g. Cells were washed once with 1× T-Base [1 mM EDTA, 15 mM (NH₄)₂SO₄, 80 mM K₂HPO₄, 44 mM KH₂PO₄, 3.4 mM sodium citrate], pelleted, resuspended in 50 μl of T-Base containing 5 μg/ml Alexa Fluor 488 C₆ maleimide dye (Molecular Probes), and incubated for 5 min at room temperature. Cells were washed three times with 500 μl of 1× T-Base and suspended in 50 μl of 1× phosphate-buffered saline (PBS). Two microliters of the cell suspension was spotted onto a thin matrix of 1.5% agarose in water (Ultrapure Agarose; Invitrogen) contained in a 1.7×2.8-cm Gene Frame (AB-0578; ABGene House, Epsom, Surrey, United Kingdom) mounted on a standard microscope slide (Super Premium slides; VWR). Each slide was prepared as follows: the gene frame was filled with molten 1.5% agarose and covered firmly with a standard microscope slide to flatten the agarose surface. Following solidification of the agarose, the slide was carefully removed, and the cell suspension was added. Once the cell suspension was dry, the gene frame was sealed with a coverslip (thickness number 1.5; VWR), and images were immediately acquired. Imaging was performed using a DeltaVision Core wide-field microscope (Applied Precision) mounted on an Olympus IX71 inverted stand with an Olympus 100×/1.4 numerical aperture [NA] lens and a CoolSNAP HQ camera (Photometrics) with differential interference contrast (DIC) and fluorescence optics. GFP was imaged using a 100 W Mercury lamp and a fluorescein isothiocyanate (FITC) filter set (excitation, 490/20 nm; emission, 528/38 nm) with an exposure time of 200 ms. DIC images were illuminated with an LED-transmitted light source.

To monitor Phag-yfp expression, cells were grown at 37°C in LB medium to an OD₆₀₀ of 1.0, 0.5 ml of the culture was harvested, and cells were washed and resuspended in 1× PBS. The cell suspension was prepared for microscopy and imaged as described above. Yellow fluorescent protein (YFP) fluorescence was imaged using a 100 W Mercury lamp and an FITC filter set (excitation, 490/20 nm; emission, 528/38 nm) with an exposure time of 50 ms. The threshold used to define activation of the transcriptional reporter Phag was set as a YFP fluorescence intensity value greater than 2 standard deviations above the mean background fluorescence. All images were rendered and analyzed postacquisition using OMERO software (www.openmicroscopy.org) (46).

Flow cytometry analysis. The fluorescence of strains harboring yfp or gfp transcriptional promoter fusions was measured in single cells extracted from planktonic cultures grown to mid-exponential phase and analyzed as described previously (47).

Whole-cell analysis of Hag. Proteins were extracted from planktonic cultures grown to mid-exponential phase. Briefly, cells were harvested by centrifugation at 4,700 × g. Cells were suspended in 1× Bugbuster (Novagen) and lysed according to the manufacturer’s instructions. Seven micrograms of protein was resolved by SDS-PAGE and stained with Coomassie brilliant blue. Hag was identified by comparison with the bph strain (DS1677) and confirmed by mass spectrometry (FingerPrints Proteomics and Mass Spectrometry Facility, University of Dundee).

Western blot analysis. Cellular proteins were extracted as for whole-cell analysis of Hag. Extracellular proteins were extracted from the culture supernatant and processed as detailed previously (48) and suspended in 50 μl of 4× SDS loading dye. Seven micrograms of cellular proteins or 7 μl of extracellular proteins was separated by SDS-PAGE prior to transfer onto polyvinylidenefluoride membrane (PVDF) membrane (Millipore) by electroblotting. Antibodies raised against Hag (a kind gift from Kursad Turgay) were used at 1:40,000, anti-FigE (a kind gift from Daniel Kearns) was used at 1:20,000, anti-α4 was used at 1:50, and goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (both from Pierce) were used at 1:5,000.

β-Galactosidase assays. The β-galactosidase activity of strains harboring lacZ promoter reporter fusions was measured as described previously (37, 49). The values presented are the average β-galactosidase activities in Miller units (50) determined from at least three independent samples. Error bars represent the standard errors of the means.

Enrichment of flagellar hook basal bodies. The flagellar HBB fraction of NCIB3610 was enriched for as described previously (31, 52). Briefly, 1 liter of cells was grown to early exponential phase and harvested by centrifugation at 6,000 × g for 45 min at 4°C. The cell pellet was resuspended in 100 ml of sucrose solution (0.5 M sucrose, 0.15 M Tris) with a protease inhibitor tablet (Roche), and cells were homogenized with a loose pestle on ice. To lyse cells and allow spheroplast formation, lysomzyme was added to the cell suspension at a final concentration of 0.1 mg ml⁻¹, and samples were incubated at 4°C with stirring for 40 min. Spheroplasts were lysed by addition of Triton X-100 to a final concentration of 1%, and viscosity was decreased by stirring at room temperature for 30 min, allowing endogenous DNases to degrade cellular DNA. Unlysed cells were removed by centrifugation at 4,000 × g for 10 min, and EDTA was added to the suspension at a final concentration of 10 mM. To aid removal of contaminating membrane proteins, the pH of the lysate was raised to 10 by addition of NaOH. The lysate then underwent high-speed centrifugation (60,000 × g for 60 min), and the pellets were resuspended in alkaline solution (0.1 M KCl-KOH, 0.5 M sucrose, 0.1% Triton X-100, pH 11.0) and centrifuged again. The pellet was then resuspended in 90 ml of TET buffer (10 mM Tris-HCl, 5 mM EDTA, 0.1% Triton X-100, pH 8.0), and a 36% CaCl₂ gradient was established. The solution was centrifuged using a Beckman SW41Ti swinging-bucket rotor at 55,000 × g for 1 h. The flagellar fraction was visible as a band approximately 2 cm from the bottom of the tube and was collected with a Pasteur pipette and dialyzed against TET buffer. In an attempt to dissociate the flagellar filaments, the flagellar fraction was suspended in acidic solution (glycine-HCl, 0.1% Triton X-100, pH 3.0) for 1 h, and HBB complexes were collected by centrifugation at 100,000 × g for 1 h. HBBs were resuspended in TET buffer and analyzed by mass spectrometry. Results were searched against the Bacillus subtilis Mascot database (FingerPrints Proteomics and Mass Spectrometry Facility, University of Dundee). The top 12 proteins as identified by their Mascot scores are listed in Table S4 in the supplemental material.

RESULTS

YvyG of B. subtilis shares secondary-structure homology with the Salmonella protein FlgN. Previous bioinformatic analysis postulated that YvyG of B. subtilis was an orthologue of FlgN (27). FlgN is an essential component of the flagellar type III secretion machinery (26, 36, 53). FlgN has been most extensively studied in...
S. Typhimurium (26, 36), but homologues have been recognized in a broad range of bacterial species (27, 53). Three criteria were employed by Pallen et al. (27) to identify FlgN homologues in a broad range of bacterial species: (i) the protein must be encoded by a gene adjacent to an flgM homologue (Fig. 1A and B), (ii) the gene should be of a similar length to FlgN of *Salmonella* (Fig. 1C), and (iii) the protein must be recognizable (however distantly) by PSI-BLAST analysis as “FlgN-like.” These criteria were set as the primary amino acid sequence of FlgN from *S. Typhimurium* (B). For primary-sequence alignments, an asterisk indicates a fully conserved amino acid, a colon indicates a highly conserved amino acid, and a period indicates a weakly conserved amino acid. Gaps indicate no homology. Dashed lines indicate a break in the sequence. For secondary-structure alignments light gray boxes indicate α-helices, solid lines indicate coiled coils, and dashed lines indicate a break in the sequence alignment. 

On the *B. subtilis* chromosome, the flgM, flgN, flgK, and flgL coding regions are adjacent. To ascertain whether all four genes were part of the same operon in the NCIB3610 strain, reverse transcription-PCR analysis was used. RNA was extracted from wild-type *B. subtilis*, and cDNA was synthesized using a primer specific to *yvyE*, the gene proximal to *flgL* at the 3′ end (Fig. 1B). The cDNA generated was used as a template for PCR with primer pairs specific to the internal coding regions of flgM, flgN, flgK, and flgL. Regions of DNA internal to the coding regions of flgM, flgN, flgK, and flgL could each be amplified from cDNA generated using a primer at the 3′ end of flgL (just beyond the termination codon) (Fig. 1D), demonstrating that all four genes are cotranscribed.

**FlgN is essential for swarming and swimming motility of *B. subtilis***. The *flgN* gene is located in a region of the chromosome known to be required for motility (9). To test if *flgN* is required for motility, a *B. subtilis* strain carrying an in-frame deletion of *flgN* was constructed, and its phenotype was assessed in swimming and swarming assays. A strain containing a deletion in the *hag* gene (DS1677) was used as a nonmotile control. While the wild-type strain was able both to swim and swarm efficiently (Fig. 2A, C, and E), this behavior was lost in the Δ*flgN* strain (NRS3570) (Fig. 2A, C, and E). Using reverse transcription-PCR analysis, we confirmed that transcription of the other genes in the operon was not impacted by the *flgN* deletion (Fig. 2F). To further ensure that this loss of motility was specific to the deletion of *flgN*, the motility of a strain where the coding region of *flgN* was reintroduced at the *amyE* locus under the control of an IPTG-inducible promoter (P*amyE*) was tested. Both the swimming (Fig. 2B) and swarming (Fig. 2D and E) phenotypes of the Δ*flgN* strain could be restored by the reintroduction of *flgN* on the chromosome upon induction with IPTG. These data demonstrate for the first time *in vivo* that the protein product of the *flgN* gene is required for both swimming and swarming motility in *B. subtilis*. It is notable that higher levels of *flgN* transcription are required to complement the swarming defect presented by the Δ*flgN* strain than are needed for the swimming defect (compare Fig. 2B and D). This is most likely attributable to a higher demand for flagellum biosynthesis in swarming than swimming motility (54).

**Deletion of *flgN* results in a loss of bimodal transcription of *hag***. To dissect the role of *flgN* in motility, the effect of deletion of *flgN* on the transcription of the flagellar filament gene *hag* was tested. Due to the heterogeneity in *sigD* transcription, transcription of *hag* is bimodal, and thus single-cell techniques are ideally suited for analysis (11, 12). To this end, a P*hag*-GFP transcriptional reporter was integrated at a heterologous location on the chromosome. Flow cytometry and single-cell microscopy were used to assess the transcription profile using the fluorescence generated by YFP as a reporter. In the wild-type strain, the bimodality of *hag* transcription in the cell population is clearly evident (Fig. 3A and C). Strikingly, upon deletion of *flgN* bimodality is lost as *hag* transcription was observed for all cells within the population, albeit at a slightly lower level than in the wild type (Fig. 3A and C).
alteration in the hag transcription profile can be complemented upon reintroduction of flgN under the control of an IPTG-inducible promoter at the heterologous amyE locus, confirming the requirement of flgN for bimodal hag transcription (Fig. 3B). However, as the hag gene is still transcribed in the absence of flgN, these findings indicate that lack of motility in the /H9004 flgN strain is not due to a lack of hag transcription. Loss of flgN is associated with a defect in flagellar biosynthesis. Given that the hag coding region is transcribed (Fig. 3), a lack of motility in the absence of flgN could arise from lack of translation of the hag transcript. To assess hag translation, a Phag’ lacZ translational reporter fusion (under the control of the hag promoter, the hag leader region, Shine-Dalgarno sequence, and start codon) was constructed and introduced into B. subtilis at the amyE locus. When the β-galactosidase activity levels in the flgN mutant were compared with the wild-type levels, Hag translation was found to be 2-fold lower (Fig. 4A). However, a strain carrying an in-frame deletion of flgE, which encodes the main protein component of the flagellar hook, showed a 100-fold decrease in Hag translation (Fig. 4A)(55). It is likely that the observed decrease in the translation of hag in the ΔflgN mutant strain is a result of hag being transcribed at a slightly lower level in all cells than in the wild-type strain (Fig. 3). Alternatively, it is technically possible that translation itself may be regulated, as occurs in the absence of flgE (see Discussion) (55). While there is a statistically significant (P = 0.01) decrease in Hag translation in the absence of flgN, it did not appear to be sufficient to account for the complete lack of motility demonstrated in Fig. 2 (i.e., the severity of the motility defect does not match the small decrease in translation of Hag). A lack of motility in the presence of hag transcription and translation could be due to a lack of Hag polymerization. To test if Hag was secreted but not assembled into a flagellar filament in the /H9004 flgN strain, proteins were extracted from the cellular and supernatant fractions of cells grown to mid-exponential phase. The presence or absence of Hag was detected by Western blotting, with the cytoplasmic sigma factor, /H9268 A, used as a loading and fractionation control. For the wild-type strain Hag is detected in the cellular fraction (which includes assembled flagella) and in the supernatant fraction (including unassembled and sheared flagella) (Fig. 4B). However, for the /H9004 flgN strain Hag is present only in the supernatant fraction at a lower molecular weight (Fig. 4B). This is likely to be unpolymerized Hag or the products of proteolytic degradation resulting from the action of the extracellular proteases (13). As a positive control for Hag secretion, a strain carry-

FIG 2 ΔflgN strains are nonmotile. Swim expansion assays (A and B) and swarm expansion assays (C and D) were performed. Wild-type (3610; filled circle), Δhag (DS1677; filled triangle), and ΔflgN (NRS3570; open circle) strains were used for the experiments shown in panels A and C. Wild-type (3610; filled circle) and ΔflgN (NRS3570; open circle) strains along with the ΔflgN amyE: P_{hy-spank} flgN-lacI (NRS3578) strain without (filled triangle) and with (open triangle) 10 μM IPTG induction were used for the experiments shown in panels B and D. Each graph is representative of three independent biological replicates. (E) Photographs of swarm expansion plates taken at the end of the assay, after 6 h of incubation at 37°C. (F) RT-PCR analysis of transcription of rRNA, flgN, flgK, and flgL in wild-type (NCIB3610), ΔflgN (NRS3570), and ΔflgK-flgL (NRS4060) strains. Genomic DNA (gDNA) and H2O are shown as positive and negative controls for amplification, respectively. Reaction mixtures were incubated with (+) or without (−) reverse transcriptase (RT).
ing an in-frame deletion of fltD, which encodes the filament cap protein, was also assessed. As shown previously (13), this strain phenocopies the Hag secretion profile seen in the ΔflgN strain, thus supporting our conclusions.

Loss of filament polymerization in the ΔflgN strain was confirmed by single-cell fluorescence microscopy of strains where the codon for threonine at position 209 of the hag gene was mutated to cysteine to enable labeling with an Alexa Fluor 488 C5 maleimide.

FIG 3 Deletion of flgN results in a loss of bimodal hag transcription. (A and B) Flow cytometry analysis of hag transcription in strains carrying the P_{hag-yfp} transcriptional reporter fusion. Strain 3610 was used as a nonfluorescent control. Shown are the wild-type (WT; NRS3076), ΔflgN (NRS3570), and ΔflgN amyE::P_{hy-spank-flgN-lacI} (NRS3713) strains without and with induction with 50 μM IPTG. (C) Fluorescence microscopy analysis of the wild-type (NRS3076) and ΔflgN (NRS3708) strains carrying the P_{hag-yfp} transcriptional reporter (false-colored green). Scale bar, 5 μm. Asterisks indicate examples of cells that do not transcribe hag. Max, maximum; AU, arbitrary units.

FIG 4 Deletion of flgN is associated with a decrease in hag translation and a block in filament assembly. (A) β-Galactosidase assays of strains carrying the Phag^{−} lacZ translational reporter fusion. Shown are the wild-type (WT; NRS4795), ΔflgN (NRS4796), ΔflgK-flgL (NRS4799), and ΔfliE (NRS4798) strains. Data are plotted as the averages of at least three independent replicates. Error bars represent standard errors of the means. Asterisks denote significance as calculated by a Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001. (B) Western blot analysis of cellular (including assembled flagella) and supernatant (including sheared and unassembled flagella) fractions of the wild-type (3610), Δhag (DS1677), ΔflgN (NRS3570), ΔflgK-flgL (NRS4060), and ΔfliD (NRS4041) strains, separately probed with anti-Hag and anti-σ^{A} primary antibodies. MW, molecular weight in thousands; α, anti. (C and D) Fluorescence micrographs of strains carrying the Hag T209C point mutation labeled with Alexa Fluor 488 C5 maleimide (false-colored green). Shown are the wild-type (NRS3719) (C) and ΔflgN (NRS3718) (D) strains. Scale bar, 5 μm.
Deletion of the hook junction genes generates a strain which phenocopies the flgN mutant. A lack of filament polymerization in the absence of flgN is consistent with the hypothesis that B. subtilis FlgN is an orthologue of ST-FlgN; i.e., if FlgK and FlgL are not properly localized to the hook-filament junction, the flagellar filament cannot be assembled (26, 53). We proposed that if Bs-FlgN were indeed an orthologue of ST-FlgN, then a B. subtilis strain lacking FlgK and FlgL might phenocopy the ΔflgN strain. A strain carrying an in-frame deletion of flgK-flgL was constructed and found to be unable to swarm (Fig. 5A). In addition, flow cytometry and microscopy analyses revealed that, like deletion of flgN, deletion of flgK-flgL resulted in a loss of bimodality with respect to transcription of the Δhag strain. (Fig. 5B) and a 2-fold decrease in Hag translation compared with the wild-type strain (Fig. 5A). Furthermore, upon deletion of flgK-flgL, Hag could not be detected in the cellular fraction but was instead found in the extracellular milieu (Fig. 4B). Using reverse transcription-PCR analysis, we confirmed that transcription of the other genes in the operon was not impacted by the flgK-flgL deletion (Fig. 2F). All of the phenotypes were proven to be specific to deletion of the flgK-flgL coding region as the mutant strain could be complemented by replacement of the flgK-flgL coding region at a heterologous location on the chromosome under the control of the P_{hy-spank} promoter (Fig. 5A and B). In conclusion, a double deletion of flgK and flgL generates a strain that phenocopies the flgN mutant, as demonstrated by physiological, biochemical, and single-cell analyses. This is consistent with Bs-FlagN functioning as an orthologue of ST-FlagN.

Overexpression of flgK-flgL cannot compensate for the absence of flgN. In Salmonella deletion of flgN can be compensated for by overexpression of flgK and flgL (16). This is because FlgN is not exclusively required for the secretion of its substrates but rather protects FlgK and FlgL from proteolysis and ensures that the substrates are efficiently transported to the export machinery (16, 17). To test if this was the case for the B. subtilis flgN deletion, the coding regions of flgK and flgL were integrated in the ΔflgN strain at a heterologous site on the chromosome under the control of an IPTG-inducible promoter. Induction of the flgK-flgL coding region was unable to restore motility to the ΔflgN strain, as determined by assaying swarming motility (Fig. 6A). To confirm that the flagellar filament was not polymerized upon overexpression of flgK-flgL, cellular protein samples (which include assembled flagella) were separated by SDS-PAGE and stained with Coomassie brilliant blue. Hag appears as a dominant protein band at 36 kDa (Fig. 6B) and can be easily identified by comparison with proteins harvested from the Δhag and wild-type strains (56). Moreover, the identity of the Hag protein was confirmed by mass spectrometry (see Fig. S1 in the supplemental material). Compared with the wild type, analysis of the cellular proteins for the ΔflgN strain indicated that Hag was not associated with the cell fraction (Fig. 6B). This is entirely consistent with the data pre-
FIG 6 Overexpression of flgK-flgL cannot complement a ΔflgN mutant. (A) Photographs of swarm expansion plates taken after 6 h of incubation at 37°C. Shown are the wild-type (3610), Δhag (DS1677), ΔflgN (NRS3570), and ΔflgN amyE::P_{hy-spank}-flgK-flgL-lacI (NRS4043) strains without and with induction with 50 μM IPTG or 1 mM IPTG. (B) Coomasie gel analysis of cellular fractions of the 3610, Δhag (DS1677), ΔflgN (NRS3570), ΔflgN amyE::P_{hy-spank}-flgN-lacI (NRS3578), and ΔflgN amyE::P_{hy-spank}-flgK-flgL-lacI (NRS4043) strains without and with induction with 50 μM IPTG or 1 mM IPTG. The Hag protein was subsequently identified by mass spectrometry analysis and is marked with asterisks. MW, molecular weight in thousands.

sent above (Fig. 4 and 6A). As expected, the presence of the Hag band could be restored by the reintroduction of flgN on the chromosome upon induction with 50 μM IPTG (Fig. 6B). However, introduction of flgK-flgL in the ΔflgN background at a heterologous site could not complement the ΔflgN mutant with respect to Hag polymerization, even in the presence of 1 mM IPTG. The inability of flgK-flgL overexpression to compensate for deletion of flgN could be due to disruption of flagellar biosynthesis at an earlier stage. However, this possibility was ruled out as we demonstrated by Western blotting that the flagellar hook protein, FlgE, was detected in whole-cell lysates (which include assembled flagella) for both the wild-type and ΔflgN strains (see Fig. S2 in the supplemental material).

The inability of heterologous flgK-flgL expression to complement the ΔflgN mutant strain is suggestive of a strict dependence on FlgN for FlgK-FlgL protein stability or secretion in *B. subtilis*. In an attempt to test if FlgK was unstable in the absence of flgN, strains were constructed to enable detection of FlgK by fusing FlgK to a poly histidine epitope tag. However, the presence of such an epitope tag at either the N or C terminus of the protein rendered FlgK nonfunctional, as determined by a nonmotile phenotype (see Fig. S3A in the supplemental material). As a method of attempting to assess FlgK assembly at the flagellar hook junction, we aimed to purify flagellar hook-basal bodies from both the wild-type and ΔflgN strains and to analyze the protein components of the complex by mass spectrometry. While we were successfully able to enrich the flagellar fraction of the wild-type strain (see Fig. S3B and Table S4 in the supplemental material), we were unable to do so for the ΔflgN strain. This is most likely because this methodology is dependent on an intact flagellar filament for isolation of the complex, and the flgN mutant does not form a flagellar filament (Fig. 4). Finally, in *S. Typhimurium*, FlgN interacts directly with FlgK and FlgL to protect the proteins from proteolytic cleavage (25). To test if FlgN could interact with either FlgK or FlgL to perform a similar role in *B. subtilis*, bacterial two-hybrid experiments were undertaken. However, an interaction could not be detected (data not shown), which could be due to inactivity of the fusion protein. Thus, despite extensive efforts, we were unable to determine the stability of FlgK in the absence of flgN or if there was an interaction between the proteins.

**DISCUSSION**

In this work we report that yyyG is required for the motility of *B. subtilis*. We demonstrate that the main role for YyyG is to enable flagellar filament polymerization. The data presented allow us to conclude that YvyG is indeed an orthologue of FlgN from *S. Typhimurium*, but in *B. subtilis* it would appear that there is a strict reliance on YvyG for the secretion and placement of FlgK and FlgL.
and by interaction with the anti-sigma factor, FlgM (11). There-

at 37°C. Shown are the wild-type (3610), /H9004

utility. (A) Photographs of swarm expansion plates taken after 6h incubation

results is

FlgN might act as a phosphate sink or store to remove free phos-

FIG 7 Mutation of identified FlgN phosphorylation sites does not affect moti-

lity. (A) Photographs of swarm expansion plates taken after 6 h of incubation at 37°C. Shown are the wild-type (3610), Δhag (DS1677), flgN-Y49A (NRS3571), flgN-Y49E (NRS3724), flgN-R60A (NRS4063), and flgN-R60E (NRS4017) strains. (B) Coomassie gel analysis of cellular fractions of the 3610, Δhag (DS1677), flgN-Y49A (NRS3571), flgN-Y49E (NRS3724), flgN-R60A (NRS4063), and flgN-R60E (NRS4017) strains. The Hag protein is marked with asterisks. MW, molecular weight in thousands.

at the hook-filament junction. In light of these data, we suggest that YvyG be referred to as FlgN.

The role of FlgN in the regulation of flagellum biosynthesis.

This work suggests that in B. subtilis FlgN partially mediates flagellum biosynthesis through its ability to regulate hag transcription and translation. In wild-type B. subtilis the sigma factor σD (sigD) needed for hag transcription is transcribed only in a subpopulation of cells, resulting in bimodal expression of hag (11, 12). Deletion of flgN results in hag being transcribed in every cell, albeit at a lower level (Fig. 3), indicating that σD is active in every cell in this genetic background. Consistent with this, we did not observe any cell chaining in the absence of flgN, indicating that the σD-dependent autolysins (10) are also transcribed in all cells. It is known that σD can be regulated by transcription of the sigD gene and by interaction with the anti-sigma factor, FlgM (11). Therefore, the change in hag transcription observed upon deletion of flgN could be explained by a change in regulation by the anti-sigma factor, FlgM. In S. Typhimurium FlgM regulates the transcrip-

tion of late-class σ28-regulated flagellar genes by both sequester-

ing free σ28 and destabilizing the σ28 RNA polymerase holoenzyme complex (57). Upon completion of HBB assembly, FlgM is secreted, and σ28 is able to activate target promoters (58).

It has been previously reported that FlgN is able to regulate the translation of FlgM in S. Typhimurium (59), therefore raising the possibility that in the B. subtilis ΔflgN strain translation of FlgM is decreased, allowing σD to trigger transcription of hag in all cells. The regulation of FlgM in B. subtilis is poorly understood, and so whether this is the case has yet to be determined.

Deletion of flgN in B. subtilis also results in a 2-fold decrease in hag translation (Fig. 4A). This effect could technically be due to translational regulation, as is seen for the ΔflgE strain (Fig. 4A) (55). Indeed, recent studies in B. subtilis have identified the RNA binding protein CsrA, the CsrA regulatory protein FliW, and the molecular chaperone FlIS as having roles in controlling Hag translation or secretion (13, 55, 60). When cellular levels of Hag are depleted, FliW binds to CsrA, leaving it unable to occlude the hag Shine-Dalgarno sequence, allowing translation to proceed. However, when Hag protein accumulates in the cytoplasm, it is able to interact with and sequester FliW, resulting in CsrA-mediated repression of translation (55). Therefore, inhibition of translation by CsrA relies on accumulation of Hag within the cell. However, the data presented in Fig. 4B show that in the absence of flgN, Hag accumulates not in the cytoplasm but in the extracellular milieu. This not only suggests that CsrA is not responsible for the observed decrease in Hag translation but also is in keeping with the hypothesis that FlgN is required for the assembly of FlgK and FlgL; in the absence of the hook-filament junction, flagellin cannot be properly assembled and so accumulates in the extracellular milieu (53). For this reason we favor the hypothesis that the change in the transcriptional profile of hag is responsible for the decrease in translation observed.

The role of FlgN phosphorylation. Tyrosine and arginine phosphorylation events have been implicated in the control of diverse biological processes in B. subtilis, including biofilm formation (38, 61), DNA replication (62), exopolysaccharide synthesis (63), the heat shock response (64), and potentially the regulation and/or assembly of the flagellar filament (30). The motility protein FlgN has been shown to be both tyrosine and arginine phosphor-

ylated (29–31). Moreover, the subcellular localization of FlgN was reported to be impacted by deletion of the tyrosine kinase PtkA (31). However, site-directed mutagenesis of the reported FlgN tyrosine and arginine phosphorylation sites in vivo failed to im-

pact the motility of B. subtilis (Fig. 7). These findings led us to con-

clude that a dominant role for phosphorylation of these residues does not exist. When these findings are considered in a wider context of the function of posttranslational modifications, they may not be surprising. For instance, in eukaryotes, it has been suggested that many phosphorylation events are nonfunctional or may occur at a very low stoichiometry such that they do not im-

pact the function of the protein (65, 66). Indeed, two tyrosine kinases (61, 63) and one arginine kinase (30) have been identified in B. subtilis, but many more proteins have been identified as being phosphorylated, thereby suggesting that each kinase is promiscuous. This may imply that random encounters between kinases and phosphorylatable sites on different proteins might result in non-

specific and nonfunctional phosphorylation events. Alternatively, FlgN might act as a phosphate sink or store to remove free phos-

phate from the system (67).

Concluding remarks. The regulation and biosynthesis of the bacterial flagellum are best understood for Gram-negative bacte-

rial species such as S. Typhimurium. However, recent work on B.
Gram-positive and Gram-negative bacterial species. Overall, this work further emphasizes the previously un-

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