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The structure and regulation of flagella in *Bacillus subtilis*

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Abstract

Bacterial flagellar motility is among the most extensively studied physiological systems in biology but most research has been restricted to using the highly similar Gram negative species *Escherichia coli* and *Salmonella enterica*. Here we review the recent advances in the study of flagellar structure and regulation of the distantly-related and genetically-tractable Gram positive bacterium *Bacillus subtilis*. *B. subtilis* has a thicker layer of peptidoglycan and lacks the outer membrane of the Gram negative bacteria; thus, not only phylogenetic separation but also differences in fundamental cell architecture contributes to deviations in flagellar structure and regulation. We speculate that a large number of flagella and the absence of a periplasm makes *B. subtilis* a premier organism for the study of the earliest events in flagellar morphogenesis and type III secretion system. Furthermore, *B. subtilis* has been instrumental in the study of heterogeneous gene transcription in subpopulations, and flagellar regulation at the translational and functional level.

Keywords

motility; flagella; *Bacillus*; bistability; homeostasis

INTRODUCTION

B. subtilis has two forms of active movement, swimming and swarming motility that are powered by rotating flagella (73, 113). Swimming motility takes place as individual cells moving in 3-dimensions of a liquid volume. Swarming motility, by contrast, takes place as groups of cells moving in 2-dimensions atop solid surfaces (75). Commonly used domesticated laboratory strains have lost the ability to swarm at two genetic loci: one mutation disrupts Sfp involved in the synthesis of a lipopeptide surfactant that reduces surface tension to facilitate spreading and the other mutation disrupts the putative master regulator of flagellar synthesis, SwrA (118). Simultaneous repair of both mutations restores various laboratory strains to swarming motility that is indistinguishable from undomesticated ancestral strains (72). The ancestral strains not only swim and swarm but also spread over surfaces by a process called sliding (77). Sliding is the product of colony growth, hydration of an extracellular polysaccharide capsule, and reduced surface tension conferred by the surfactant (77, 126). In this review, we focus on the structure and regulation

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of *B. subtilis* flagella and motility to highlight the depth, breadth, and complexity of hierarchical structural assembly in bacteria.

Flagellar structure is complex and is considered to have three architectural domains: the basal body, the hook, and the filament (Figure 1A). The basal body is embedded in the cell envelope, houses the secretion apparatus that exports the more distal components, and provides the power for flagellar rotation. The hook is a flexible universal joint connected to the basal body that changes the angle of rotation to the long helical filament that acts as a propeller. Thirty-two genes required for basal body synthesis are concentrated in the large 27 kb *fla/che* operon expressed by RNA polymerase and the vegetative sigma factor sigma A (σ^A). Once hook assembly is complete, the alternative sigma factor sigma D (σ^D) is activated and expresses another set of genes dedicated to filament assembly and rotation (Figure 1B). Much of the work on flagellar structure has been conducted in the Gram negative model organisms and recently reviewed (28). We will discuss structural domains, prior to regulation, starting from the most distal components and working towards the cell interior by comparing the Gram negative paradigm to studies in *B. subtilis*.

FLAGELLAR STRUCTURE

Filament

The filament is a helical propeller composed of a repeating protein monomer called flagellin (151). Flagellin is polymerized by interaction between its N-terminal and C-terminal domains into 11 protofilaments that form a hollow cylinder (20 nm in diameter with a 2 nm channel). While the filament is considered rigid, it can slide through various polymorphic forms in response to environmental conditions, torsional load, and importantly, during direction reversals which govern motile behavior (33, 145). *B. subtilis* encodes two homologs of flagellin, *yvzB* and *hag*. The *yvzB* gene would produce a partial C-terminal domain of flagellin insufficient for polymerization, is not required for motility, has no known function and may be a pseudogene, but its expression is occasionally reported in transcriptional profiling experiments perhaps due to the near identity of sequence it shares with the other flagellin gene *hag* (74, 118). The *hag* gene, by contrast, is essential for flagellar assembly and encodes the flagellin monomer protein Hag (short for H-antigen) (86).

Hag monomers are secreted in an unfolded state through the hollow flagellar rod and hook by the flagellar type III secretion (T3S) apparatus within the basal body (42, 138). Flagellin readily oligomerizes into filaments *in vitro*, but *in vivo*, polymerization is restricted by intracellular and extracellular chaperones. Secretion is enhanced by the intracellular chaperone FliS that binds and delivers Hag to the T3S apparatus (4, 107). Once Hag monomer emerges from the secretion conduit, it encounters the extracellular chaperone FliD that catalyzes flagellin folding and ushers flagellin assembly at the tip of the nascent filament (39,64). FliD serves as the filament cap, which when absent, results in secretion and accumulation of flagellin in the extracellular medium (63, 107). FliD polymerizes atop two structural transition proteins called FlgK and FlgL that bridge the flagellar hook and filament.

Hook

The hook is a curved hollow cylinder situated between the basal body and the filament that acts as a universal joint (11, 36, 81). The FlgE protein constitutes the primary structural subunit of the hook consistent with its high abundance in biochemical preparations of purified *B. subtilis* hook-basal body complexes (30, 81). Like flagellin, the hook subunits are secreted by the flagellar basal body, through the rod, and polymerized underneath a specific capping protein, FlgD (30, 116). The three-dimensional structure of FlgE suggests that polymerized hook tolerates expansion on one side and compression on the other allowing for the structure to transmit and reorient motor torque on the helical filament (125).

The length of the polymerized hook is regulated by FliK. In the absence of FliK, hook polymerization goes on unchecked resulting in extended structures of FlgE called “polyhooks” (30). Intermittent secretion of the extended FliK protein results in export of the N-terminus that interacts with the nascent hook, an intermediary domain that straddles the flagellar conduit, and the C-terminus is transiently cytoplasmic (40, 97, 105). The length of the FliK intermediary domain is proportional to the length of the hook and hook polymerization is terminated when the FliK C-terminus activates autoproteolysis of the membrane protein FlhB by direct contact (46, 69, 130). FlhB proteolysis causes the secretion apparatus to switch substrate specificities such that filament class proteins become recognized and exported. Thus, the length of the FliK primary sequence controls the duration of FlgE secretion at individual basal bodies (105). Accordingly, the lengths of *B. subtilis* FliK and flagellar hook are each roughly 25% longer than that of *S. enterica* (30).

Basal body

The basal body is the part of the flagellum that is anchored in the cell membrane and transits the peptidoglycan (Figure 1A). Each basal body serves as the structural anchor, polymerization platform, secretion conduit for the hook and filament structural proteins and the rotor for torque generation. Relatively little research has been done on the Gram positive basal body save that it was purified from *B. subtilis* and was found to be both similar in structure and contains protein related to those found in flagella of *S. enterica* (81, 82). As such, most of what will be discussed here summarizes research in Gram negative model organisms and differences in the *B. subtilis* basal body structure or function will be specifically emphasized.

Flagellar basal body assembly likely begins with the type III secretion apparatus composed of FliO, FliP, FliQ, FliR, FlhA, and FlhB (89, 98). Besides FliO, the other five components are conserved in the type III needle complex/injectisome of pathogens but are less understood than the cargo proteins that they secrete. Flagellar secretion requires the proton motive force so presumably one or more of the proteins forms a proton channel (99, 119). FliO seems to be specific to flagellar secretion systems and may stabilize FliP (10). FlhA is the best understood protein in the complex and it interacts with the FliHJ chaperone-stripping complex to control the secretion of filament-class flagellar proteins (6, 76, 141). FlhA may also form the nucleation center around which the basal body protein FliF assembles (89).

The base of the basal body is a membrane-anchored ring polymerized from multiple subunits of the protein FliF (68, 146). FliF has two transmembrane segments and a large central extracellular domain that, along with a protein of unknown function FliE, likely forms a fitting for the rod (109, 138, 146). Beneath FliF sits FliG that polymerizes into a cytoplasmic gear-like rotor (48, 88, 140). Beneath FliG are two rings of protein made of FliM and FliY that interact with the chemotaxis system and control the direction of flagellar rotation (120, 131). Residues throughout the FliG primary sequence are required for flagellar biosynthesis for unknown reasons, but charged residues particularly in the C-terminus of FliG interact with MotA to generate the torque for flagellar rotation (65, 90, 91). Torque is then imparted through FliF to the flagellar rod.

The rod serves as an axle to transmit basal body rotation to the hook and filament. Rod structure is complex and is assembled from four related proteins with the inferred assembly order from cell proximal to distal: FlgB, FlgC, FlgF and FlgG (59, 82). FlgB is thought to be adjacent to FliF as FlgB was found to interact with FliE (82, 100, 109). FlgG is thought to be adjacent to the hook as FlgG was the only rod protein to be co-purified with the hook when rods were sheared and FlgG was separately implicated in outer membrane transit (29, 117). Rod assembly order was difficult to observe directly in *E. coli* as mutations of any of the rod genes abolished the assembly of all of the rod proteins leading to the assumption that the entire structure was metastable depending on completion (82). Alternatively, the absence of a subunit may render partial rod structures susceptible to proteolytic degradation in the Gram negative periplasm (58). Recent cryo-EM tomography studies of partial rod structures enabled the determination of rod assembly order in *Borrelia burgdorferi* suggesting that rod instability/proteolysis may be particular to *E. coli* (154).

B. subtilis encodes four putative rod structural proteins annotated as FlgB, FlgC, FlhO and FlhP due to their similarity to each other and their homology to the rod/hook structural class. Furthermore, all four putative rod proteins co-purified with the *B. subtilis* hook-basal body (81). FlgB and FlgC have not been directly investigated but FlhO and FlhP are likely part of the rod, upstream of hook assembly, as mutation of either protein abolishes hook synthesis and results in secretion of hook subunits into the supernatant (30). Whereas the rod genes are often co-expressed with the other basal genes, in *B. subtilis* only the *flgB* and *flgC* are encoded in the *fla/che* operon and the *flhO* and *flhP* genes, by contrast, reside as a dicistron elsewhere in the genome expressed at a very low level and amplified by a σ^D -dependent promoter (30). The unusual genetic architecture of the *B. subtilis* rod genes may be pertinent to complex regulation of flagellar assembly particular to Gram positive bacteria.

The *B. subtilis* basal body differs from that of *S. enterica* and *E. coli* in two critical ways. First, *B. subtilis* appears to lack bushing proteins. The bushings in Gram negative bacteria are two different proteins that form separate rings in the peptidoglycan layer and outer membrane respectively that allow the rod to transit and spin freely in the context of the cell envelope (135). The bushings may also function as a torque stabilizer by direct interaction with the proton channel stators (57). *B. subtilis* does not encode homologs of the bushing proteins and electron microscopy of purified basal bodies does not seem to indicate ring-like densities that could potentially be attributed to bushing-like structures (38, 81). Perhaps *B. subtilis* encodes as-yet-undiscovered bushing proteins that do not resemble those of Gram

negative bacteria. Alternatively, the Gram positive envelope structure alone may serve as a sufficient bearing and stabilizer for flagellar rotation.

The second structure that the *B. subtilis* basal body appears to lack, when compared to Gram negative bacteria, is the rod cap. In *S. enterica*, the rod cap protein FlgJ has two functions conferred by separate domains. It is thought that FlgJ is loaded first as a cap on the nascent rod such that the N-terminal domain acts as a chaperone to usher rod protein polymerization (56). *B. subtilis* lacks a homolog of FlgJ. Perhaps *B. subtilis* encodes an as-yet-undiscovered rod cap but it is noteworthy that *B. burgdorferi*, a relative of *B. subtilis* phylogenetically closer than *S. enterica*, encodes a homolog of FlgJ that is not essential for rod assembly (153). Thus, a rod cap may be unnecessary but why this would be is unclear as polymerization of two other flagellar structures, the hook and the filament, require specific cap chaperones.

The C-terminal domain of FlgJ in Gram negative bacteria is a peptidoglycan hydrolase oriented such that as the rod extends, FlgJ contacts and cleaves the peptidoglycan to permit rod penetration (111). Peptidoglycan remodeling is thought to be essential for flagellar assembly as the diameter of the rod (8–14 nm) exceeds the pore size of peptidoglycan (4 nm) (35, 138, 154). The absence of FlgJ in *B. subtilis*, therefore, creates complications for cell envelope transit of the flagellum. Whereas FlgJ mounted on the end of the rod provides an ideal way to spatially restrict peptidoglycan hydrolysis to the precise place and time of flagellar assembly in *S. enterica*, *B. subtilis* could encode a related hydrolase with a different mechanism of spatio-temporal control. *B. subtilis* is thought to encode at least 35 peptidoglycan hydrolases commonly called “autolysins” because when native regulation is disrupted their uncontrolled activity results in cell lysis (133).

Autolysins have been implicated in promoting *B. subtilis* motility as mutants defective in synthesis and secretion of multiple autolysins are non-motile and grow in long, unseparated chains of cells (44, 45). Although the chaining mutants defective in multiple autolysins were later found to be disrupted for the transcriptional regulator SinR (see below), three individual autolysins have been specifically implicated in the control of motility: LytC (an *N*-acetylmuramoyl-L-alanine amidase), LytD (an endo- β -*N*-acetylglucosaminidase), and LytF (a γ -D-glutamate *meso*-diaminopimelate muropeptidase) (13, 84, 87, 93, 94, 124). The genes encoding LytC, LytD, and LytF are expressed in whole or in part by the flagellar sigma factor σ^D consistent with the reasonable supposition that these enzymes facilitated flagellar peptidoglycan penetration (44, 122). Recently, however, a *lytC lytD lytF* triple mutant was shown to be proficient in flagellar biosynthesis indicating that if peptidoglycan remodeling is required, the activity is conducted by as-yet-undiscovered enzymes (25).

LytC, LytD, and LytF are nonetheless required for motility. One model suggested that the autolysins might promote motility simply by separating cells in chains which would otherwise be unable to coordinate their motility and inadvertently generate conflicting forces (13). LytF appears to be the only autolysin of the three that is necessary and sufficient for cell separation, however, and cells simultaneously lacking LytC and LytD, while poorly-motile, do not appear to have a substantial cell separation defect (25). Rather, motility could be improved to the LytC LytD double mutant by disruption of the intracellular protease

LonA (25). The target of LonA in motility regulation is unknown but the involvement of LonA suggests that LytC and LytD may play a regulatory role in addition to, or instead of, a purely structural role in peptidoglycan remodeling. Ultimately, the role of autolysins in motility is unclear but thus far no peptidoglycan hydrolase has been found to be essential for flagellar synthesis. How the flagellum transits the 30–40 nm thick Gram positive peptidoglycan remains unknown (133).

Cell biology

Cytological tools have been generated for the study of flagellar assembly in *B. subtilis* (Figure 1A). Introduction of a unique cysteine residue into exposed surfaces of Hag (Hag^{T209C}) and FlgE (FlgE^{T123C}) enables fluorescent labeling of the flagellar filament and hook respectively with a cysteine-reactive dye (15, 30, 144). Basal bodies are fluorescently labeled by fusing green fluorescent protein (GFP) to FliM (54). Labeling each structural domain of the *B. subtilis* flagellum enables determination of whether and where any particular motility mutant is defective in the process of flagellar assembly. In addition, each stage was observed sequentially and it was determined that basal bodies form within 5 minutes, hooks form within 10 minutes, and filaments begin within 15 minutes of flagellar gene induction (54). Filament elongation is slow, however, such that both long filaments and motile cells are first observed only after 40 minutes of assembly. Thus, when *B. subtilis* is growing rapidly and dividing roughly every 20 minutes, the flagella begun by a mother cell will be functional in the grand-daughter cells.

B. subtilis synthesizes approximately 20 flagellar basal bodies along the length of the 4 μ m cell (54). While the “peritrichous arrangement” is often considered to be synonymous with “random” positioning, measurements indicate that the *B. subtilis* basal bodies have a non-random distribution. Each basal body is separated from one another by a minimum distance greater than predicted by chance conferring a grid-like organization to the overall pattern (54). Furthermore, although the basal bodies are more symmetrically distributed with respect to the midcell than predicted by chance, they also appear to be anomalously underrepresented at the cell poles (54). The overall pattern of flagellar position in *B. subtilis* is yet to be fully characterized but basal body distribution is genetically controlled and may be disrupted by mutation of either FlhF or FlhG encoded within the *fla/che* operon (54).

FlhF and FlhG are known regulators of flagellar positioning in Gram negative and Gram positive bacteria with a single polar flagellum (70). FlhF is a SIMIBI family signal recognition particle-like GTPase that localizes to the site of nascent flagellar synthesis and recruits FliF in *Vibrio cholerae* (51). In *B. subtilis*, FlhF GTPase activity is stimulated on contact with FlhG, a SIMIBI family putative ATPase related to the MinD protein that governs cell division site selection in some bacteria (7, 8). Mutation of FlhF abolishes symmetrical placement of flagella in *B. subtilis* and causes flagella to accumulate at, rather than be excluded from, the cell poles (54). By contrast, mutation of FlhG abolishes flagellar spacing causing flagella to aggregate and originate from a single locus (54). FlhG seems to antagonize FlhF activity as mutation of FlhF is epistatic to mutation of FlhG in double mutants (54). The mechanism by which the interaction of FlhF and FlhG controls flagellar patterning needs further investigation.

Flagellate bacteria have a genetically programmed number and pattern of flagella that is sufficiently robust that such characteristics were considered definitive traits for numerical taxonomy (54). Presumably, each particular flagellar pattern confers a selective advantage to each particular species. The peritrichous flagellar arrangement is correlated with the ability of bacteria to swarm over surfaces but severe perturbations in flagellar patterning do not reduce swarming in *B. subtilis* (54). Rather, flagellar patterning may maintain productive motility of individual cells by ensuring the inheritance of a sufficient number of flagella after binary fission (54). Flagellar structure is an important reservoir of epigenetic information and unequal flagellar inheritance has been recently demonstrated to have pleiotropic regulatory effects (83). Thus, the function of specific flagellar patterns is poorly understood but may have consequences beyond simply the regulation of motility.

FLAGELLAR REGULATION

The regulation of motility is complex in *B. subtilis*. As with many flagellar motility systems, elaborate feedback mechanisms couple the flagellar structural assembly state to gene expression. *B. subtilis*, however, has the added complexity wherein late flagellar genes are expressed in only a subpopulation (74). During exponential growth the population bifurcates such that cells are either joined end-to-end in sessile cell chains or grow as single motile individuals (Figure 1B). The mixed population of physiologically distinct sessile chains and motile cells is perhaps advantageous for instantaneous environmental sampling: chains can colonize the current location, form biofilms, and enjoy enhanced resistance to protozoan grazing, while motile cells can disperse to new and potentially favorable niches. Because motile and sessile cells freely mix in liquid culture, population heterogeneity presents technical complications as the physiology of each subpopulation is difficult to study in isolation.

Transcriptional regulation

Population heterogeneity is under the control of the alternate sigma factor σ^D in *B. subtilis* (55, 74, 95). Cells mutated for σ^D grow exclusively as long non-motile chains, and thereby lack heterogeneity (55). Furthermore single cell analyses using transcriptional fusions of fluorescent proteins to the σ^D -dependent promoters P_{hag} and P_{lytF} indicated that these genes were expressed only in the motile subpopulation (25, 94, 101). Cells that are “ON” for σ^D -activity express Hag to complete flagellum assembly and express LytF to separate individuals from chains. Cells that are “OFF” for σ^D -activity do not express either Hag or LytF and grow as aflagellate chains (25). Thus the activity of σ^D governs the fate of individuals in each subpopulation.

The relative frequency of motile and non-motile cells in a *B. subtilis* population differs based on the strain due to their intrinsic bias in σ^D -dependent gene expression (50, 74). Commonly-used laboratory strains are biased towards OFF cells and grow predominantly as long chains whereas the ancestral strain is biased towards ON cells and grows predominantly as motile individuals (74). The frequency of the σ^D -ON state is increased by the biaser protein SwrA and laboratory strains favor the OFF state due to the inheritance of a high-frequency frameshift mutation that abolishes SwrA function (20, 72, 74, 152). Indeed, the

swrA gene was first discovered as a spontaneous mutation in laboratory strains with an improved motility allele called *ifm* that was later determined to be a revertant that restored the *swrA* open-reading frame (20, 50). The allele “*ifm*” that repaired SwrA function was so named for “increased-frequency-of-motility” further supporting its role in controlling population bias (50).

SwrA controls population bias by transcriptionally activating the *fla/che* operon. Cells mutated for *swrA* reduce expression from the $P_{fla/che}$ promoter and basal body number roughly 2-fold whereas cells that artificially overexpress *swrA* increase expression from the $P_{fla/che}$ promoter and basal body number roughly 2-fold (54, 74, 127). Furthermore, spontaneous mutations that bypass the absence of SwrA increase expression of the *fla/che* operon either by mutation of the promoter closer to consensus or by deletion of an upstream rho-independent terminator that allows transcriptional read-through (3, 74). Finally, transcriptomic analysis shows that SwrA elevates the expression of the *fla/che* operon (including the *sigD* gene) which in turn increases expression of the σ^D regulon (74). SwrA may activate the *fla/che* operon indirectly by binding to and antagonizing a repressor, the phosphorylated form of the response regulator DegU (3, 104, 114, 143).

SwrA biases σ^D -dependent gene expression by raising σ^D levels above a threshold such that motile cells contain high levels and chaining cells contain low levels of σ^D protein (31). σ^D protein levels are low in chains due to a failure to express the *sigD* gene, the penultimate gene in the 27 kb *fla/che* transcript (1, 96, 147). Transcript abundance decreases along the *fla/che* operon such that the position of the *sigD* gene is critical for bias and the number of ON cells in the population may be increased simply by moving the *sigD* gene forward in the operon, closer to the promoter (Figure 1B) (31). Further, the two subpopulations are differentiated by a 4-fold increase in expression magnitude throughout the operon in motile cells relative to chaining cells (31). Thus, there appears to be a threshold of σ^D expression that is exceeded only in the motile subpopulation and heterogeneity could arise by a combination of noise in the promoter activation and positioning of a key regulator at the end of an operon.

The mechanism that causes the gradual decrease in *fla/che* operon transcript abundance is controlled, at least in part, by SlrA (Figure 1B). SlrA is a small peptide, which when overexpressed, results in enhanced distance-dependent decrease in *fla/che* operon transcript abundance, undetectable levels of σ^D protein, an OFF state for the entire σ^D regulon, and aflagellate cell chains (32, 78). Furthermore, mutation of *slrA* increases the frequency of σ^D ON cells in a *swrA* mutant background (32). Thus, in the absence of SlrA, the gradual decrease in *fla/che* transcript abundance does not occur and reduced expression from $P_{fla/che}$, even in the absence of SwrA, is sufficient to exceed the threshold needed for σ^D expression, and thus σ^D activity. SlrA inhibits *fla/che* operon transcript through a paralogous pair of DNA binding proteins: SinR and SlrR (23, 32, 112).

SinR is the master transcriptional repressor of biofilm gene expression in *B. subtilis* that also represses its paralog SlrR (71, 78). When repression by SinR is antagonized, SlrR is expressed and a SinR•SlrR heteromer is formed (23). The SinR•SlrR heteromer appears to adopt a new, perhaps heteromeric, binding sequence and thereby retargets the regulators. For

example, the heteromeric complex, but neither protein alone, was shown to bind to and repress the promoters of the σ^D -dependent genes expressing Hag and LytF as well as LytC (which is under partial σ^D control) (23, 87). The benefit of directly repressing individual genes under σ^D control, however, is unclear since SlrA/SinR/SlrR also inhibits the accumulation of σ^D protein and as a consequence, deactivates the entire σ^D regulon (29, 74, 128). Further, low level artificial expression of σ^D was able to override Hag and autolysin repression suggesting that SlrA/SinR/SlrR primarily acts upstream of σ^D and that repression at individual downstream promoters, if relevant, is either weak or easily reversed (32). The complete set of targets and the binding site for the SinR/SlrR heteromeric complex remains to be determined.

SlrA manipulation could create a homogenously OFF population to demonstrate two properties of bistability: hysteresis and hypersensitivity. Hysteresis is a property of bistable systems in which each state, once acquired, resists switching to the other in the absence of a history-dependent stimulus. Hysteresis was confirmed when artificial ectopic IPTG induction of σ^D could override the inhibitory effects of SlrA for 20 cell generations after IPTG was removed (32). Once a system exceeds a threshold level of stimulus that differs depending on the state history, hypersensitivity ensures a rapid transition and prevents the system from dwelling in intermediate states. Hypersensitivity was demonstrated when a linear increase in the induction of the *fla/che* operon gave a non-linear response in σ^D -dependent gene expression (32). Hysteresis and hypersensitivity are often mechanistically related.

To account for hysteresis, biological bistable regulatory systems experience feed-forward regulation. Feed-forward regulation was indicated when transient expression from an artificially-induced gene encoding σ^D maintained an ON state but only when the native copy of the σ^D gene was also present in the chromosome (32). Multiple opportunities for feed-forward regulation on the native σ^D gene have been reported (Figure 1B). First, a σ^D -dependent promoter enhances expression of the activator SwrA which in turn activates the *fla/che* operon (21, 104). Second, a σ^D -dependent promoter (P_{D-3}) is located upstream of the $P_{fla/che}$ promoter, which when deleted simultaneously with SwrA, causes a severe motility defect (41, 104, 148). Third, σ^D -dependent promoter activity, P_{ylyF3} , was found upstream of genes encoding flagellar hook components within the *fla/che* operon, but the precise location of the promoter and its relevance to bistability has not been determined (32). Finally, a σ^D -dependent promoter was reported to be immediately upstream of the σ^D gene itself but subsequent work has been unable to detect the putative promoter's transcriptional activity (2, 32, 148). Either all or a combination of these σ^D -dependent promoters may contribute to raising σ^D expression above a threshold.

Hypersensitivity in a system is often mediated by cooperative protein interactions. Cooperativity is difficult to explain for sigma factors as most function as monomers bound to RNA polymerase. Unlike other sigma factors, however, σ^D has been shown to bind to promoters in the absence of RNA polymerase core enzyme (12, 27, 129). Binding of σ^D to promoter DNA shows super-shifted banding patterns suggesting multiple proteins may bind in a cooperative complex (27). In addition, σ^D activity is enhanced in response to cooperative assembly of the flagellar hook-basal body structure and certain proteins required

for hook assembly are transcribed primarily from a σ^D -dependent promoter (9, 30). Thus when σ^D becomes active, more hook-basal body complexes are completed, and σ^D activity becomes hysteretically self-amplifying.

The activity of σ^D is enhanced by hook basal body completion through antagonism of the anti-sigma factor FlgM (22). FlgM acts as an anti-sigma factor by binding to σ^D and inhibiting association with RNA Polymerase (12, 134). One way FlgM is antagonized by the completion of the hook basal body complex (Figure 2A). In *S. enterica*, the hook-basal body antagonizes FlgM when completion of the flagellar hook changes the specificity of the flagellar secretion apparatus to secrete FlgM from the cytoplasm and liberate σ^D to direct transcription (61, 85). In contrast, FlgM secretion has not been reported in *B. subtilis*. Furthermore, the N-terminus of FlgM from *S. typhimurium* is disordered to promote secretion, whereas the N-terminus of FlgM from *B. subtilis* is structured, perhaps suggesting an alternative mechanism of control that remains unknown (12, 34).

A second way in which FlgM is regulated in *B. subtilis* is that it is expressed by σ^D , the same sigma factor that it inhibits to seemingly create a regulatory conflict (60, 102). To resolve the conflict, FlgM expression is activated by the two-component system response regulator DegU (Figure 2A) (60). When phosphorylated by its cognate histidine kinase DegS, DegU-P lowers the σ^D threshold at the P_{flgM} promoter such that *flgM* expression is prioritized over other members of the σ^D regulon (60, 106). While the signal input for the soluble DegS kinase is unknown, DegS may be activated either by incomplete flagella or impedance on flagellar rotation establishing a quality control system for assembly analogous to that proposed for *Campylobacter jejuni* (17, 19, 24, 62). Phosphorylation of DegU further inhibits motility at the $P_{fla/che}$ promoter and perhaps other DegU phenotypes such as biofilm formation, hydrophobin synthesis, protease secretion, and poly-glutamate production are activated in response to the flagellar defect cue (3, 19, 110, 143).

Regulation of flagellar gene expression involves at least two other poorly understood proteins, SwrB and YmdB. SwrB is a single pass transmembrane protein expressed immediately downstream of σ^D in the *fla/che* operon (Figure 1B) (147). Mutation of SwrB specifically abolishes swarming motility and appears to reduce late class flagellar gene expression (72, 147). Although the mechanism of SwrB function is unknown, it synergizes with SwrA as a *swrA swrB* double mutant results in a uniform population of non-motile chains (74). YmdB resembles a phosphodiesterase that inhibits motility gene expression such that when it is mutated flagellin expression increases dramatically (37). How and where YmdB inhibits flagellin is unknown but YmdB mutants are also severely defective in biofilm formation perhaps suggesting that it could mediate its effects by way of SlrA/SinR/SlrR or other biofilm regulators.

Post-transcriptional regulation

Flagellar assembly is an energy-expensive process primarily due to an estimated 20,000 subunits of flagellin that are required polymerize into a single filament (92). To support the high structural demand, flagellin genes are expressed from strong promoters and flagellin proteins are translated from near-consensus ribosome binding sites. The energetic cost of flagellar synthesis is particularly high in *B. subtilis* as it synthesizes roughly 20 flagella per

cell (54). To offset the high metabolic cost, the flagellin primary sequence has evolved to contain a higher proportion of energy-efficient amino acids and flagellin expression has been shown to be highly regulated in every organism in which it has been studied in detail (132). Thus, flagellin must be expressed at a high level during filament assembly and yet flagellin levels must be held in check to prevent not only wasteful consumption of nutrients but also consumption of molecular space in the cytoplasm.

In *B. subtilis*, cytoplasmic levels of the Hag flagellin protein are homeostatically auto-inhibited by a negative feedback loop including FliW and CsrA (108) (Figure 2B). Hag binds to the FliW protein causing FliW to release its alternative interacting partner: CsrA (108, 142). CsrA in turn binds to two stem loops in the 5' UTR of the *hag* transcript, occludes the Shine-Dalgarno sequence and inhibits Hag translation (150). As Hag translation decreases, the reduced levels of Hag result in a stoichiometric excess of FliW. Free FliW in turn binds to CsrA and inhibits CsrA's ability to bind to RNA causing relief of Hag translational inhibition. The cycle is believed to rapidly repeat causing levels of the Hag protein to oscillate over a low and narrow threshold concentration in the cytoplasm (108).

Hag homeostasis is disrupted during periods of filament assembly. The flagellar filament chaperone FliS binds to Hag and ushers it for export through the flagellar secretion apparatus (107). Hag secretion results in a transient decrease in the cytoplasmic pool of Hag, allowing FliW to antagonize CsrA activity and permit high level translation of *hag* transcript for the duration of filament assembly. Conversely, it has been speculated that when filament assembly is completed, a transient increase in the Hag cytoplasmic pool sequesters FliW to release CsrA activity and repress Hag translation such that homeostasis autocorrects. Important to the model that secretion regulates homeostasis, it was shown that both FliW and FliS bind Hag monomer units simultaneously as competition between the two proteins would trigger regulatory consequences that would limit filament assembly (107).

CsrA is a highly conserved RNA binding protein and regulation of flagellar assembly may be its ancestral function. The *csrA* gene shows a remarkable phylogenetic distribution being restricted primarily to those bacterial genomes that also encode flagellin (108). The deepest branches of the bacterial tree support that FliW may be the ancestral form of CsrA antagonism as the genes encoding the two proteins are often translationally coupled and found tightly linked to filament structural genes (108). CsrA, however, is more commonly studied in γ -proteobacteria within which it pleiotropically regulates biofilm formation, carbon storage, virulence gene expression and motility. The γ -proteobacteria regulate CsrA activity, not by FliW, but by small RNAs that bind and sequester multiple CsrA dimers simultaneously (5). Perhaps some organisms acquired CsrA in the absence of its native antagonist FliW and merely evolved the most expedient regulators in the form permutations of CsrA binding stem loops to act as competitive inhibitors (108).

The role of CsrA in *B. subtilis* is thought to coordinate flagellin expression with the assembly state of the flagellar basal body, a task assigned to FlgM in *S. enterica* (61, 85). In *B. subtilis*, both FlgM and CsrA synergize to control flagellin expression (108). We note however translational regulation is more immediate than transcriptional regulation for the assembly of structures, and whereas CsrA is flagellin specific, FlgM controls the entire σ D

regulon of genes. Thus, the role of FlgM may be more directly related to coordinating other aspects of cell physiology with flagellar assembly than to coordinating filament assembly per se. Further, it can be speculated that post-transcriptional regulation is perhaps more prevalent than commonly appreciated to limit structural assemblies like flagellar rod and hook, secretion apparatus, pili, ribosomes or cell division machinery.

Functional regulation

Functional regulators control flagellar rotation at the post-transcriptional level as well. The most fundamental members of the functional regulatory class are the stators that transduce the power of chemiosmotic ion motive force into the mechanical power of flagellar rotation. Eight to 11 membrane-bound stator complexes composed of four subunits of the protein MotA and two subunits of the protein MotB surround each flagellum (14). MotB binds to peptidoglycan and accepts a proton to cause a conformational change in MotA and impart torque on the rotor FliG (79, 80). *B. subtilis* encodes two pairs of paralogous proteins MotP/MotS and MotA/MotB. MotP and MotS consume the sodium motive force and conditionally support flagellar mediated motility (24, 67, 139). MotA and MotB, by contrast, consume the proton motive force, are required to power both swimming and swarming motility and functionally require the same residues as their *E. coli* counterparts (19, 24, 103). Thus, the mechanism of flagellar force generation seems to be conserved from the proteobacteria to the firmicutes.

New functional regulators of the flagellum are being discovered in the context of biofilm formation (53). Biofilms are multicellular aggregates of bacteria held together by an extracellular matrix often composed of extracellular polysaccharide (EPS) (47). Flagellate cells lose motility during the transition to the sessile biofilm state. The motility-to-biofilm transition seems rapid and thus turning off transcription, or even translation, may be too slow to inhibit motility in the short term. To complicate matters further, flagella are often numerous and highly stable structures such that the inhibition of de novo synthesis should not be expected to stop rotation of the pre-existent flagella. By contrast, functional regulators are ideal to rapidly inhibit motility because as few as a single protein is required to interact with the basal body and inhibit rotation. In *B. subtilis*, two functional flagellar inhibitors have been discovered: DgrA and EpsE.

DgrA (aka YpfA), is a di-quanylate receptor protein that binds to the small signaling molecule c-di-GMP through a conserved PilZ domain (26, 49). When c-di-GMP is bound, DgrA inhibits flagellar rotation seemingly by direct interaction with MotA (Figure 2C) (26). Similar activity has been observed by YcgR, the DgrA homolog in *E. coli*, that slows flagellar rotation by a brake-like mechanism (16, 43, 121). DgrA may be involved in the motility-to-biofilm transition as c-di-GMP accumulation often mediates the physiological changes that accompany biofilm formation. *B. subtilis* however, mutations that abolish or artificially elevate the cytoplasmic pool of c-di-GMP has no obvious effect on biofilms (26, 49). Thus the physiological role of both c-di-GMP and motility inhibition by DgrA in *B. subtilis* is currently unknown.

A functional flagellar regulator more explicitly related to biofilm formation is EpsE (Figure 2D). EpsE is a bi-functional glycosyltransferase that requires the conserved active site

residues for synthesis of the *B. subtilis* biofilm EPS matrix and separately requires a set of residues predicted to occupy an exposed alpha helix to inhibit motility (52). EpsE inhibits motility by binding to the rotor protein FliG, and biophysical studies indicate that EpsE functions as a clutch by disengaging the rotor and stator components (15). EpsE is encoded within the fifteen gene *eps* operon for biofilm matrix synthesis the transcription of which is repressed and activated by the master biofilm regulators, SinR and RemA, respectively (71, 149). In addition, an RNA anti-terminator is required for expression of *eps* genes encoded downstream of EpsE perhaps to ensure that motility is inhibited before proceeding to matrix synthesis (66). Thus, the motility-to-biofilm transition is conceptually unified in the regulation of EpsE.

The inhibition of flagellar rotation also results in increased secretion of poly-g-glutamate (PGA), a polymer that forms a capsule in *B. anthracis* but manifests in *B. subtilis* as a slime layer (18). Mutation of MotA/MotB or inhibition of flagellar rotation by expression of EpsE results in highly mucoid colonies due to PGA overproduction (19, 24). PGA overproduction is due to activation of the operon responsible for PGA synthesis that is dependent on the two-component system DegS/DegU (19, 24). How the DegS kinase might sense flagellar rotation is unclear, and why PGA production appears to be linked to flagellar rotation is unknown. PGA, however, has been linked to surface-adhesion and DegU has been shown to interfere with SinR and SlrR (115, 136). Thus, the inverse regulation between flagellar function and PGA synthesis may be another facet of the motility-to-biofilm transition.

Chemotaxis is another form of functional regulation that controls the direction of flagellar rotation. Cells move up chemical gradients by biasing the time spent either running in a relatively straight direction or erratically tumbling to acquire a new trajectory. Running and tumbling are mediated by rotating the flagella counterclockwise and clockwise respectively and a signal transduction system controls the frequency at which flagella change direction. In *E. coli*, the CheA/CheY two-component system generates clockwise rotation for a tumble but in *B. subtilis*, the system is reversed where CheA/CheY generates counterclockwise rotation for a run. Further, *E. coli* gradually methylates its chemoreceptors in response to stimuli but *B. subtilis* rapidly demethylates its receptors before a slower remethylation step. Finally, to aid adaptation, *B. subtilis* has two proteins, a soluble CheC and the flagellar bound FliY, that deactivate CheY to restore tumbling and reset the system. The differences between the chemotactic responses of *B. subtilis* and *E. coli* have been recently reviewed in detail (123).

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SUMMARY POINTS

1. Electron microscopy of purified Gram positive and Gram negative cell flagella indicates that the two structures are different likely due to the demands of cell envelope architecture. *B. subtilis* nonetheless encodes many flagellar structural proteins that have homologs in the Gram negative bacteria but presumably with different regulation and solutions for accommodating the thick peptidoglycan and lack of outer membrane.
2. Cell biological tools have been developed for observing *B. subtilis* flagellar basal bodies, hooks and filaments. Wild type cells of *B. subtilis* produce roughly 20 flagella per cell that are distributed non-randomly over the cell surface. Flagellar number is increased by SwrA, and FlhF and FlhG control flagellar positioning.
3. Late class flagellar filament genes and genes for cell separating autolysins under the control of the sigma factor σ^D are transcribed in a subpopulation of cells. Heterogeneity maintains a subpopulation of motile cells that permit rapid response to environmental change as flagella take up to 3 generations to synthesize de novo. Bistability of σ^D -dependent gene expression is controlled by the *fla/che* operon architecture, SwrA, SwrB, DegS/DegU, YmdB, and the SlrA/SinR/SlrR system.
4. Flagellin is energetically costly and homeostatically restricted in the cytoplasm by a partner-switching mechanism involving the Hag flagellin, FliW, and the RNA binding protein CsrA. CsrA specifically inhibits Hag translation and translation inhibition is relieved during periods Hag secretion and active filament synthesis. The regulation of flagellar assembly may be the ancestral role for CsrA, a highly pleiotropic regulator of virulence in γ -proteobacteria.
5. *B. subtilis* has been an important organism for the study of functional regulators that control flagellar rotation. During biofilm formation, the bi-functional glycosyltransferase/clutch EpsE disengages the rotor from the stators to depower rotation. In response to c-di-GMP, DgrA (YpfA/YcgR) may act as a brake by binding to the flagellar stator. The inhibition of flagellar function may have cascading effects including an increase in the extracellular polymer poly- γ -glutamate.

FUTURE ISSUES

Rod

How is rod build in Gram positive bacteria? How is it polymerized, how does it penetrate peptidoglycan and is there a bushing that holds it in place? What governs rod length?

Type III secretion

How do the 6 proteins at the core of the type III secretion apparatus work? Can we use Gram positive bacteria that lack a periplasm to study the earliest secretion events?

Bistability

How do cells bifurcate into two stable subpopulations? Which feedback loops contribute to hysteresis and hypersensitivity? What is the advantage of biasing in the population towards one cell type or another?

Structural homeostasis

Are other subunits of the flagellum besides the cytoplasmic pool of filament protein regulated homeostatically? If so, what mechanisms govern homeostasis? If flagellin homeostasis is governed post-transcriptionally, what is the function of the transcriptional regulator FlgM?

Flagellar patterning

Why are flagella inserted in a non-random pattern in *B. subtilis*? What is the pattern of flagellar arrangement? How do FlhF and FlhG govern flagellar patterning? How does peptidoglycan remodeling accommodate patterned flagellar insertion?

Transcriptional Regulation

How do the regulators like SwrA, SwrB, YmdB, and the Sin/Slr system control motility? How is the 27kb *fla/che* operon transcript managed? Do autolysins have regulatory functions?

Functional regulation

How does DgrA inhibit flagellar rotation, and when? How does inhibition of flagellar rotation induce poly-glutamate synthesis, and why?

Figure 1. Flagellar structure and genetic hierarchy

A) Cartoon depicting the putative structure of the *B. subtilis* flagellum based on empirical data and similarity to *S. enterica*. Peptidoglycan is indicated in light gray. Membrane is indicated in dark gray. Flagellar components are colored and labeled. Micrographs are super-resolution fluorescence micrographs of the indicated structures in wild type cells: filament (maleimide-stained Hag^{T209C}), hook (maleimide-stained FlgE^{T123C}) and basal body (FlIM-GFP fusion protein). B) Cartoon depicting genetic hierarchy of flagellar genes in *B. subtilis*. Open arrows are genes. Bent arrows are promoters. Closed arrows indicate activation; T-bars indicate inhibition. Genes are color coded to match structures in panel A. Micrograph

indicates bistable gene expression found in a *swrA* mutant of *B. subtilis*. Membranes colored red (stained with FM 4–64 dye). P_{hag} expression colored green cytoplasmically (P_{hag}-YFP). Filaments colored green extracellularly (maleimide-stained Hag^{T209C}). Only a subpopulation of cells expresses the filament gene *hag* and assembles filaments. Scale bars are 2μm.

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Figure 2. Flagellar regulation

A) FlgM (red) antagonizes σ^D (cyan) but σ^D activates FlgM expression prioritized by DegU-P. The autoinhibitory loop on σ^D is broken by completed hook-basal body structure by an unknown mechanism and *hag* transcript (green arrow) is made. B) CsrA (red triangle) binds to the RBS (open box) of the *hag* transcript and inhibits translation. When Hag (green barbell) is secreted aided by FliS (orange pentagon), FliW (blue hexagon) switches partners and antagonizes CsrA allowing high level Hag translation for the duration of filament assembly. C) EpsE (red circle) is relieved from SinR repression during biofilm formation and acts as a clutch by binding to FliG and disengaging the rotor (purple) from MotA (brown). D) DgrA (red octagon) is activated by binding c-di-GMP and interacts with MotA to inhibit flagellar rotation.