Coordinating assembly of a bacterial macromolecular machine

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Abstract | The assembly of large and complex organelles, such as the bacterial flagellum, poses the formidable problem of coupling temporal gene expression to specific stages of the organelle-assembly process. The discovery that levels of the bacterial flagellar regulatory protein FlgM are controlled by its secretion from the cell in response to the completion of an intermediate flagellar structure (the hook–basal body) was only the first of several discoveries of unique mechanisms that coordinate flagellar gene expression with assembly. In this Review, we discuss this mechanism, together with others that also coordinate gene regulation and flagellar assembly in Gram-negative bacteria.

Proton motive force
(PMF). The energy source of the cell. The PMF is a combination of the membrane electrical potential and the pH gradient that is produced from the breakdown of carbohydrates by cellular catabolic pathways.

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Bacteria swim up chemical gradients, propelled by some of the tiniest, complex motors in the biosphere. The bacterial flagellum is one of the most remarkable structures in nature: a complex self-assembling nanomachine that allows bacteria to move in their environment. Many Gram-positive, Gram-negative and archaeal bacteria use flagella for motility. Flagella from Gram-positive and Gram-negative bacteria are essentially identical, except that flagella from Gram-negative bacteria extend through a second, outer membrane that is absent in Gram-positive bacteria. Archaeal flagella have a different mechanism of flagellin secretion and a high number of post-translational modifications to the flagellin subunits[1]. The arrangement and number of flagella varies among bacterial species (FIG. 1). Other types of bacterial motility include the extension and retraction of pili to produce twitching motility[2], such as in *Neisseria gonorrhoeae*. Adventurous motility in *Myxococcus* spp. seems to be powered by movement along tracks that are held down by transient adhesion complexes on the cell surface[3]. *Mycoplasma mobile* seems to 'walk' on surfaces using protein appendages that protrude from the cell surface[4]. Recently, *M. mobile* was engineered to drive a 20-μm-diameter silicon dioxide rotor on a silicon track[5]. The potential for bacterial macromolecular machines in nanotechnology is still in its infancy. The use of bacterial motility in robotic devices, however, is on the verge of leaving the stage of being only an exciting possibility to becoming a fantastic reality.

From a structural perspective, dozens of proteins, many of which have intrinsic self-assembly properties, need to come together in an ordered assembly process to complete these molecular nanomachines (BOX 1). The structures of several flagellar proteins and composite structures have been solved to an atomic level and important insights into the assembly process have been gained[6–10]. To function properly, each component of the final molecular machine that assembles by protein polymerization must achieve an accurate size and subunit composition[11–13]. Coordination of this assembly process is aided by gene-regulatory mechanisms that manage the logistics of component production[14], whereas other mechanisms regulate the timing of the specific subunits that are being exported[15].

Flagellar biology ranges from the study of nanostructures that are used to form rigid propeller structures and molecular motor-stator complexes to understanding the coupling of the proton motive force (PMF) to generate torque from ion power. This article focuses on the regulatory mechanisms that couple gene expression to flagellar assembly in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). Many regulatory checkpoint mechanisms of flagellum assembly have been elucidated, and these present a sophisticated strategy for coordinating gene expression to the assembly process.

Bacterial flagellum

The bacterial flagellum is a long, thin filament that protrudes from the cell body (FIG. 1). Three main structures form the bacterial flagellum: an engine, a propeller and a universal joint that connects them (FIG. 2). The engine, or basal body, includes a rotor and a stator, both of which are embedded in the cytoplasmic membrane; a rod that acts as a driveshaft and extends from the rotor

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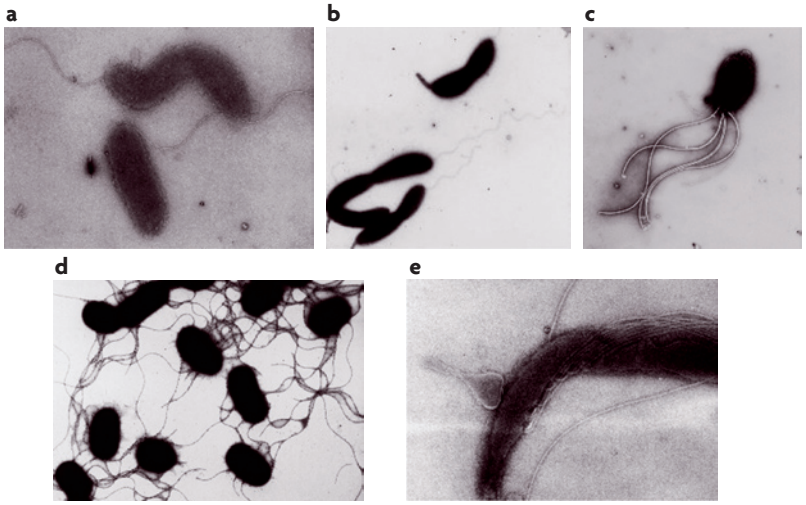

Figure 1 | Electron micrograph images illustrating the different types of flagellar arrangement in bacteria. A single flagellum can be present at one end of the cell (monotrichous); for example, in *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Idiomarina loihiensis* (**a**) and *Caulobacter crescentus* (**b**). Many bacteria have numerous flagella and, if these are co-located on the surface of the cell to form a tuft, the bacterium is lophotrichous; for example, *Vibrio fischeri* (**c**) and *Spirillum* spp. Peritrichous flagella are distributed all over the cell; for example *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (**d**). For spirochaetes, such as species of *Borrelia* (**e**), *Treponema* and *Leptospira*, a specialized set of flagella are located in the periplasmic space, the rotation of which causes the entire bacterium to move forward in a corkscrew-like motion. Images kindly provided by S.-I. Aizawa, Prefectural University of Hiroshima, Japan.

through the peptidoglycan layer to the outer membrane; and a bushing complex that assembles around the distal rod to form a pore in the outer membrane. The propeller is a long, helical filament that is composed of up to 20,000 subunits of a single protein that is capped by a scaffold, which permits the folding and polymerization of secreted filament subunits as they reach the tip of the structure following secretion[16]. The universal joint, known as the hook, permits articulation between one angular direction of rotation for the rod and a different angular rotation for the filament[17]. Most of the flagellar subunits are assembled external to the cell, and therefore have to be exported. This export uses a secretion mechanism that is part of a family of type III secretion systems (T3SSs) that are also used to secrete virulence factors by Gram-negative plant and animal pathogens[18,19]. T3SSs are characterized by key features that include: amino (N)-terminal peptide secretion signals that are often structurally disordered[20]; substrate-associated secretion chaperones that stabilize or target their cognate secretion substrates[15]; and ATP hydrolysis that is coupled to the delivery of a substrate for PMF-dependent secretion from the cell[21–23]. The flagellar- and virulence-associated T3SSs have analogous structures, with many proteins that are similar in sequence or function[18,24,25]. The main components of the flagellum are described in BOX 1.

Chaperone
A type III secretion chaperone binds a secretion substrate to prevent its degradation before secretion and pilots the substrate to the secretion apparatus to facilitate export.

Flagellar export apparatus

Many of the subunits of the bacterial flagellum are external to the cell, and therefore must be exported. By defining external structure as any structure that is outside of the cytoplasmic membrane, 14 proteins can be counted that are required for the structure and assembly of the external portions of the bacterial flagellum (FIG. 2). The external proteins are: proximal rod (FliE, FlgB, FlgC and FlgF), distal rod (FlgG) and rod cap (FlgJ), hook (FlgE), hook cap (FlgD) and hook-length control (FliK), hook-filament junction proteins (FlgK and FlgL), filament (FliC or FljB) and filament cap (FliD), and the regulatory protein (FlgM). All of these proteins cross the cytoplasmic membrane, and all, except for those that form the P and L rings, which are secreted though the Sec pathway, use the flagellar-specific T3SS. The T3SS is a narrow channel with a diameter of approximately 2.5 nm[9], which means that the translocated proteins are most likely to be exported in an unfolded or partially folded state. Both virulence-associated and flagellar type III secretion (T3S) were initially characterized by the lack of substrate signal-peptide cleavage, and in both systems it was simultaneously proposed that the N-terminal secretion signal was structural in nature[26,27]. Secretion requires at least 11 components, of which 7 are membrane proteins (FlhA, FlhB, FliF, FliO, FliP, FliQ and FliR) and are thought to be located within the flagellar basal body[28,29]. Three other proteins are soluble components that form the ATPase complex: the ATPase that drives export (FliI), a negative regulator of the ATPase (FliH) and a general flagellar-secretion chaperone (FliJ)[29].

The FlhA, FlhB, FliF and FliPQR proteins are conserved in all T3SSs. It was thought that the FliHIJ ATPase complex shuttled secretion substrates to the integral-membrane gate complex in the MS ring for ATP-dependent secretion. However, we now know that the FliHIJ complex and its associated ATPase activity are not essential for export, but serve to make the secretion process more efficient. Secretion is completely dependent on the PMF. The integral-membrane secretion complex is now known to use proton influx to derive the secretion of flagellar substrates[22,23]. ATP hydrolysis in virulence T3S is known to unfold secretion substrates[21]. This could indicate that the primary role of the FliHIJ ATPase complex is to shuttle substrates to the integral-membrane secretion system and present them as unfolded substrates for efficient PMF-dependent secretion. Thus, the PMF is required for both flagellar secretion and rotation, whereas ATP hydrolysis might be limited to substrate delivery, which would increase the efficiency of assembly. Finally, it was recently shown that FliH and FliJ act independently of the FliI ATPase to inhibit and enhance secretion, respectively, which suggests that previous assumptions of the role of the FliHIJ complex in secretion are incomplete[23].

Recent investigations have suggested roles for the ATPase and secretion chaperones in sorting secretion substrates to promote an increased efficiency in the assembly process[30,31]. A problem of secretion-substrate selection arises from the fact that a given cell has multiple flagella at different stages of the assembly process. Therefore, flagellin subunits need to be directed to structures that are growing filaments, whereas hook subunits need to be directed to structures that

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Box 1 | **Structure of bacterial flagella**

The bacterial flagellum has been best-studied in *Salmonella enterica* serovar Typhimurium, and comprises an engine and a propeller that are joined by a flexible hook (FIG. 2).

The engine

The basal body. The basal body initiates with the formation of an integral-membrane ring structure, the MS ring, that is attached to the rotor — a cytoplasmic-facing ring structure (the C ring or switch complex)[63]. The type III secretion system (T3SS) is thought to assemble in the centre of the MS ring, followed by rod assembly in the periplasm[94]. Unlike rod subunits, the P and L rings, which form the rod bushing, are not secreted by flagellar type III secretion (T3S), but through the Sec secretion pathway. The membrane-embedded MS ring is formed from approximately 26 copies of FliF. The rod is composed of distinct proximal and distal rod structures. The proximal rod contains 6 subunits each of FlgB, FlgC and FlgF, whereas an estimated 26 subunits of FlgG form the distal rod[65,95]. Another rod-associated protein, FliE, is required for efficient subunit secretion[96,97], and strains that are defective in FlhE are defective in swarming[98]. Finally, FliL was recently shown to affect the integrity of the proximal–distal rod junction. Flagella that are deficient in FliL break at high frequency on swarm agar at the proximal–distal rod junction[99].

The motor-force generators and flagellum rotation. Flagellum rotation is driven by an ion-powered rotary motor, which includes rotor and stator protein complexes. The integral-membrane proteins MotA and MotB form the ion-conducting stator complexes[100,101], which are necessary for motor rotation but not for flagellum assembly. The motor can alternate between clockwise and anticlockwise directions through an interaction between a phosphorylated component of the chemosensory system (CheY) and the three protein subunits that make up the rotor: FliG, FliN and FliM. Estimates for the stoichiometry of FliM, FliN and FliG are approximately 34,110 and 25, respectively[102,103]. FliG is most directly involved in rotation of the motor[104,105], and is known to interact with the stator protein MotA and the MS-ring protein FliF[106–108]. FliM has a large role in switching between anticlockwise and clockwise rotation by binding to CheY phosphate[109,110]. FliN plays a part in rotation and switching[35,104], and probably contributes to export by interacting with the secretion substrate ATPase-complex protein FliH[33–35].

The flagellar propeller

Bacterial motility relies mainly on rotation of the flagellar propeller, a 20-nm-thick and 10 to 15-μm-long hollow tube[16]. A single filament can consist of as many as 20,000 subunits of a single protein: the flagellin (FliC or FljB in *Salmonella* species). The flagellar motor drives the rotation of this helical propeller. When the motor rotates anticlockwise, several flagellar filaments with a left-handed helical shape form a bundle that propels the bacteria. The bacteria change direction or 'tumble' by quick reversal of the motor to the clockwise rotation[111], which transforms the filament structure such that it is right-handed, thereby causing the bundle to fall apart[112,113].

The hook, capping proteins and junction proteins

The hook. The main feature of the hook is its flexibility; it helps the flagella that are emerging from different positions in the cell (peritrichous) to function properly as a bundle to move forward. It is composed of approximately 120 copies of FlgE and is built in a similar way to the filament[8,114]. The length of the hook is controlled by the secretion apparatus proteins FlhB and FliK[44].

Capping proteins. The capping protein FliD is essential for filament polymerization[64,115], as it selectively permits certain proteins to pass, while enabling the filament proteins to polymerize. Five FliD molecules assemble to form an annular pentameric structure with a flat-top domain and flexible leg domains[60]. At an earlier stage of assembly, a capping structure (FlgD) also forms at the tip of the growing hook. At an even earlier stage of assembly, the same is probably true for the rod with the protein FlgJ[96,108].

Junction proteins. The junction or hook-associated proteins are structural adaptors between the flexible hook and the rigid filament structure. Once the hook has reached its mature length, the hook cap (FlgD) is discarded and replaced by three successive zones of proteins: the first hook-filament junction protein FlgK, the second hook-filament junction protein FlgL and the filament-capping protein FliD[116–119]. Whereas the hook-filament junction proteins stay in place, the filament-capping protein moves outward as flagellin monomers polymerize.

are growing hooks. It has been demonstrated that the FliJ component of the FliHIJ complex interacts with FliM of the C ring and FlhA of the integral membrane T3SS, whereas FliH interacts with FliN of the C ring[32–35]. Neither late secretion substrates nor their cognate chaperones alone interact with the FliHI complex, whereas the substrate-chaperone complex does[36]. The general secretion chaperone FliJ interacts with unbound chaperones, but not free substrates or substrate–chaperone complexes[30]. This suggests that a cycling mechanism directs substrates to FliI during secretion and removes released chaperone from the ATPase after secretion through FliJ to transfer the chaperone to another secretion substrate.

PMF and secretion

In both flagellar and non-flagellar T3S, the process of secretion is remarkably fast. In the early stages of filament growth, 55-kDa flagellin subunits are delivered at a rate of several molecules, which corresponds to ~10,000 amino acid residues per second[37]. The non-flagellar injectisome of *S. typhimurium* can transfer several thousand molecules of the protein SipA into a host cell only minutes after contact[38]. This rate of secretion cannot be accounted for by the activities that have been measured for T3S-associated ATPases, which led to the realization that T3S is a PMF-dependent process[22,23]. The fundamental components that are shared among all T3S machines are homologues of

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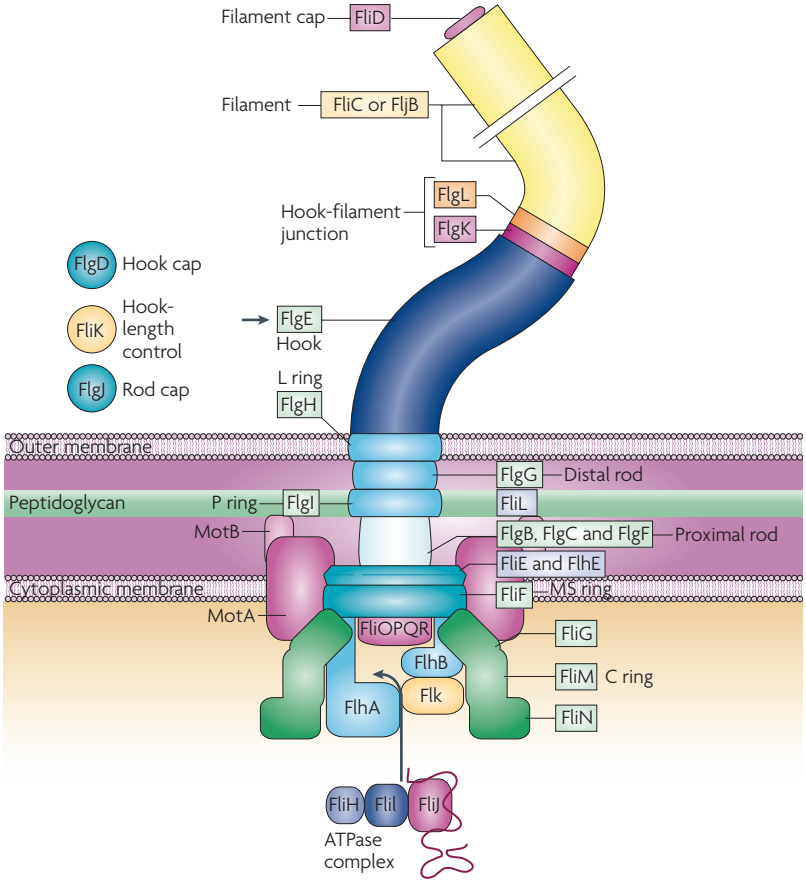

Figure 2 | **Flagellar components of *Salmonella enterica* serovar Typhimurium.** Shows the structure of the bacterial flagellum as it resides within the cell wall and membranes (BOX 1). Soluble cytoplasmic components include the FliH–FliI–FliJ ATPase complex, which is thought to deliver a number of the secreted substrates and help determine the order of substrate secretion. The filament protein consists of either FliC or FljB, which are alternately transcribed. The rod cap (FlgJ) and the hook cap (FlgD) are transiently associated with the flagellum during rod and hook polymerization, respectively. FliK is secreted during rod–hook polymerization as a molecular ruler that couples rod–hook length to the flagellar secretion specificity switch at FlhB.

the integral membrane components FliF, FlhA, FlhB, FliP, FliQ and FliR. The assembly of the first FliF ring structure (MS ring) is the base into which the rest of the membrane-localized machine localizes, whereas the other membrane components constitute the basic secretion apparatus. Homologues of the cytoplasmic component FliN and the ATPase complex components FliH, FliI and FliJ are probably involved in the localization of secretion substrates. Because the FliN homologues assemble onto the FliF ring before the integral-membrane components are assembled, it is likely that this cytoplasmic-facing component, which is found in all T3S machines, is necessary for the localization of the integral-membrane components. Thus far, only the stoichiometry of FliF and FliN are known. The number of integral membrane components within a given T3S apparatus and how they are assembled into a functional secretion machine remain to be determined.

Sigma factor
The subunit of RNA polymerase that recognizes promoter sequences.

Bypass mutant
A mutant that works in the presence of an antagonist protein, whereas the wild-type protein is normally inhibited.

Secretion substrate-specificity switch

When the hook reaches 55 nm in length, the substrate specificity switches from rod or hook substrates to filament substrates, which means that proteins that form the rod and the hook are exported before proteins that form the filament. This switch in export specificity results in the export of FlgM, which inhibits transcription of late genes by sequestering σ^{28}, a flagellar-specific sigma factor. After the export of FlgM, the production of filament substrates can begin.

The three main proteins that have an active role in the substrate-specificity switch are FlhB, FliK and Flk (also known as RflH or Fluke, to avoid confusion with FliK). FlhB is an integral member of the export apparatus and undergoes autocleavage into two subdomains as part of the specificity switch. FliK functions as a molecular ruler that measures hook-length completion, and must interact with FlhB to switch export specificity to late secretion substrates. Fluke functions as a second lock on the switching processes to prevent premature substrate-specificity switching before rod or hook completion[39,40]. As two comprehensive reviews[41,42] of the roles of FliK and FlhB in flipping the T3S substrate-specificity switch have recently been published, the role of these proteins will not be further discussed here.

Polyhooks (uncontrolled hook growth) were first observed in *fliK* loss-of-function mutants[43]. These mutants fail to switch from rod or hook substrate secretion to late or filament secretion. Motile revertants of *fliK* null alleles were located in the *flhB* locus, which indicates that FliK acts on FlhB to catalyse the secretion substrate-specificity switch[44,45]. However, these FliK-bypass mutants in *flhB* have lost normal hook-length control, which indicates that FlhB is a primary candidate for the switch, whereas FliK couples the FlhB switch to hook-length control. But how does FliK access FlhB after completion of the hook? The current models that are under investigation are discussed below.

The ruler model. FliK is a monomer in solution, has an elongated structure[46] and is also secreted as a rod or hook-type substrate[47]. The N terminus of FliK, in common with all T3S substrates, encodes the primary T3S signal. Suppressor analysis suggests that the carboxyl (C) terminus of FliK interacts with FlhB to flip the secretion specificity switch[46,48]. The basic tenet of the ruler model is that FliK is secreted during hook elongation, thereby making temporal assessments of hook length. A pause in FliK secretion is proposed to occur through an interaction of the FliK N terminus with the assembled hook and hook cap[12]. If the C terminus of FliK is near to the FlhB protein in the flagellar T3S apparatus, the switch is flipped to late secretion mode. Presumably, the C terminus of paused FliK is only near to FlhB when the hook has reached its complete length of 55 nm.

The ruler model resulted from the work of Guy Cornelis and colleagues[49,50] on the *Yersinia enterocolitica* type III needle-length control system. Insertions and deletions in the *Y. enterocolitica* FliK homologue of

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this system, YscP, resulted in longer and shorter needle structures, respectively[49]. A domain was identified within the C terminus of YscP (T3S4) that was responsible for the secretion specificity switch, and this domain is also present in FliK[50]. When a related study was performed on FliK of *S. typhimurium*, similar results were observed[13]. It should be pointed out that most FliK deletions resulted in a loss-of-function (polyhook) phenotype. However, the FliK of *Salmonella* spp. is among the shortest FliK proteins[42], and therefore most of the protein might be essential for function.

The C-ring cup model. The C ring forms a cup-like structure at the cytoplasmic base of the flagellum[51]. The C ring is composed of FliG, FliM and FliN, and is also known as the switch complex because these proteins interact with phosphorylated CheY protein to change the direction of rotation of the flagellar motor from anticlockwise to clockwise[52]. Some mutations in *fliG*, *fliM*, or *fliN* had a unique phenotype of shorter hook structures[53]. FliG, FliM and FliN assemble in 3 protein layers of approximately 30, 30 and 120 subunits, respectively, to make up the C-ring cup, and the number of assembled hook subunits is approximately 120 (REF. 52). It was proposed that the FliGMN C ring binds hook subunits by multiples of 30 so that the loss of a hook-subunit binding site within the C ring would shorten the hook by 30 or 60 subunits.

The three-dimensional solution of the hook protein provided a volume of hook subunits that allows, at most, 50 FlgE subunits in the C-ring cup[8] (BOX 2). This calculation is an overestimate at best, as we do not know the mechanism of FlgE folding or whether FlgE has an associated chaperone. Furthermore, this calculation does not take into consideration the space that is required for the FliHIJ ATPase complex to coexist in the cup during hook secretion. Thus, for the cup model to work and account for the reduction in hook lengths of 30 hook subunits in the *fliG*, *fliM* and *fliN* mutants, the cup would need to fill and empty 4 times with 30 subunits to produce a 120-subunit-length flagellum. This suggests that the C ring is more than a simple measuring-cup device to control hook length and raises the question of how mutations in C-ring subunit proteins (FliG, FliM or FliN) result in shorter hook structures. Recent data suggest that any mutations which slow down the assembly process result in shorter hook structures (by the molecular-clock model discussed below). Therefore, the short-hook mutants in FliG, FliM or FliN might be too slow to assemble a functional T3S apparatus or might be defective in recruiting the hook subunits to be secreted, which, according to the molecular-clock model, would result in shorter hook structures.

The molecular-clock model. The most recent model to explain hook-length control is the molecular-clock model. This model is based on three observations: first, overexpression of the hook protein results in longer hook structures; second, a hook mutant that is defective in polymerization makes shorter hook structures; and third, overexpression of a polymerization-defective hook mutant results in hooks of normal length[12]. These observations led to a model that the initiation of hook polymerization starts a 'countdown' for the specificity switch that is coupled to the FliK ruler.

A fundamental problem in our understanding of the secretion specificity switch that remains to be solved is whether cessation of hook polymerization is directly coupled to the secretion substrate-specificity switch (from rod or hook substrates to late secretion substrates: filament, FlgM and hook-associated proteins) or whether they are two separate events. There is evidence to suggest that they are separate events. For example, loss of FliK results in polyhook structures, but a detailed measurement of hook lengths in the polyhook mutant revealed that hook length peaked at the wild-type hook length of 55 nm[54]. This would imply a mechanism of hook-length control that is independent of FliK: possibly a molecular clock. A primary candidate for the molecular clock would be the FlhB autocleavage event. The auto-cleavage of FlhB from *S. typhimurium* was shown to have a half-life of approximately 7 minutes, and a mutant in FlhB that was unable to autocleave produced polyhooks, although the distribution of hook lengths in this mutant was not determined[55]. It is not known whether the FliK interaction with FlhB catalyses autocleavage. One model for how the FlhB molecular clock works is that FlhB autocleavage occurs with a 7-minute half-life after FlhB synthesis and assembly into the flagellar T3S apparatus, thereby resulting in the cessation or slowing down of hook polymerization[12]. The FliK ruler would both catalyse FlhB autocleavage upon the interaction with assembled FlhB during its secretion and catalyse a conformational change in cleaved FlhB to change the secretion specificity switch. The combination of FlhB autocleavage to stop hook polymerization and the FliK ruler to change secretion specificity after the hook reaches 55 nm would reduce the number of FliK subunits that would need to be continuously secreted to measure hook length. If only one protein can be secreted through the flagellum at a time, FliK secretion would compete with hook secretion. Therefore, secreting rulers to measure the flagellum could have the negative effect of slowing down flagellar assembly. The competition between hook-length control (to maximize optimum hook length) and the efficiency of flagellar assembly would be under intense selective pressure.

Box 2 | **Capacity of the C ring**

Based on an inner radius of 150 Å and a depth of 150 Å, an approximate inner C-ring volume would be $3.14 (Pi) \times 150^2 \times 150$, which is equal to 1.06×10^7 Å^3. The volume of a hook subunit based on the diameter of the hook at 180 Å and the axial length for the 2 turns of the 1-start helix, which contains 11 subunits of 46 Å × 2 (which equals 92 Å), gives a volume for a hook subunit of $3.14 \times 90^2 \times 92/11$, which equals 2.1×10^5 Å^3.

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Fluke: cork at the base of the flagellum

Prior to completion of the hook–basal body (HBB), the flagellar T3SS is specific for rod or hook substrates. Upon completion of the HBB, the interaction of FliK with FlhB results in a switch to late secretion specificity. The interaction of FliK with FlhB that is coupled to hook-length control requires that FliK is secreted by the flagellar T3SS. However, overexpression of a non-secretable mutant form of FliK that lacks a secretion signal sequence can complement an *fliK* mutant. Therefore, FliK does not need to be secreted to interact with FlhB and change secretion specificity, although the switch modulated by the mutant FliK is no longer coupled to hook-length control[56]. This suggested that a barrier exists to prevent cytoplasmic FliK from interacting with FlhB. The Fluke gene (*flk* or *rflH*) was identified by loss-of-function alleles that allowed the switch to late substrate secretion in strains that lacked hook or hook-cap proteins, P- or L-ring proteins, and rod or rod and cap proteins[39,57,58]. There is evidence to suggest that this barrier is the Fluke protein, which prevents the FliK-mediated secretion specificity switch before HBB completion[40]. Fluke (333 amino acids) is anchored in the cytoplasmic membrane by a C-terminal hydrophobic transmembrane domain[40] that is essential for its function.

The frequency of switching to late secretion in the absence of Fluke varied among the different rod or hook mutant strains[58]. Strains that were defective in rod or hook assembly genes switched to late secretion in the absence of Fluke at a low frequency, but P- or L-ring mutants (with functional rod and hook assembly genes) switched at a much higher frequency. One explanation for this is that the localization of FliK to the growing basal body brings the FliK C terminus near to FlhB. FliK is known to interact strongly with the hook-capping protein FlgD and to a lesser extent with FlgE (hook protein)[12]. These interactions could provide a pause in secretion to allow the FliK C terminus to interact with FlhB[12]. FliK-dependent switching occurs at high frequency in strains that do not have P and L rings, but only if Fluke is also missing. As FlgD assembles after rod completion, an FlgD-dependent pause in FliK secretion would only occur after rod completion, FlgD assembly and initiation of hook elongation. If Fluke were sequestered into the flagellum to form a cork-like structure, it would prevent access of the FliK C terminus during the secretion pause until the hook reached a length such that the FliK C terminus was beyond the Fluke cork. Bypassing the Fluke cork would require the presence of a flagellar T3S signal, but might also occur through an association with a flagellar T3S chaperone.

Rod to hook transition

The flagellum is an axial structure that consists of 11 protofilaments that are arranged in a helical pattern[59]. Each layer of the flagellar helical structure grows by the addition of 5.5 residues per turn, with 2 turns per helical layer of subunits[60]. The axial components polymerize first, with 6 subunits each of the proximal rod proteins FlgB, FlgC and FlgF, followed by an estimated 26 subunits of the FlgG subunits of the distal rod[61–63]. Hook subunits (120) polymerize on the distal rod, followed by 11 subunits each of the hook-filament junction proteins FlgK and FlgL (also called hook-associated proteins)[8]. Finally, up to 20,000 FliC or FljB filament subunits polymerize onto the assembled hook-filament junction proteins to complete the flagellum[16]. The axial components can be divided into three types, based on their intrinsic polymerization properties. The proximal rod proteins FlgB, FlgC and FlgF polymerize to six subunits, which is one turn or half a layer of the helix[61]. The hook-filament junction proteins FlgK and FlgL polymerize to form a thin layer, which is probably 2 turns or 1 full layer (11 subunits) of the helix[64]. FlgG (distal rod), FlgE (hook) and FliC (filament) polymerize to 2 (REF. 65), 12 (REFS 8,44) or up to ~2,000 helical layers[16], respectively. However, FlgG, FlgE and FliC all have the intrinsic ability to polymerize indefinitely, and different mechanisms are used to control the final length of these structures[11,43,66,67].

In vitro, the length of filament polymerization is limited by subunit availability and is not limited to the 10 μm length that is observed for filaments that grow *in vivo*[67]. This suggests that final filament length *in vivo* is controlled by hindered diffusion. This is supported by the observation that the rate of *in vivo* polymerization exponentially slows with increasing length[37], whereas there is no effect of increasing length on the rate of *in vitro* polymerization[67]. Hook polymerization normally terminates at a length of 55 nm[44] but, similar to filament polymerization, in the absence of FliK, hook polymerization can continue and is only limited by the availability of hook synthesis *in vivo*[68].

FlgG has recently been shown to be capable of continuous polymerization, similar to the hook and filament[11]. Mutants in *flgG* resulted in filamentous rod structures that grew to approximately 80 nm, which is the normal rod–hook length in the HBB structure. Similar to normal rod–hook length control, the FliK protein now regulated rod length in the filamentous rod strains. Loss of FliK in filamentous rod strains resulted in polyrod structures of up to 1 μm in length that seemed to be limited only by the length of the cell. As rod growth normally stops after two helical layers of FlgG are assembled, FlgG seems to be controlled by an intrinsic mechanism that allows only two stacks of FlgG subunits to assemble in an individual protofilament.

The *flgG* mutants that resulted in filamentous rod structures had two other interesting properties: first, they produced longer rod structures with multiple P rings and, second, the resulting structures failed to penetrate the outer membrane, resulting in flagella that grew in the periplasm[11]. Thus, the mechanism of outer-membrane penetration is coupled to proper rod-growth termination. It is likely that the normal rod length of 25 nm positions the tip of the rod at the outer membrane. A two-layer stack of FlgG subunits is the substrate for P-ring formation. As P-ring subunits are secreted through the Sec pathway, whereas FlgG subunits are secreted through the flagellum, rod completion and P-ring formation are not coupled. Rod-growth termination

Periplasm
The region between the inner and outer membranes of Gram-negative bacteria.

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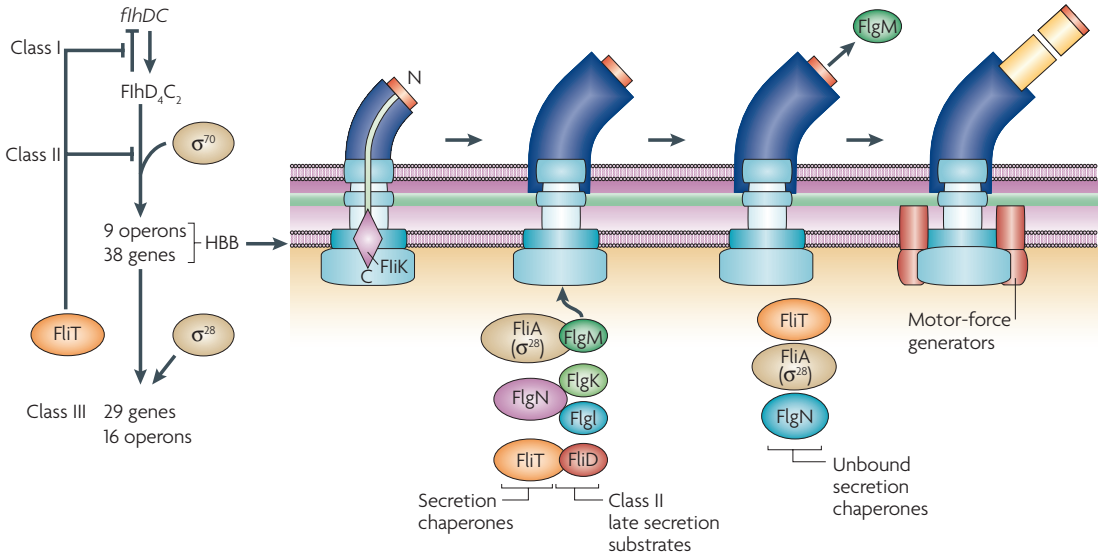

Figure 3 | **Coupling of flagellar gene regulation to flagellum assembly.** The *flhDC* operon, or flagellar master operon, is under the control of numerous global regulatory signals that lead to the expression or inhibition of flagellar gene expression. Induction of the class I *flhDC* operon (class I on) produces FlhD and FlhC, which form a heteromultimeric complex, $FlhD_4C_2$, that acts to direct σ^{70}-dependent transcription from class II flagellar promoters and auto-represses *flhDC* transcription (class I off; class II on). Class II promoters direct the transcription of genes that are necessary for the structure and assembly of the hook–basal body (HBB) substructure. Upon HBB completion, late secretion substrates are exported from the cell and their cognate chaperones are released to regulate gene expression. FliT is an $FlhD_4C_2$ factor and prevents both $FlhD_4C_2$ auto-repression and the activation of class II promoters. The σ^{28} transcription factor directs the transcription of class III promoters, which include the filament structural genes and the genes of the chemosensory pathway (class I on; class II off; and class III on). Activation of class I transcription would re-initiate the flagellar regulon for a new round of flagellar gene expression. As drawn, the FliK and FlhA proteins are meant to reside within the C ring. The stoichiometries of Fluke, FlhA, FlhB, FliO, FliP, FliQ and FliR within the C ring are not known.

at 25 nm would allow time for the P ring to form around a completed rod structure. In turn, a P ring that is formed on a two-layer stack of FlgG is the substrate for formation of the L ring, which results in an outer-membrane pore at the tip of the rod. This is probably the signal for the initiation of hook polymerization outside of the cell, although this remains to be determined.

Flagellar gene hierarchy

The flagellar regulon of *S. typhimurium* includes more than 60 genes[69]. The transcripts of the flagellar regulon of *S. typhimurium* are organized into a transcriptional hierarchy that is based on three promoter classes that are temporally regulated in response to assembly[70,71] (FIG. 3). This system is well conserved among Gram-negative enteric bacteria with peritrichous flagella, but other bacteria use different modes to coordinate flagellar gene regulation with assembly. For example, the flagellar regulon in *Caulobacter* spp. is intimately coupled to the cell cycle and translation plays an important part in regulation[72], whereas *Pseudomonas aeruginosa* and *Helicobacter pylori* use a four-tiered regulatory hierarchy[73,74].

In *S. typhimurium*, the flagellar master operon *flhDC* is at the top of this hierarchy and controls the fundamental decision of whether to produce flagella. The *flhDC* operon is expressed from a class I promoter. The FlhD and FlhC proteins form a heteromultimeric complex ($FlhD_4C_2$) that functions as a transcriptional activator to promote σ^{70}-dependent transcription from the class II flagellar promoters[75]. The class II promoters direct transcription of the genes that are needed for the structure and assembly of the flagellar motor structure, which is also known as the HBB. Upon HBB completion, class III promoters are transcribed by σ^{28} RNA polymerase[71], which is specific for flagellar class III promoters[76]. Prior to HBB completion, σ^{28} RNA polymerase is inhibited by the anti-σ^{28} factor FlgM. Upon HBB completion, FlgM is secreted from the cell, presumably through the completed HBB structure, and σ^{28}-dependent transcription ensues. In this way, genes such as the flagellin filament genes, the products of which are needed after HBB formation, are only transcribed when there is a functional motor onto which they can be assembled.

Transcription and anti-transcription factors

The FlgM anti-σ^{28} factor is secreted in response to completion of the HBB[71]. Research over the past few years has focused on the purpose of the σ^{28}–FlgM regulatory mechanism. The *flgM* gene is expressed from both class II and class III promoters. Class-II-expressed FlgM is an internal checkpoint in the regulation of flagellar gene expression. It was shown that during initiation of flagellar biosynthesis, class-II-expressed FlgM (which represents 20% of total FlgM) is produced in the cytoplasm, binds to σ^{28} (FliA) and prevents the association of σ^{28} with RNA polymerase, thereby inhibiting flagellin gene transcription[77–79]. However, once the HBB structure is

Regulon
A group of genes that are coordinately regulated.

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assembled, the flagellar T3SS changes specificity from rod or hook substrates to late assembly substrates. FlgM is a late secretion substrate[80,81], and therefore FlgM signals that a functional HBB is present in the cell by participating as a substrate for secretion through the flagellum-specific T3S pathway. The onset of FlgM secretion results in a decrease in the ratio of FlgM to σ^{28} in the cell[82]. Free σ^{28} is then available to associate with RNA polymerase and enable class III flagellar gene transcription. The σ^{28}–FlgM regulatory system is complicated by the fact that both the *flgM* gene and the σ^{28} structural gene (*fliA*) are expressed from class II and III promoters. Thus, excess FlgM would inhibit class III *fliA* and class III *flgM* gene transcription, whereas excess σ^{28} would increase the transcription of class III *fliA* and *flgM*.

Another layer of autoregulation occurs at the first step of the flagellar regulatory hierarchy. The FlhDC activators of class II transcription are also autoinhibitors of their own transcription[83]. However, it is not known how much FlhDC class II transcription occurs relative to the level of FlhDC autoinhibition.

FliT is a second regulator that inhibits both the FlhDC activation of class II promoters upon HBB completion and FlhDC autoinhibition[84]. HBB genes are expressed from class II promoters, and therefore when the HBB is completed class II transcription is no longer required. FliT is inhibited by an interaction with the filament-capping protein FliD, a late secretion substrate[85,86]. Therefore, the secretion specificity switch removes the inhibitors of both σ^{28} (FlgM) and FliT (FliD). The following scenario (FIG. 3), could be envisioned to couple flagellar gene expression to the cell cycle. Class II transcription produces FlhDC, which accumulates, resulting in the activation of class II transcription and inhibition of class I (*flhDC*) transcription. Subsequently, completion of the HBB results in the secretion specificity switch and removal of FlgM and FliD. This releases FliT, which inhibits FlhDC, preventing it from autoinhibition, to restore class I transcription. FliT also inhibits class II transcription, whereas free σ^{28} transcribes class III promoters. Thus, completion of the flagellar transcriptional cycle (initiation of class III transcription) coincides with re-initiation of class I transcription.

Secretion chaperones as regulatory proteins

Virulence-associated and flagellar T3SSs use T3S chaperones to facilitate the secretion of effector proteins in the virulence T3SSs and secretion of late assembly substrates in the flagellar T3SSs[15]. Another role for the T3S chaperones is to target secretion substrates to the correct secretion apparatus. For example, in the absence of their chaperone-binding domains, the *S. typhimurium* virulence-associated T3S substrate proteins SptP and SopE are no longer targeted for secretion through their cognate *Salmonella*-pathogenicity-island-1 T3SS needle structure, but are instead secreted by flagellar T3S[87]. In addition, T3S chaperones were also shown to mask a localization domain of the effector that is required for localization inside the host, whereas the effector is present in the bacterium[88].

Flagellar T3S chaperones fall into three classes: those that prevent proteolysis of a secretion substrate in the cytoplasm before secretion; those that play a part in localizing the secretion substrate to the secretion apparatus to facilitate secretion; and those that do both. In addition, many of these proteins regulate gene expression. It was initially shown in *Yersinia* spp. that T3S chaperones link gene regulation to the secretion of substrates[89]. Since then, a general mechanism has emerged in which a dual role of secretion chaperone and regulator of gene expression allows gene regulation to be timed to the secretion of specific proteins in flagellar and virulence-associated T3SSs[15,90]. In the *Escherichia coli* and *S. typhimurium* flagellar systems, HBB completion corresponds to the initiation of late substrate secretion, which includes the hook-filament junction proteins FlgK and FlgL, the filament-cap protein FliD, the flagellins FliC or FljB, and FlgM. Three of the corresponding T3S chaperones, FlgN (FlgK and FlgL chaperone), FliT (FliD chaperone) and σ^{28} (FlgM chaperone)[85,91,92] function in their substrate-unbound states as regulators of gene expression. FlgN increases translation of FlgM, FliT inhibits both the autoinhibition and class II transcriptional activation of FlhDC and σ^{28} promotes transcription from class III flagellar promoters[71,84,92]. A regulatory role for the flagellin chaperone FliS has not yet been determined. Unexpectedly, σ^{28} was found to facilitate secretion of the anti-σ^{28} factor FlgM, which means that σ^{28} is both a transcription factor and a T3S chaperone[92]. The dual roles of substrate-bound secretion-chaperone activity and substrate-free regulatory activities are common to both the flagellar and virulence T3SSs. Virulence T3S also uses secretion chaperones that function as regulators to coordinate gene expression to the secretion of their cognate substrates[15].

Outlook

The dynamic field of flagellar biology is moving forward, and there are specific areas in which progress can soon be expected, as outlined below.

The structures of the filament and hook have been solved by X-ray crystallography and, to a great extent, so have the C-ring components FliG, FliM and FliN[93]. The related axial components in the rod are probably similar to the core of the hook and filament. Importantly, the membrane-anchored structures of the secretion apparatus and the motor-force generator (MotAB) complexes remain to be solved. We do not know how MotA and MotB interact with the rotor (presumably with FliG), and therefore we do not know how proton flow through the MotAB complexes makes the motor turn, nor do we know how the motor changes direction instantaneously. Although the molecular details of export in T3SSs remain unclear, the recent finding that the process is PMF dependent allows us to pose more accurate mechanistic questions. If secretion is fuelled by the PMF, then the outward movement of protein substrates must be accompanied by an inward flow of protons. Future studies should address both how and where protons move through the export apparatus and the

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stoichiometry of the process (the number of H^+ that are translocated per amino acid residue exported). Invariant acidic and basic residues of the integral-membrane components could be of particular importance, as they might function directly in proton-linked processes that are essential for transport. Bioinformatics could uncover these residues, but reconstitution of T3S using individual components will be essential for the full mechanistic characterization of this fundamental process.

The T3S-associated ATPase plays some part in secretion selectivity, but it might primarily function as an unfoldase in the final presentation of substrates[21]. The mechanisms of secretion substrate selection and order of delivery of individual substrates, including the roles of substrate-specific chaperones and of the general, conserved, ATPase-associated secretion chaperone FliJ, remain to be determined. In at least one case described for *S. typhimurium*, increasing the cellular concentration of the cognate secretion substrates (FlgK and FlgL) eliminated the requirement for the substrate-specific chaperone FlgN[92].

There is abundant evidence for localized translation to individual T3S apparatuses within a cell. For flagellar T3S, localized translation could provide a mechanism to independently regulate flagellar gene expression for individual flagellar structures within the same cell that are at different stages of the assembly process.

Many bacteria, such as species of *Caulobacter* and *Vibrio*, switch between non-flagellated and flagellated states. This can occur upon transition from a free-swimming state to a surface-associated swarming state. The mechanism that underlies this switch is unknown, and we still do not know what determines the highly variable number and placement of nascent flagella in the membrane of the cell (FIG. 1).

Finally, it seems that the bacterial flagellum is a structure of great complexity. In an attempt to understand why, it is not necessary to resort to intelligent designers, because surely a designer would have fashioned a simpler structure and gene regulation system. We only need to be reminded that evolution demands that changes occur on the existing structure — no starting from scratch. It is fair to say that we are at long last making a dent in our understanding of how this evolutionary process might have occurred for the reducibly complex bacterial flagellum and the beautiful result it has produced.

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DATABASES

Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Caulobacter crescentus | *Escherichia coli* | *Helicobacter pylori* | *Idiomarina loihiensis* | *Mycoplasma mobile* | *Neisseria gonorrhoeae* | *Pseudomonas aeruginosa* | *S. typhimurium* | *Vibrio cholerae* | *Vibrio fischeri*
Entrez Protein: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=protein
FlgB | FlgC | FlgD | FlgE | FlgF | FlgG | FlgI | FlgK | FlgM | FlhA | FlhB | FliA | FliC | FliD | FliE | FliF | FliH | FliI | FliJ | FliK | FliO | FliP | FliQ | FliR | Flk | SopE | SptP

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