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The Role of the FliK Molecular Ruler in Hook-length Control in *Salmonella enterica*

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Abstract

A molecular ruler, FliK, controls the length of the flagellar hook. FliK measures hook length and catalyzes the secretion-substrate specificity switch from rod-hook substrate specificity to late substrate secretion, which includes the filament subunits. Here, we show normal hook-length control and filament assembly in the complete absence of the C-ring thus refuting the previous “cup” model for hook-length control. Mutants of C-ring components, which are reported to produce short hooks, show a reduced rate of hook-basal body assembly thereby allowing for a premature secretion-substrate specificity switch. Unlike *fliK* null mutants, hook-length control in an autocleavage-defective mutant of *fliH*, the protein responsible for the switch to late-substrate secretion, is completely abolished. FliK deletion variants that retain the ability to measure hook length are secreted thus demonstrating that FliK directly measures rod-hook length during the secretion process. Finally, we present a unifying model accounting for all published data on hook-length control in which FliK acts as a molecular ruler that takes measurements of rod-hook length while being intermittently secreted during the assembly process of the hook-basal body complex.

Keywords

Flagellar assembly; Type III protein export; Hook length control; Molecular ruler

Introduction

In order to propel themselves in their living environments towards nutrients, bacteria, such as *Escherichia coli* and *Salmonella enterica*, have developed a sophisticated ion-powered rotary machine called the flagellum (Kojima & Blair, 2004). The bacterial flagellum extends from the cytoplasm to the cell exterior and is made from about 25 different proteins each in multiple copies from a few to many thousands (Macnab, 2003). The flagellum is a motor organelle that includes a protein secretion apparatus, which is a member of the type III family of bacterial secretion systems (Macnab, 2004).

Typically, the bacterial flagellum is composed of three main structures: an engine, a propeller and a universal joint that connects them (Figure 1) (Berg & Anderson, 1973). The engine, or basal body, includes a rotor and stator embedded in the cytoplasmic membrane, a rod that acts as a drive-shaft and extends from the rotor through the peptidoglycan to the

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outer membrane; a bushing-like complex that assembles around the distal rod forming a pore in the outer membrane (Macnab, 1996). The propeller is a long helical filament composed of up to 20,000 subunits of a single protein capped by a scaffold that permits the folding and polymerization of secreted filament subunits as they reach the tip of the structure following secretion (Yonekura *et al.*, 2003). The universal joint, also known as the hook, allows for the transmission of torque energy generated at the cytoplasmic rotor to rotational energy of the external filament (Samatey *et al.*, 2004).

The three structures that comprise the axial component of the flagellum, the rod, the hook and the filament, are all capable of continuous polymerization. However, each is under a different length control mechanism (Chevance & Hughes, 2008). The rod extends from the cytoplasmic membrane through the outer membrane, a distance of about 22 nm, the hook extends from the surface of the cell 55 nm and the filament extends about 10 microns from the hook or about 10 times the length of the cell. Filament growth decreases exponentially with length suggesting that terminal filament length is determined by hindered diffusion, so after about 10 microns in length subunits are no longer able to diffuse out to the filament tip. Recent evidence suggests that terminal rod length occurs by a stacking mechanism that allows distal rod subunits to polymerize onto identical protein subunits only once (Chevance *et al.*, 2007). Hook-length has been reported to rely on multiple factors including molecular cups, clocks and rulers (Ferris *et al.*, 2005, Makishima *et al.*, 2001, Minamino & Pugsley, 2005, Moriya *et al.*, 2006, Shibata *et al.*, 2007).

Loss of hook-length control was first observed in strains defective in the *fliK* gene (Patterson-Delafield *et al.*, 1973). The absence of a functional FliK protein produces hooks with a wide length distribution up to about a micron in length, called polyhooks. A measurement of the distribution of hook-lengths in the polyhook mutant showed that the population of lengths peaked at near wild-type length followed by a tail of increasing size and decreasing numbers (Koroyasu *et al.*, 1998). This hook-length distribution study suggested that a mechanism is in place, in the absence of FliK, to ensure that most hooks are of the wildtype length and that inclusion of FliK added another layer of regulation to control hook length by preventing the polyhook structures from forming.

Ultimately, hook length is controlled at the level of substrate secretion. Upon formation of the flagellar type III secretion (T3S) apparatus at the cytoplasmic base of the basal structure, secretion is specific for rod and hook subunits. The hook-basal body (HBB) is complete when the hook reaches 55 nm (Hirano *et al.*, 1994). At this point in the assembly process, an interaction between FliK and an integral membrane component of the flagellar T3S system, FlhB, results in a change in secretion substrate specificity from rod-hook subunits to late secretion substrates (Ferris *et al.*, 2005, Minamino & Macnab, 2000, Minamino *et al.*, 2006, Kutsukake *et al.*, 1994, Williams *et al.*, 1996). Late secretion substrates include the hook-filament junction proteins and the filament cap, historically referred to as hook-associated proteins (HAPs), the filament proteins, FliC or FljB, and a transcriptional inhibitor FlgM.

In addition to interaction with FliK, FlhB also undergoes an autocleavage event with a 5 minutes half-life *in vitro* (Minamino & Macnab, 2000). The 383 amino acid protein FlhB is composed of a 211 amino acid, membrane-embedded N-terminal domain followed by a 172 amino acid cytoplasmic C-terminal domain (Minamino *et al.*, 1994). Cleavage of the C-terminal cytoplasmic domain of FlhB between amino acid residues N269 and P270 in addition to interaction with FliK is required for the secretion-specificity switch to occur. Mutants of amino acid residue N269 and P270 thus remain in rod-hook-type secretion mode. In the *fliK* null background, hook growth is fast, starting at 40 nm/min slowing until wild-type hook length of 55 nm is achieved and followed by a steady growth rate of 8 nm/min (Koroyasu *et al.*, 1998).

The fundamental problem has been to determine how FliK measures a hook length of 55 nm beyond the cell surface and then interact with FlhB in the inner, cytoplasmic membrane to flip the secretion specificity switch. Initially, the possibility that FliK acted as a molecular ruler was argued against because deletions of the FliK protein resulted in long, polyhook structures rather than shorter hook structures (Kawagishi *et al.*, 1996). Later, a cup model was proposed suggesting that the components that make up the flagellar rotor, FliG, FliM and FliN act as a measuring cup (Makishima *et al.*, 2001). Electron micrograph pictures show that these proteins make a cup-like structure, called the C-ring at the base of the flagellum (Thomas *et al.*, 2006). It was proposed that the C-ring fills with a cup-full of hook subunits, which upon emptying the cup results in hooks of proper size and exposure of the cytoplasmic component of FlhB to interact with FliK. This model was based on the observation that mutants in *fliG*, *fliM*, and *fliN* produce shorter hook structures (Makishima *et al.*, 2001). However, the dimension of the C-ring suggests that it has the capacity to contain at most 50 of the 130 hook subunits required (Chevance & Hughes, 2008).

Evidence has now accumulated to support a molecular ruler model that was originally discarded. The FliK ruler model was revised based on findings in the *Yersinia enterocolitica* virulence-associated type III system (Journet *et al.*, 2003). Virulence-associated T3S systems utilize needle-like structures, which resemble flagellar hook-basal bodies, to secrete virulence determinants into host cells (Cornelis, 2006, Galan & Wolf-Watz, 2006). A FliK functional homolog, YscP, functions to control needle length. Secretion of YscP through the needle structure is necessary for its function (Agrain *et al.*, 2005). Loss of YscP resulted in needles of uncontrolled growth and insertions and deletions of YscP resulted in needle lengths that directly corresponded to the length of YscP. Recently, a study by Wagner *et al.* (Wagner *et al.*, 2009) further supports the ruler model by modeling the structure of YscP. The authors showed that functional YscP likely has a helical structure. Based on the results with YscP, insertions and deletions in FliK were constructed and resulted in longer and shorter hooks, respectively, that directly corresponded to the increase or decrease in FliK length (Shibata *et al.*, 2007).

FliK is secreted through the flagellar basal structure as a rod-hook substrate even though it is not incorporated into the flagellar structure (Minamino *et al.*, 1999). Recently, it has been shown that an interaction between FliK and the hook proteins are needed for an efficient secretion specificity switch. It has been suggested that a temporary interaction of FliK with the hook subunits, within the secretion channel during FliK secretion, resulted in a pause in FliK secretion. That pause would allow for interaction of the C-terminal domain of FliK with FlhB thereby catalyzing the secretion specificity switch (Minamino *et al.*, 2009). In addition, a strong interaction was reported between the N-terminus of FliK and the hook capping protein FlgD. It was proposed that after FliK was secreted, the N-terminus would interact with the hook cap pulling FliK into the secretion channel as the hook elongated until the C-terminus of FliK was in vicinity of FlhB at the base to catalyze the secretion-specificity switch (Minamino & Pugsley, 2005). However, the secretion channel is too narrow to allow secreted hook subunits to pass by a FliK molecule that is maintained within the channel. The average width of an α -helix is about 1 nm and the inner diameter of the filament has been shown to be 2.0 nm (Yonekura *et al.*, 2003), whereas the inner diameter of the hook channel is even smaller (Shaikh *et al.*, 2005). In the *Yersinia* needle case, the YscP ruler is estimated to have a maximum width of 1.3 nm (Wagner *et al.*, 2009). Thus, the retention of a ruler while subunits pass by would be physically improbable in both the *Yersinia* needle-length and flagellar hook-length control systems.

In this work, we present data that FliK is a molecular ruler that directly measures hook length in a temporal manner. We present a model proposing that intermittent FliK secretion during hook polymerization results in temporal measurements of hook length to produce the

wild-type spectrum of hooks that range from 35 – 75 nm peaking at 55 nm (Hirano et al., 1994).

Results and Discussion

Hook-filament assembly in the absence of the C-ring

Here, we address the assembly of hook-filament structures *in vivo* in the complete absence of the C-ring. In order to facilitate type III-dependent secretion and maximize late substrate concentrations, we combined an F₀F₁ ATP synthase mutant to increase the proton-motive force as described previously (Paul *et al.*, 2008), and additionally *flgM* null as well as *flhD** promoter-up mutations. The removal of the negative regulator of late substrate gene expression, FlgM, and the promoter-up mutation of *flhD* promoter both increase availability of flagellar secretion substrates (M. Erhardt, T. Hirano, K.T. Hughes, unpublished results). We analyzed those mutants lacking components of the C-ring (*fliG* or *fliGMN*) by fluorescent microscopy and found that in the absence of the ATP synthase a significant fraction of the analyzed cells assemble flagella (Figure 2A+B). It is of interest that almost all analyzed mutant cells possessed only one, unusually long flagellum despite the probable presence of multiple hook-basal body structures within each cell. This would indicate a possible mechanism of preferentially localized secretion of flagellar components. However, due to the preparation and staining procedures involved in visualizing flagella for fluorescent microscopy, we are unable to quantify the exact fraction of cells producing flagella. The flagella are sheared easily during the slide preparation and therefore most of the flagella are not attached to cells anymore. It also seems possible that the long flagella on the mutant cells are more sensitive to shearing than those on wild type cells.

Additionally, we measured the hook-length in mutants lacking the C-ring and found a hook-length distribution with a mean of about 71 ± 26 nm and a peak at the wildtype length of 55 nm (Figure 2C+D). We employed non-linear fitting of the Gaussian distribution for hooks of lengths below 100 nm corresponding to 88% of the total analyzed hooks (Figure 2C, dashed red line). Accordingly, the average hook-length of the majority of hooks in the C-ring deletion mutant is 58 ± 18 nm, closely following the average hook-length of 55 ± 6 nm of the wildtype (Hirano et al., 1994).

By utilizing the combination of *atpA*, *flgM* and *P_{flhD}**, the capability of the cell for type III secretion is increased substantially by both excess substrates and energy. This is consistent with our recent finding where we screened for transposon insertions that allowed for type III secretion in the complete absence of the C-ring. We found that any condition that increased levels of the flagellar master regulatory proteins, FlhDC, bypassed the C-ring requirement in flagellar type III secretion (Erhardt & Hughes, 2009). Accordingly, we conclude that the C-ring is not essential for flagellar type III secretion under excess secretion substrate conditions. This conclusion is also supported by the fact that we primarily observed unusually long filaments (Figure 2A+B), which indicates that the secretion process *per se* is not impaired. Hook-length, however, seems to be only partially controlled in the C-ring null mutant. If the C-ring acts under wildtype conditions as an affinity cup-like structure for secreted substrates, then we would presume that in the C-ring deletion mutant, targeting of secreted proteins is impaired. Thus, secretion of proteins is now only dependent on their concentration and an increase in the ratio of FlgE to FliK subunits secreted during hook growth would account for the longer hook structures observed in the absence of the C-ring. Less secreted FliK molecules during hook elongation will result in longer hooks because of fewer measurements.

C-ring subunits do not interact with the hook subunit FlgE

A prediction of the C-ring cup model is that the FliG, FliM and FliN subunits that make up the C-ring structure interact with FlgE subunits. We tested for possible interactions between purified GST-FlgE and purified FliG, FliM and FliN proteins using standard pull-down assays. Importantly, the FliG, FliM and FliN constructs used here are able to fully complement respective *fliG*, *fliM* and *fliN* deletion strains (Supplemental Figure 3). As a positive control, FliK, which was previously shown to interact with FlgE *in vitro* (Moriya et al., 2006), was also tested. As shown in Figure 2E, GST-FlgE did interact with FliK, but not with FliG, FliM or FliN. Thus, if FlgE interacts with the C-ring proteins *in vivo*, then it is likely to interact only after these proteins are assembled into the C-ring. Alternatively, these results suggest an alternative model in which the *fliG*, *fliM*, and *fliN* mutants produce short hooks by a mechanism distinct from the measuring cup model.

These results are consistent with recently published data showing that the formation of filaments in mutants partially deleted for the C-ring occurred under conditions where the flagellar type III secretion system-specific ATPase FliI was overproduced (Konishi *et al.*, 2009). Recently, the C-ring was shown to act as an affinity cup-like structure that is not essential to the secretion process, but does facilitate secretion. The C-ring appears to increase the efficiency of the secretion process by locally increasing secretion substrate concentrations prior to secretion or preventing non-substrates interactions with the type III secretion apparatus (Erhardt & Hughes, 2009). Together, these results refute the measuring cup model and support a temporal, molecular ruler model of hook-length determination as described below.

C-ring mutants producing short hooks are defective in HBB assembly

A clue to explain how mutants in the C-ring structural genes *fliG*, *fliM*, and *fliN*, could affect hook length came with the discovery of a polymerization-defective hook mutant that also produced shorter hook structures (Moriya et al., 2006). This led to the idea that once hook formation was initiated, a molecular clock prevented or slowed hook elongation after a given amount of time. The molecular clock may result from the FlhB autocleavage event (Ferris et al., 2005, Minamino & Macnab, 2000). The FlhB protein was shown to undergo autocleavage with a 5 min half-life. A FlhB mutant protein that is defective in autocleavage stays in the rod-hook secretion mode. Thus, the cleavage of FlhB is required to switch to the late secretion mode. It is therefore possible that FlhB autocleavage might result in an inability or reduced ability to secrete hook subunits as proposed by Moriya et al. (Moriya et al., 2006).

Thus, we decided to assay whether C-ring mutants in the *fliG*, *fliM*, or *fliN* genes are slow to assemble HBB structures because they are defective in HBB assembly. We analyzed i) temporal secretion deficiencies of the C-ring mutants by determining the necessary time for induction of the *motA* promoter, which indicates completion of the HBB complex, and ii) additionally deficiencies in cumulative secretion by determining ampicillin resistance conferred by secreted FlgE-Bla fusion protein. Previously, we have shown that we could synchronize the flagellar assembly pathway by placing the flagellar master control operon under a tetracycline-inducible promoter (Karlinsey *et al.*, 2000). Upon HBB completion, an inhibitor of late flagellin gene expression, FlgM, which is also a flagellar late secretion substrate, is secreted from the cell. Secretion of FlgM, releases a transcription factor (σ^{28}) that is specific for transcription of the flagellin genes, *fliC* or *fliB*, the genes encoding the motor force generators (flagellar stator complex), *motA* and *motB*, and the genes of the chemosensory response system.

To monitor the time for completion of HBB structures upon induction of the flagellar master operon, we used a fusion of the *motA* promoter to the luciferase operon, *luxCDABE* of *Photorhabdus luminescens* (Goodier & Ahmer, 2001) in a strain with the flagellar master operon under tetracycline (Tc) control (Karlinsky et al., 2000). As shown in Figure 3, we determined a half-maximal induction time of 82 ± 3.5 minutes for the *motA* promoter by non-linear regression analysis, which was consistent with half-maximal P_{motA} induction times reported in an earlier study (Brown et al., 2008). Importantly, in a previously described hook polymerization-defective mutant the half-maximal P_{motA} induction time was greatly increased to 106 ± 3.0 minutes (Figure 3D). Thus, variation in the time it takes for HBB completion is readily observed in our assay system.

We analyzed the half-maximal P_{motA} induction times for various C-ring mutants (Makishima et al., 2001) and found two different classes of mutants based on the time for HBB completion, hook length, and secretion of FlgE-Bla, respectively (Table 1). The first class of C-ring mutants displayed significantly longer half-maximal P_{motA} induction times compared to the wildtype. Similarly to the hook polymerization-defective mutant FlgE (T149N), the hook-length of the class I C-ring mutants has previously been measured by Makishima et al. (Makishima et al., 2001) to be only slightly shorter than the wildtype (45 nm compared to 55 nm). We additionally determined if the class I C-ring mutants have impaired secretion by analyzing secretion of an FlgE-Bla fusion protein. The β -lactamase protein (Bla) must be secreted into the periplasm to confer resistance to ampicillin (Ap^R). In a strain deleted for the flagellar proximal rod genes *flgB* and *flgC*, the FlgE-Bla fusion is secreted into the periplasm and the cells are Ap^R (Lee & Hughes, 2006). By measuring the minimal inhibitory concentration (MIC) to ampicillin, one can determine the amount of FlgE-Bla secretion into the periplasm for the short-hook, C-ring mutants as compared to a wild-type C-ring strain (Table 1 and supplemental Table 2). Compared to the wildtype (MIC >400 μ g/ml) we found that four of seven of the first class of C-ring mutants indeed showed severely impaired secretion as judged by a low MIC of 200 μ g/ml, whereas the remaining three mutants displayed a lower MIC of 400 μ g/ml (Table 1).

The second class of C-ring mutants displayed no significant difference in P_{motA} induction times and additionally no significantly impaired FlgE-Bla secretion (supplemental Table 2). However, this class of C-ring mutants is reported to have very short hooks with an average length of 25 nm (Makishima et al., 2001). Importantly, a re-examination of the hook-length of several of the class II C-ring mutants revealed no significant difference in hook length compared to wildtype (N. Moriya, T. Minamino and K. Namba, unpublished results). As shown in Supplemental Figure 2, we analyzed hook length of the *fliM1813* allele (Class I mutant based on P_{motA} induction time; reported hook length 42 ± 7 nm (Makishima et al., 2001)) and the *fliM2309* allele (Class II mutant based on P_{motA} induction time; reported hook length 26 ± 6 nm (Makishima et al., 2001)) and found a hook length of 45 ± 9 nm in case of *fliM1813* and 51 ± 5 nm in case of *fliM2309*. Accordingly, we presume that the originally described short-hooks, class II C-ring mutants are unstable and accumulated secondary mutations that restored normal hook-length control.

In summary, the class I C-ring mutants took longer to build the HBB and produced shorter hooks because the mutants displayed a defect in the secretion process. We hypothesized that the incorporation of FlhB into a functional secretion apparatus started a countdown for the FlhB autocleavage event that ultimately resulted in cessation or slow-down of hook-polymerization. Accordingly, the secretion-impaired class I C-ring mutants produced shorter hooks because of inefficient secretion, therefore allowing a premature FlhB autocleavage event. As discussed below, independent from FlhB autocleavage, a change in the ratio of secreted FlgE/FliK molecules would result in shorter hooks. This would be the case e.g. if secretion of hook subunits, but not FliK ruler molecules, was impaired in the class I C-ring

mutants. To test if FlhB autocleavage indeed was responsible for the termination of hook subunits secretion, we next analyzed the hook-length distribution in an autocleavage-defective FlhB mutant.

Hook-length distribution in a *flhB* mutant that is unable to undergo autocleavage

FliK is generally referred to as the hook-length control protein. Loss of FliK results in polyhook structures (Patterson-Delafield et al., 1973). However, a hook-length distribution analysis in a *fliK* null strain demonstrated that hook length still peaks at the wild type hook length (Koroyasu et al., 1998). Furthermore, hook growth was shown to be initially fast followed by an exponential reduction in growth rate up to 55 nm in length after which the hook grows at a constant rate. This result suggests a hook-length control mechanism that controls hook growth up to 55 nm followed by a FliK-dependent inhibition of further hook elongation to prevent the “long monotonic tail” of extended polyhook structures, which extend up to 1.7 μ m on cells missing FliK.

A mechanism to explain the biphasic hook growth rates is based on the autocleavage of FlhB. The secretion specificity switch from rod-hook to late-type substrates requires a cleavage of the C-terminal cytoplasmic domain of FlhB (FlhB_{CC}) and the interaction of this domain with the C-terminus of FliK (Minamino et al., 2006). As with FlhB, the EscU and SpaS homologs of virulence-associated type III secretion systems undergo autocleavage (Zarivach et al., 2008). These systems are different in that FlhB undergoes autocleavage with a 5 min half-life after folding, while EscU and SpaS undergo autocleavage immediately after folding. However, in all three systems failure to autocleave prevents the secretion-specificity switch. All systems require both autocleavage followed by a conformational change in their cleaved C-terminal domains to switch to late secretion mode. In the flagellar system, this conformational change in FlhB_{CC} can occur spontaneously at a low frequency, and at higher frequencies in mutant strains, but is believed to be catalyzed efficiently through an interaction between FlhB_{CC} and FliK_C.

If the 55 nm peak of hook-length observed in the *fliK* null mutant were due to FlhB autocleavage, then we would not expect the same hook-length distribution in the *flhB* autocleavage mutant. Accordingly, hook length distribution was measured in the *flhB* autocleavage mutant. Figure 4A shows the measurements of hook structures in a strain harboring the N269A autocleavage-defective FlhB mutant. This mutant does not show a peak at 55 nm as was shown for the *fliK* null strain and instead gives a broad length distribution of hook structures. Importantly, a *fliK flhB*_{N269A} double mutant also shows no hook length control at wildtype 55 nm, demonstrating that the autocleavage-defective FlhB mutant is dominant over the *fliK* deletion and that hook length control is completely abolished in the absence of FlhB autocleavage (Figure 4B). We conclude that upon initiation of HBB assembly, hook secretion stops or is substantially slowed after a certain time. FlhB autocleavage could control hook-length in the absence of FliK, if cleaved FlhB is unable or has a reduced ability to secrete FlgE.

FliK deletion variants that retain hook-length control are secreted

The *Y. enterocolitica* YscP protein was proposed to act as a molecular ruler that physically measured the distance from the tip of the needle to the cytoplasmic base of the secretion apparatus (Journet et al., 2003). The *S. enterica* FliK protein was proposed to act as a ruler that could somehow measure the final hook length in the cytoplasm, presumably prior to hook secretion (Shibata et al., 2007). This conclusion was based on the finding that some of the FliK deletion variants retained the ability to measure hook length, but were not found in the external medium. A problem with this interpretation is the fact that the FliK deletions, which were not in the secreted fraction, were also absent in the cellular fraction. This

suggests that these variants were simply unstable and not present in sufficient quantities to be readily detected in the external medium. We repeated the secretion experiments using anti-FliK antibodies that are more sensitive than those used in the previous study. As shown in Figure 5, the FliK deletion variants were less stable in the cytosolic fraction than wildtype FliK, but in all cases, the FliK deletion variants were secreted from the cell. Thus, the instability of the FliK deletion variants resulted in reduced amount of protein in the cytoplasm and even less in the secreted fraction, such that they were not detected in the secreted fractions in the previous study. With the improved detection method that includes a more sensitive anti-FliK antibody preparation we were able to detect the FliK truncated proteins, which retained hook-length control, in the secreted fraction. These results refute the internal (cytoplasmic) ruler model.

Concluding remarks

Previously, several alternative models have been proposed for the mechanism of flagellar hook-length control: i) the measuring tape model (Figure 6A), where FliK constantly measures hook length during hook subunit secretion while being attached to the hook cap FlgD (Minamino & Pugsley, 2005); ii) the internal ruler model, where FliK somehow measures the final hook length in the cytoplasm (Shibata et al., 2007); iii) the measuring cup model (Figure 6B), where the C-ring is filled with FlgE subunits thereby preventing a FliK-FlhB interaction (Makishima et al., 2001); or iv) the molecular clock model (Figure 6C), where autocleavage of FlhB is the timing device that switches secretion specificity after a certain time (Moriya et al., 2006). Importantly, all previously proposed models of flagellar hook-length control conflict with published data of hook lengths under conditions where the ratio of secreted FliK to FlgE molecules is changed and thus those models cannot explain the mechanism of flagellar hook-length control sufficiently (see below).

We refute previous models for hook-length control (Figures 6A–C) and hereby propose a unifying, new model that accounts for all published data on hook-length control where FliK acts as a molecular ruler taking temporal measurements of rod-hook length throughout the assembly of the external axial component of the HBB (Figure 6D), thus providing experimental evidence for a similar model previously proposed for the YscP needle-length control (Cornelis, 2006). We propose that the ratio of FliK rulers secreted per hook monomers secreted would determine the average hook length. Thus, any situation that results in an increase in FliK measurements per time it takes to complete the HBB will produce shorter hooks and the converse, any situation that results in a decrease in FliK measurements per time it takes to complete the HBB will produce longer hooks.

In this temporal ruler model, FliK acts as a molecular ruler that takes measurements of rod-hook length while being intermittently secreted throughout the assembly process of the HBB complex and the number of secreted FliK ruler molecules per time it takes to complete the HBB defines the ultimate length of the flagellar hook (Figure 6D).

Here, we show that this temporal ruler model in conjunction with FlhB autocleavage accounts for the short-hook mutants in *fliG*, *fliM* and *fliN*, the structural genes for the C-ring complex (Makishima et al., 2001), as well as for the polymerization-defective hook mutants (Moriya et al., 2006). Similar to the hook polymerization-defective mutants, we found that one class of the short-hook mutants in *fliG*, *fliM* and *fliN* take longer to build the HBB structure and switch to late secretion specificity. In both the hook polymerization-defective and C-ring mutants, the more frequent FliK measurements per time it takes to complete the HBB result in shorter hooks.

In summary, our model of FliK taking temporal measurements of hook lengths is consistent not only with the FlgE polymerization-defective mutants (Moriya et al., 2006), but also

previous findings of effects of over/under-expression of FlgE or over/under-expression of FliK on flagellar hook length (Minamino et al., 1999, Muramoto *et al.*, 1999, Muramoto *et al.*, 1998). It has been reported that over-expression of FlgE or under-expression of FliK resulted in longer hooks (Muramoto et al., 1999, Muramoto et al., 1998). These results can be explained with fewer FliK measurements per time it takes to assemble the HBB because of the changed ratio of secreted FliK to secreted FlgE molecules, therefore resulting in longer hooks. On the contrary, under-expression of FlgE or over-expression of FliK produced shorter hooks (Muramoto et al., 1999, Muramoto et al., 1998). As it is the case for C-ring mutants or the hook polymerization-defective mutants (Moriya et al., 2006), the under-expression of FlgE or over-expression of FliK changes the ratio of secreted FliK to FlgE molecules in a way that FliK measures hook length more frequently in the time it takes for HBB completion. This produces shorter hooks. Consistently, over-expression of the hook polymerization-defective mutants rescued the short-hook phenotype and resulted in hooks of wildtype length (Moriya et al., 2006). This can be explained with the changed ratio of secreted FliK rulers to over-expressed FlgE mutant subunits. Because the FlgE mutants are defective in polymerization, in this case the over-expression of mutant FlgE produces wildtype hooks because of fewer FliK measurements per time it takes for HBB completion.

Experimental procedures

Bacterial strains, plasmids and media

All bacterial strains and plasmids used in this study are listed in Supplemental Table 1. Cells were grown in either Luria broth (LB) or TB broth (1% Tryptone and 0.5% NaCl) and, when necessary, supplemented by chloramphenicol (12.5 mg/ml) or tetracycline (15 mg/ml). The generalized transducing phage of *S. typhimurium* P22 *HT105/1 int-201* was used in all transductional crosses (Sanderson & Roth, 1983).

SDS-PAGE and Western blotting

Whole cell fractions and culture supernatant fractions were prepared by described methods (Hirano *et al.*, 2005). Silver staining of the gel was performed as described (Morrissey, 1981). Anti-FliK antibody was kindly provided by Keiichi Namba. Specific protein detection was performed using ECL plus Western blotting detection reagents (Amersham Biosciences).

Binding assays

Binding assays of FlgE to FliN, FliM, FliG or FliK were measured using a GST pull-down assay (Paul *et al.*, 2006) with minor modifications as outlined in the Supplemental Experimental procedures.

Luciferase assays

Plasmid pRG19 harboring *Salmonella enterica* serovar Typhimurium *motA::luxCDABE* was used for monitoring flagellar class 3 gene expression (Goodier & Ahmer, 2001). The flagellar master operon was induced using a *tetA* promoter placed upstream of *flhDC* (*flhDC::T-POP* (Karlinsey et al., 2000)), and the cultures were grown in 96-well microtiter plates (Greiner Scientific) at 30 °C. OD₅₉₅ and luciferase light production was measured over time using a PolarStar Optima microplate reader (BMG labtech). After background correction, the relative light units (RLU) were calculated as follows:

$$RLU = \text{light} \cdot \left(\frac{OD_{595}^{t=0}}{OD_{595}^{t=n}} \right).$$

Half-maximal induction times ($t_{\frac{\max}{2}}$) of each mutant strain were determined from at least three independent, biological samples and normalized to the wildtype control of the same

microtiter plate. The incomplete gamma function $f_{(t/b,a)} = \frac{1}{\Gamma(a)} \int_0^{t/b} x^{a-1} e^{-x} dx$ was used for parameterizing the individual gene expression trajectories as described previously (Brown et al., 2008). Non-linear curve fitting was performed using the *nlinfit* function of the Optimization Toolbox of Matlab 7.4 (Mathworks). Subsequently, the half-maximal induction time was calculated using $t_{\frac{\max}{2}} = a \cdot (b - 1)$ with given estimates for *a* and *b*. One-way ANOVA with Dunnett's post test was employed to compare statistical significances between half-maximal induction times of the C-ring mutants relative to the respective wildtype control. All statistical analysis were performed using GraphPad Prism 5.0b for Macintosh (GraphPad Software, San Diego California USA, www.graphpad.com).

Hook-length measurement

For hook-length measurements, flagellar structures were isolated and the hook-basal bodies were prepared as described (Hirano et al., 1994).

Fluorescent microscopy

For fluorescent microscopy analysis, cells were grown to mid-log phase and immobilized with poly-L-lysine treated coverslips. Cells were fixed by addition of formaldehyde (5% final). DNA and membrane staining was performed using DAPI and FM-64 (0.5 µg/ml). Flagella were stained using polyclonal anti-FliC antibodies (rabbit) and anti-rabbit-Alexa488 secondary antibodies (Invitrogen). Images were collected using an Applied Precision optical sectioning microscope and deconvolved using softWoRx v.3.4.2 (Applied Precision).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic overview of the bacterial flagellum

The structure of the flagellum can be divided in three parts: 1) a basal body consisting of the MS-ring in the inner membrane (IM), the cytoplasmic C-ring, the P-ring in the peptidoglycan layer (PG), the L-ring in the outer membrane (OM), the rod spanning the periplasmic space and the type III secretion apparatus assembled within the MS-ring in the inner membrane; 2) a flexible hook with hook-associated proteins; 3) the rigid filament and the filament cap.

Dashed boxes indicate proteins that function in flagellar type III secretion, either in the membrane-bound part of the secretion apparatus or in delivery and unfolding of the secretion substrate. Flagellar components that depend on export are indicated in light gray (early substrates) or dark gray (late substrates). Export-dependent components are structural proteins that form the rod, hook, and filament, the negative regulator of late substrate gene expression, FlgM, and the hook-length regulator FliK.

Figure 2. Filament assembly and hook length in the absence of the C-ring. Interaction of C-ring subunits with the hook

(A + B) Fluorescent microscopy of C-ring null mutants (*fliG*) that assemble one, unusual long flagellum under excess secretion substrate conditions (*flgM*, P_{flhD}^*) and increased protonmotive force (*atpA*). Filament was detected by anti-FliC polyclonal antibodies (rabbit) and stained using anti-rabbit-Alexa488 (green), membranes were stained using FM-64 (red) and DNA was stained using DAPI (blue). Scale bar = 2 μ m. Staining of wild type cells is shown in Supplemental Figure 1. (C) Hook-length peaks around 58 nm in the absence of the C-ring. Hook-length distribution in the C-ring null mutant that assembles filaments (*fliG flgM*, P_{flhD} *atpA*) as measured by electron microscopy. Overall hook length is not completely controlled (71 ± 26 nm, $n = 43$), but peaks around wildtype 55 nm hook length. Non-linear fitting of the Gaussian distribution was employed for hook-lengths below 100 nm (dashed red line) and the mean hook-length of the majority of hooks is 58 ± 18 nm ($n = 38$) compared to 55 ± 6 nm of the wildtype (Hirano et al., 1994). (D) Electron-microscopic images of hooks and filaments isolated from the C-ring null mutant (*fliG flgM*, P_{flhD} *atpA*). Scale bar = 200 nm. (E) C-ring subunits do not interact with the hook. The interaction of the C-ring with the hook subunit FlgE was assayed using GST-pulldown assay and immunoblotting using specific antibodies against the C-ring subunits FliG, FliM and FliN, as well as FliK respectively. As a control, the previously reported interaction of FlgE with FliK was tested (Moriya et al., 2006).

Figure 3. Time for hook-basal body completion is prolonged in short-hook mutants
 (A–D) Dynamic comparison of *motA* promoter induction times in (A) *fliN1784*, *fliN1809*, *fliN1810*, (B) *fliG2834*, (C) *fliM1764*, *fliM1806*, *fliM1813* and (D) *flgE2219* (T149N) short-hooks producing mutants. *flgE2219* (T149N) is defective in hook-polymerization (Moriya et al., 2006). Completion of hook-basal bodies (= corresponds to induction of the *motA* promoter) was monitored over time following the induction of flagellar gene expression as described in Experimental procedures. The dashed line at 82 min indicates the calculated half-maximal *motA* induction time of the wildtype control. Dashed red line = wildtype control, RLU = relative light units. Data shown are the average of at least three independent, biological replicates. (E) Relative *motA* half-maximal promoter induction

times of the first class of short-hooks producing mutants. Half-maximal *motA* promoter induction times were calculated using the incomplete gamma function as described in Experimental procedures and normalized against the wildtype control. Data shown are mean $\pm$ SD.

Figure 4. Hook length is not controlled in an *flhB* mutant defective in autocleavage
 (A) Hook-length distribution of the *flhB*_{N269A} autocleavage-defective mutant. Hook-length control is completely abolished and does not peak at the wildtype length of 55 nm. (B) Hook-length distribution of the *fliK flhB*_{N269A} double mutant. Hook-length control is completely abolished in the combination mutant and does not peak at the wildtype length of 55 nm compared to the *fliK* deletion mutant (Koroyasu et al., 1998).

Figure 5. FliK deletion variants are secreted

FliK truncation mutants that were previously reported to produce short hooks (Shibata et al., 2007) are secreted outside of the cell. A Δ *flgE* deletion mutation was introduced to facilitate FliK secretion. (A) Silver-staining of supernatant fractions. Proteins secreted into the culture supernatant were precipitated by 10% TCA. White arrowheads indicate positions of FliK variants. (B) Anti-FliK immunoblotting of the same culture supernatant fractions as in (A). (C) Anti-FliK immunoblotting of cellular fractions. Non-specific bands are only visible in the cellular fraction and can be used to compare equal loading. Whole cell fractions were disrupted by sonication and mixed with 5 $\times$ SDS-sample buffer.

Figure 6. Comparison of flagellar hook-length control models

(A–C) Previously suggested models for flagellar hook-length control. (A) FliK acts as a measuring tape that constantly measures hook-length during hook subunit secretion while being attached to the hook cap FlgD (Minamino & Pugsley, 2005). (B) The C-ring acts as a measuring cup filled with hook subunits that prevent FliK-FlhB interaction prior to hook completion (Makishima et al., 2001). (C) FlhB autocleavage as a molecular clock controlling hook-length in a temporal manner (Moriya et al., 2006). (D) Temporal, molecular ruler model. The molecular ruler FliK and hook subunits FlgE are intermittently secreted during the hook-basal body assembly process. Accordingly, FliK takes several, independent measurements of rod-hook length in a temporal manner. Interaction of the FliK N-terminus with the hook subunits FlgE and the hook cap FlgD at the end of the growing hook structure results in a pause that enables the C-terminus of FliK to interact with FlhB and thereby catalyzing the secretion specificity switch if the hook has reached the proper length. The red star at the N-terminus of FliK signifies a potential interaction with the hook cap as the N-terminus reaches the exit portal and the red star at the C-terminus of FliK a potential interaction with FlhB that stimulates the secretion-substrate specificity switch. Exported flagellar components are indicated in light gray (early substrates) or dark gray (late substrates). Dashed boxes represent components of the type III secretion apparatus. The hook-length regulator FliK is highlighted in orange. OM = outer membrane; PG = peptidoglycan; IM = inner membrane.

Class I C-ring mutants producing short hooks

Table 1

Summary of the properties of the first class of C-ring mutants *flhG2834*, *flhM1813*, *flhN1784*, *flhN1809*, *flhN1810* and the polymerization-deficient mutant *flgE2219* (T149N). Mutation sites of the mutants were confirmed by sequencing and hook lengths were taken from (Makishima et al., 2001). The ability of the mutants to secrete FlgE-Bla was assayed as described in Supplemental Experimental Procedures and is reported as the minimal-inhibitory concentration of ampicillin (µg/ml). Half-maximal induction times $\pm$ SD of the *motA* promoter indicate hook-basal body completion and were compared to the wildtype (82 $\pm$ 3.5 min) as described below. The half-maximal *P_{motA}* induction times of the analyzed C-ring mutants were significantly different compared to the wildtype control (P value < 0.01). One-step ANOVA with Dunnett's post test was employed to compare statistical significances to the wildtype control. SD = standard deviation.

gene	allele	mutation	reported hook length (nm) (Makishima et al., 2001) or (Hirano et al., 1994)	MIC (amp µg/ml)	Half maximal <i>P_{motA}</i> induction time (min)
WT	-	-	55 $\pm$ 6	>400	82 $\pm$ 3.5
<i>flhG</i>	<i>flhG2834</i>	R160H	44 $\pm$ 7	400	90 $\pm$ 2.5
<i>flhM</i>	<i>flhM1764</i>	F131L	46 $\pm$ 4	200	92 $\pm$ 1.3
	<i>flhM1806</i>	G133D	45 $\pm$ 5	200	92 $\pm$ 0.5
	<i>flhM1813</i>	H106P	42 $\pm$ 7	200	91 $\pm$ 1.4
<i>flhN</i>	<i>flhN1784</i>	G103V	47 $\pm$ 7	400	96 $\pm$ 1.2
	<i>flhN1809</i>	L78Q	44 $\pm$ 6	400	88 $\pm$ 0.8
	<i>flhN1810</i>	N24 (1bp)	46 $\pm$ 5	200	94 $\pm$ 1.6
<i>flgE</i>	<i>flgE2219</i>	T149N	45 $\pm$ 10	n.d.	106 $\pm$ 3.0