Flagellar Hook Length Is Controlled by a Secreted Molecular Ruler

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In 1973, when I was still in high school, the flaR gene of Salmonella was described (30). Null alleles of the flaR gene produced unusual flagellar structures. Instead of the normal basal body-hook-filament structures, flaR mutants resulted in basal bodies with attached hooks of uncontrolled lengths. The paper contained beautiful electron micrographs showing structures, termed “superhooks,” extending hundreds of microns from the cell surface. An important observation from that paper was that about 1% of flaR mutant cells were motile due to the presence of a single flagellum extending from a “superhook” structure. Thus, the presence of extended hook structures did not prevent filament formation; rather, filament formation did not occur at high frequencies in flaR mutants for some other reason. During the next 20 years, the names of the structures and the associated gene changed from “superhook” to “polyhook” and flaR to fliK. Null mutants in fliK proved useful for the physical and biochemical analysis of the hook structure due to their size relative to wild-type hooks, which are about 55 nm long.

The reason fliK mutants do not produce polyhooks with attached filaments has to do with a secretion-specificity switch in the flagellar-associated type III secretion (T3S) system. Flagellar biogenesis follows an ordered assembly pathway (4). The first structure assembled is the flagellar rotor, sometimes called the C-ring structure, which is anchored to the cytoplasmic membrane at the membrane-embedded MS ring. The remaining basal body structures assemble outside the cytoplasm. This requires the assembly of the T3S apparatus for their secretion (24). The flagellar T3S system is composed of integral membrane proteins that are believed to assemble in the center of the rotor. A rod structure next assembles that extends the basal body about 23 nm to the outer membrane (33). The basal body penetrates the outer membrane with the attachment of the PL-ring structure, which is thought to form a pore in the outer membrane (5). Following PL-ring formation, the hook polymerizes on the rod tip, extending from the cell surface about 55 nm (11). It is at this point in hook polymerization that FliK exerts its effect, which is to stop hook polymerization and allow for a transition of the secretion of rod-hook substrates to the secretion and polymerization of the long flagellar filament, whose rotation results in motility. Thus, FliK catalyzes the secretion-specificity switch in the flagellar T3S system at a hook length of about 55 nm from the rod-hook secretion class to the filament or late class of secretion substrates.

A clue as to how FliK catalyzed the secretion-specificity switch came from work in the laboratories of Shin-ichi Aizawa and Kazuhiro Kutsukake. In 1994, both laboratories reported the isolation of suppressor mutants in the flhB component of the flagellar T3S system that allowed a high frequency of filament formation in fliK null mutants (11, 19). These strains still produced polyhooks, but many of these polyhooks had attached filaments. Motility was not great relative to that of the wild type but was markedly better than that of the fliK null strain. The implications of these findings and subsequent follow-up work on fliK and flhB were that FlhB, an integral membrane component of the flagellar T3S system, was the secretion-specificity determinant and that FliK was responsible for communicating hook completion to FlhB and catalyzing the secretion-specificity switch.

In 1999 and 2000, two papers, from the laboratory of Robert Macnab, with Tohru Minamino as primary author of both, reported two remarkable discoveries (23, 25), first, that FliK is secreted as a rod-hook secretion substrate and, second, that FlhB is cleaved into a membrane-anchored N-terminal domain and soluble C-terminal domain. The secretion of FliK provided a mechanism by which FliK could somehow assess hook-length completion, and the cleavage of FlhB could explain the secretion-specificity switch. Indeed, mutants in FlhB, unable to undergo cleavage, failed to transition from rod-hook substrate secretion to late substrate secretion, suggesting that FlhB cleavage was part of the switch. However, coexpression of the cleaved halves of the flhB gene showed significant complementation of an flhB null allele (about 50% wild-type phenotype on motility agar). This suggested that FlhB cleavage was not a complete secretion-specificity switch but was probably an extension of substrate secretion from the rod-hook to the rod-hook-filament type and that cessation of rod-hook secretion might occur through a mechanism different from that of FlhB-dependent substrate recognition.

By the end of the year 2000 we were left with a model in which secreted FliK sensed hook completion and signaled FlhB cleavage to allow filament polymerization on completed hook structures. It was ruled out that FliK could act as a molecular ruler (15); at least, it could not do so by the mechanism by which lambda H protein acts as a molecular ruler to control tail length in bacteriophage lambda (14). Deletions of the lambda H gene corresponded to shorter phage tails, suggesting that lambda H protein acts as a physical scaffold for tail protein subunits to assemble onto. In the case of FliK, deletions resulted in longer hook structures, suggesting that FliK measures hook length by a mechanism completely different from that by which lambda H controls phage tail length.

Then came the battle of the Science papers, the first from the Aizawa laboratory in 2001 (20) and the second from the Cornelis laboratory in 2003 (13). These papers provided evidence for two competing and seemingly incompatible models to explain how FliK might coordinate hook completion with the FlhB-dependent secretion-specificity switch. Both models were elegant.

The Aizawa laboratory published what became known as the “cup model” for hook-length control (20). They obtained mu-
tants in the C-ring genes, *fliG*, *fliM*, and *fliN*, which produced shorter hook structures. The C-ring is composed of roughly 30 subunits of FlgE, 30 subunits of FliM, and 120 subunits of FliN. The hook is composed of roughly 120 subunits of FlgE hook subunits. The “cup model,” as I understood it, proposed that the C-ring proteins when assembled provided sites of interaction for the FlgE subunits that made up the hook. Upon rod and PL-ring completion, the C-ring “cup” that was filled with FlgE subunits emptied to produce a hook of a defined length of about 55 nm. Once emptied, FliB was exposed to FliI to allow FliK-dependent catalysis of the secretion-specificity switch to late subunit secretion. The *fliG*, *fliM*, and *fliN* shorter-hook mutants were presumed to have lost a last of FlgE binding sites so that these C-ring “cups” had fewer bound FlgE subunits and, when these cups were emptied, they produced shorter hook structures. An initial flaw with this cup model was the result that strains defective in *flgD*, the hook-cap gene, secreted FlgE subunits continuously but did not undergo the secretion-specificity switch (18).

The Cornelis laboratory published what became known as the molecular ruler model (13). Their system was the *Yersinia* type III injectisome system. In this system, the injectisome needle structure is controlled by a FliK functional homolog, YscP. Similar to the effect of FliK on flagellar hook-length control, loss of YscP resulted in uncontrolled, continuous needle growth. I visited the Cornelis laboratory in the fall of 2003 and was presented these results by Laura Journet and Céline Agrain, a postdoctoral fellow and a graduate student who worked on the needle length control project. Their study had built upon the observation that complementation of a *Yersinia enterocolitica* yscP null mutant by the *Y. pestis* yscP gene resulted in shorter needle lengths than complementation by the *Y. enterocolitica* yscP+ gene. Because the YscP protein from *Y. pestis* is 455 amino acids in length compared to 515 for the *Y. enterocolitica* YscP, they inferred that YscP might work as a molecular ruler: a shorter ruler resulted in shorter needles. They made a series of insertions and deletions in YscP and found that needle length increased or decreased by 0.19 nm per amino acid inserted or deleted. They even inserted an amino acid sequence from FliK and had the same 0.19-nm increase in length per amino acid inserted. The Cornelis laboratory also showed that FliK and YscP were more than just functional homologs; they and other needle length control proteins all contained a conserved C-terminal structural domain that they named the type III secretion substrate specificity switch or T3S4 domain (1). This domain had been implicated as involved in control of the secretion-specificity switch in the flagellar system by earlier work in the Macnab laboratory, whereas the N-terminal domain had been shown to include the secretion signal (15, 23, 35). Based on the work of the Cornelis laboratory, a model emerged where the YscP ruler was secreted during needle polymerization, with the N terminus of YscP interacting with the needle cap. Eventually, needle growth would bring the YscP T3S4 domain within the vicinity of the FliB homolog of the *Yersinia* T3S system to flip the secretion-specificity switch and terminate needle growth (6). By analogy, in the flagellar system, secreted FliK would interact with the hook cap during hook polymerization; thus, when the hook polymerized to a length of about 55 nm, the FliK T3S4 domain would be in proximity to FliB to catalyze the secretion-specificity switch, terminate hook polymerization, and initiate filament assembly (27).

After I met with Laura Journet and Céline Agrain and saw their data on the molecular ruler mechanism for needle length control, I decided that my laboratory should do a similar study with FliK. There was a void in the flagellar field, as Robert Macnab had passed away a month prior to my visit to Basel, Switzerland, in 2003, so I decided to initiate work on FliK and test if it could act as a molecular ruler in the manner that the Cornelis laboratory had shown was the case for YscP. I asked my research scientist, Joyce Karlinsky, to make a series of insertions of YscP sequence at different sites between the N-terminal secretion signal of FliK and the T3S4 domain, which she did, and we sent them to Shin-Ichi (Chi) Aizawa to determine if any resulted in increased hook length. We waited a year before the Aizawa laboratory had time to look at our FliK inserts. Then, finally, in the early months of 2005, the email came. It turned out that all the FliK inserts resulted in increases in the hook lengths of about 0.17 nm per amino acid, similar to the YscP insertion results (32). At that time, Fabienne Chevance joined the laboratory, and over the next year Fabienne and Joyce made construct after construct according to Chi Aizawa’s specifications to exhaustively determine which regions of FliK could be deleted to produce shorter hooks. While all insertions we tried made longer hooks of controlled lengths, there were just two small regions in the 405-amino-acid FliK protein where deletions could be tolerated, amino acids 161 to 202 and 238 to 278. All other deletions resulted in the *fliK* null phenotype of polyhook formation.

At this time, differences in opinions between Chi and me on the meaning of our study emerged. I was convinced that our data supported a model similar to what the Cornelis group proposed for YscP, while Chi was convinced that FliK was a ruler that somehow measured the C-ring cup. In my mind the cup model was losing ground. I had obtained the C-ring mutants that resulted in shorter hook structures and made a series of double mutants, thinking that double mutants might result in even shorter hook structures than the single mutants if multiple FlgE binding sites in the cup were lost in the double-mutant constructs. However, I sent the double mutants to Chi and he reported to me that they produced hooks of wild-type length. Furthermore, the crystal structure of the hook protein was then solved in the laboratory of Keiichi Namba at Osaka University (31). The volume of the C-ring cup at most could not be more than 40 FlgE subunits, not the more than 120 that make up a 55-nm hook (4).

In the fall of 2006, Keiichi Namba had invited me to spend 3 months in his laboratory at Osaka University as a visiting professor. Tohru Minamino had joined the Namba laboratory after his postdoctoral work with Robert Macnab and, along with a graduate student, Nao Moriya, continued working on, among many other things, FliK and hook-length control. I was asked to edit a paper of Nao’s work (29). There were two major findings reported in that paper. The first major finding was that the N terminus of FliK interacted strongly with the hook cap, FlgD, and, to a lesser extent, with hook protein FlgE. The second was that polymerization-defective hook mutants produced shorter hook structures, except when overexpressed, in which case they produced hooks of wild-type length. It was as though there existed a timing device, a “molecular clock”: once hook polymerization began, the clock started, and after a certain time period, hook polymerization stopped and the secretion-specificity transition occurred. The strong interaction between the FlgD hook cap and the N terminus of FliK supported the Cornelis molecular ruler model where the FliK N terminus would interact with the hook cap and, when the hook length reached 55 nm, the FliK T3S4 domain would be in
the vicinity of FlhB to catalyze the secretion-specificity switch. The “molecular clock” idea from the polymerization-defective flgE mutant studies would later explain the results of the C-ring mutants associated with the cup model that resulted in shorter hooks.

By the end of the first decade of the 21st century, a pile of data had accumulated on the role of FliK in catalyzing the secretion-specificity switch in FlhB in coordination with hook completion. The Aizawa laboratory showed that FliK secretion was not required for the switch to occur; however, they showed that FliK secretion was required for the switch to occur in coordination with hook length control (10). Overexpression of a nonsecreted form of FliK could catalyze the secretion-specificity switch, but hook-length control was lost. I interpreted this to imply simple mass action: secretion of limiting amounts of FliK was required to get the FliK T3S4 domain to communicate efficiently with FlhB in timing with hook completion, but by massively overexpressing FliK deleted for the N-terminal secretion signal, an interaction would be forced independently of secretion and regardless of hook length. More data from Minamino, Namba, and colleagues supported the idea of the role of the FliK T3S4 domain in the secretion-specific switch (22, 28), and the thesis work of Hedda Ferris showed that the FlhB cleavage reaction could occur independently of interaction with FliK (8). This suggested that FliK probably stimulated an autocleavage of FlhB but also induced a conformational change in the cleaved cytoplasmic C-terminal domain of FlhB to allow late substrate secretion (9). I believe that this explains the early finding that about 1% of cells with fliK null alleles produce polyhooks with attached filaments. Presumably, the FlhB conformational change following an autocleavage event occurs at a low frequency spontaneously, which accounts for the low frequency of the motile cells in a fliK null strain.

Our 2007 collaboration paper with the Aizawa laboratory showed that FliK worked as a molecular ruler. The title named FliK an internal ruler. For Chi, “internal ruler” meant that it is internal to the rod hook during FliK secretion (12). Chi’s main argument against FliK being a molecular ruler was that this explains the early finding that about 1% of cells with fliK null alleles produce polyhooks with attached filaments. Presumably, FliK conformational change following an autocleavage event occurs at a low frequency spontaneously, which accounts for the low frequency of the motile cells in a fliK null strain.

Our 2007 collaboration paper with the Aizawa laboratory showed that FliK worked as a molecular ruler. The title named FliK an internal ruler. For Chi, “internal ruler” meant that it is internal to the cytoplasm and functions to measure the cup (2, 3). For me, “internal ruler” meant that it is internal to the rod hook during FliK secretion (12). Chi’s main argument against FliK directly measuring rod-hook length came from FliK deletions that controlled hook length but were not secreted. However, with better FliK antibody, we later showed that these deletion mutants were, in fact, secreted, effectively killing his argument. As a coup de grâce to the C-ring physical cup model, we had created a strain that would grow flagellar structures without any C-ring at all. We sent this strain to Chi, asking him to measure hook lengths, and the result came back that strains devoid of C-ring structures produced hook filaments with controlled hook lengths averaging 58 nm. Furthermore, we showed that the C-ring cup mutants, which produced short hooks, were defective in hook secretion. Thus, as Nao Moriya had shown with polymerization-defective flgE alleles (the molecular clock model), the length of time it takes to grow a hook corresponds to an increasing probability that the secretion-specificity switch would occur at hook lengths shorter than 55 nm.

Our most recent publication on the role of FliK in hook length control was published in 2011 in The EMBO Journal (7). There we teamed up with Jim Keener to produce a paper whose results support what I believe is a complete mechanism to explain all published data on FliK-dependent hook length control. Jim is a mathematician at the University of Utah who has an interest in biological questions and made important contributions to the human genome project. When I gave my job talk at the University of Utah in October of 2003 just after my visit to Basel, I mentioned FliK and hook length control in my talk. Jim became intrigued by my talk and began working on modeling hook length control by FliK (16). On the basis of all the available data, Jim predicted that FliK must work after hooks reach a length specified by the length of FliK (17). What Jim predicted, and we then verified experimentally, was that FliK is secreted continuously during hook polymerization and that, regardless of how long the hooks are, after they reach a certain length, the next FliK molecule that is secreted through the rod-hook channel catalyzes the secretion-specificity switch. This explains why overexpression of FliK results in hooks of 43-nm average lengths and not 55 nm. It is because there are more FliK measurements taken during hook polymerization, allowing the switch to occur at a shorter length. The same is true for the C-ring cup mutants and flgE polymerization-defective molecular clock mutants: more FliK measurements are taken during hook polymerization, resulting in shorter hooks. We also showed that the rate of FliK secretion determined the ability of the 75S domain of FliK to interact with FlhB to flip the switch. We know that in a strain deleted for the hook gene, FliK is continuously secreted and yet fails to flip the FlhB secretion-specificity switch. That is because prior to hook completion FliK secretion occurs at a high rate, effectively preventing a productive interaction between the FliK T3S4 domain and FlhB. However, once the hook has reached a certain length, due to interactions of the N terminus of FliK with the hook cap and hook subunits (26), FliK secretion slows substantially, which presumably allows a productive interaction between the FliK T3S4 domain and FlhB to then flip the secretion-specificity switch (7).

There is only one final point to be cleared up. The Cornelis group proposes that YscP acts as a static ruler which stays in the channel during needle polymerization. Support for this model comes from the fact that simultaneous expression of short and long forms of FliK results in a bimodal production of short and long needles (34). If YscP rulers are continuously secreted during needle polymerization, then I would have expected that the short ruler would always flip the switch before needles became long enough for the longer ruler to act. We repeated this experiment in the flagellar system, and in our case, coexpression of short and long FliK rulers always yielded short hooks, as we expected, for a ruler that was continuously secreted during hook polymerization (7). We did not think that a static ruler mechanism could account for hook length control, because the channel is too narrow to accommodate a static ruler and allow hook subunits to pass by. I had thought that in the needle system, the needle subunits polymerized at the tip. However, if needle subunits polymerize at the base, then that would account for the findings of the Cornelis group. Polymerization at the needle base would also account for the work from Jorge Galán’s laboratory on the Salmonella Spi1 injectosome needle system and Scott Lloyd’s group on the Yersinia needle system that demonstrated a role for the inner rod protein in needle length control (21, 36). Needle polymerization from the base rather than the tip could account for the role of the inner rod in needle length control and still include a role for a static molecular ruler. Time will tell.

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New ideas keep coming to satisfy all data accumulated regarding hook length control (1). Let us look at some of them and see if they are well brushed up as concept or just whim (3, 4).

(i) **The molecular clock** (8). Length \( L \) and time \( T \) are independent parameters in physics. However, in our case, hook length \( L \) (in nanometers) and time \( T \) (in seconds) are intricately related to each other by the equation \( L = \nu T \), where \( \nu \) is the growth rate of the hook (in nanometers per second). The rate is possibly time-dependent, as we have previously shown by a statistical method (6). Thus, the molecular clock is a tautology. Then why do we bother with time? Let us stick to length alone (1, 3).

(ii) **The statistical ruler** (5). This model assumes that FliK is continuously secreted into the culture medium. OK. The Cornelis group showed that probably only one FliK molecule is required to determine length (8a, 9). OK. We showed that a FliK variant which lacked the N-terminal region and thus was not secreted under any circumstances still controlled length when it was overproduced (2). What is the truth?

(iii) **The proximal growth** (7, 10). Can a flagellum grow from the bottom? No! It was well established by the Asakura group in 1970s that the flagellum grows from the distal end of the filament. The concept called distal growth actually led us to further important discoveries such as the cap proteins and the type III secretion system (3, 4).

New ideas are welcome, but please do not forget the old concepts.

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Mystery of FliK in Length Control of the Flagellar Hook

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A long history of the long hook: the first paper regarding hook length control in the flagellum appeared about 40 years ago. Silverman and Simon (1972) found that a flaE (the previous name of fliK in Escherichia coli) mutant produced extraordinarily long hooks, which they named polyhooks, and concluded that the flaE gene "functions to control the length of the hook" (22). Around the same time, Patterson-Delafield et al. (1973) found that a flaR (the previous name of fliK in Salmonella enterica serovar Typhimurium) mutant similarly produced extraordinarily long hooks, which they named superhooks (20). "Polyhook" connotes a repeated end-to-end polymer of the hook, whereas "superhook" simply connotes a long hook. Retrospectively, "superhook" might have been the proper name, since there is no evidence so far to show that the lengths of long hooks occur as integral multiples of the wild-type hook length. But we respect history and call it a polyhook today. Ironically, later on, we found extraordinary long polyhooks and named them super-polyhooks (19).

Because this topic has a long history and has involved contradictory explanations of similar data from many research groups, it is not easy to present all experimental data in chronological order. Many ideas and models have come and gone, but none of them is fully satisfactory. In this article, I discuss the currently most popular model from other laboratories and present our new model, which naturally opposes the current model. Because of limited space, I have to skip most of the biochemical and genetic data which oppose the current model. Instead, I focus on physical aspects of the hook length control and begin with the statistics of hook length.

AN IMPORTANT MESSAGE FROM LENGTH DISTRIBUTION

In order to discuss length, it is crucial to begin with quantitative measurements of the hook length. Since the hook has a characteristic curved shape, it was difficult to measure the length of the hooks even with computer software for measuring curves without introducing errors. To make matters worse, the hook is thick (20 nm) in comparison with its length (55 nm), which would have caused further errors in measurements. Thus, it was desirable to measure straightened hooks. We modified the usual method of preparation of hooks for electron microscopy by staining grids using phosphotungstic acid at pH 4, because low pH and low temperatures cause a polymorphic transition of the hook shape from curved to straight.

The length distribution of the wild-type hook is rather broad, with an average of 55 nm and a standard deviation of 6 nm (5). This standard deviation is not a result of measurement errors. We find hooks as long as 80 nm, or as short as 30 nm, indicating that control of hook length is not tight at all. Furthermore, the broad, symmetric shape of the distribution suggests a stochastic process like diffusion rather than a deterministic one as seen with a physical ruler. Despite the large standard deviation, people tend to use the averaged value of length only, claiming that hook length is "well-controlled" at ca. 55 nm. The claim is misleading.

FIK IS THE KEY FACTOR IN HOOK LENGTH CONTROL

Since 1972, we have found all the proteins that play a role in hook length control. They are FlgE, FlgG, FlgK, FlgL, FliI, FliK, FliM, FliN, and FliB (3, 5, 12, 19, 23, 25) (Fig. 1). FliK is the most mysterious among them. (i) FliK is not incorporated into the flagellar structure, while the others are structural proteins of the flagellum (1). (ii) FliK is soluble and secreted (13). When it is properly secreted, the average length of the hook is kept to ca. 55 nm. When it is not properly secreted, hook length is uncontrolled and increases. (iii) FliK is bifunctional, controlling the hook length and changing secretion substrates (so-called substrate specificity switching) (6, 25). (iv) The most striking feature of FliK is that the molecular size of FliK is proportional to the hook length (21). These facts urge people to believe that the FliK molecule directly measures the length of a growing hook as if it were a physical ruler.

In addition to the unique features, FliK has a characteristic structure. (v) The N-terminal half of FliK (FliK\textsubscript{N}) is largely unstructured, while the C-terminal half (FliK\textsubscript{C}) contains a compactly folded domain that interacts with FliB to switch the secretion modes (16). These distinct structures are responsible for two separate functions: FliK\textsubscript{N} is directly involved in length control, while FliK\textsubscript{C} controls the hook length only by switching the substrate specificity of the secretion apparatus. It should be noted that these two functions are not independent of each other. When FliK\textsubscript{C} is absent, the hooks keep elongating into polyhooks even in the presence of FliK\textsubscript{N}. In contrast, as long as FliK\textsubscript{C} is present, the hooks elongate to produce polyhooks with filaments attached, the so-called polyhook-filament phenotype. Moreover, when FliK\textsubscript{N} is absent, overproduction of FliK\textsubscript{C} fragments gives rise to shorter hooks in the polyhook filaments (6). Therefore, it seems that FliK\textsubscript{C} plays a crucial role in ending hook elongation. What then is the role of FliK\textsubscript{N}?

CRITICISM OF THE PHYSICAL RULER MODEL

The physical ruler model claims that the FliK molecule measures hook length \textit{per se} (4, 9, 17, 24). It works like this: FliK\textsubscript{N} goes through a channel of the flagellar filament and interacts with FlgD, the cap protein of a growing hook, while FliK\textsubscript{C} forms a globular structure and stays in the cytoplasm (Fig. 2). When FliK\textsubscript{N} reaches
we have previously shown by a statistical method (11). We cannot measure the length of time it takes each hook to grow. For now, let us stick to measurable length rather than introducing immeasurable time (2).

The physical ruler model is popular at the moment, perhaps because the mechanism is simple and easily grasped. However, the physical ruler model goes against my understanding of the physical properties of proteins and peptides. First, it is difficult for me to imagine a stretched peptide working outside and inside the cell at the same time, and FliK$_{\Sigma}$ is not large enough to be a sensor on the order of, e.g., chemoreceptors in the membrane. Second, there is inconsistency in the proposed mechanism: when a growing hook is still shorter than the FliK$_{\Sigma}$ length, FliK$_{C}$ must not work, and instead FliK may be exported and another FliK molecule may wait for the hook to reach a proper length. But when a growing hook is at the proper length relative to the length of FliK$_{\Sigma}$, FliK$_{C}$ must act immediately to stop the hook elongation. This activity of FliK$_{C}$ is thus purposely altered according to hook length. People who favor the ruler model often use phrases such as “when the hook reaches its mature length of 55 nm” or “when the hook elongates to its defined length” (14). The phrases do not really explain the model, because they do not say how the proper length is detected. Indeed, it is impossible for a FliK molecule to detect the hook length before it is secreted in this model.

The molecular clock model (17) is a variant of the physical ruler model. Length ($L$) and time ($T$) are independent parameters in physics. However, in our case, hook length $L$ (in nanometers) and time $T$ (in seconds) are intricately related to each other by the equation $L = v'T$, where $v'$ is the growth rate of the hook (in nanometers per second). The rate is possibly time-dependent, as we have previously shown by a statistical method (11). We cannot yet measure the length of time it takes each hook to grow. For now, let us stick to measurable length rather than introducing immeasurable time (2).

HOW MANY FliK MOLECULES ARE SECRETED?

It is important to know the number of FliK molecules secreted during assembly of a mature hook. In the original ruler model, FliK is assumed to be secreted several times until the hook length comes close to the FliK$_{\Sigma}$ length. I and others previously showed that a FliK variant which lacked the FliK$_{\Sigma}$ and thus did not secrete FliK$_{C}$ at all produced normal hook lengths when the variant was overproduced (6). We also showed that amounts of the FliK variants, which have small deletions at various points in FliK$_{\Sigma}$ vary from an undetectable level (probably one FliK molecule per hook) to a very high level, although all the variants give rise to controlled hook lengths (21).

PROBLEMS WITH THE MEASURING-CUP MODEL

We previously proposed a model in which the C ring measures the amount of FlgE and subsequently determines hook length (12). Remember that a mutant devoid of the fliK gene produces polyhooks. The length distribution of polyhooks, however, shows a peak at 55 nm with an exponentially decreasing tail. Analysis of the two-phase curve suggested that hooks quickly grow to a length of ca. 55 nm and then continue to grow at a low but constant rate (11). If FliK were sporadically secreted, the likelihood would be high for FliK to go through the gate when the hook length is around 55 nm, and whether it does so could be determined by the amount of FlgE initially accumulated in the cup.

To test if the C ring could form a measuring cup, we constructed mutants lacking each component of the C ring. The mutants cannot form an intact C ring and thus cannot produce intact flagella. They, however, can produce a few intact flagella, if FliI (ATPase for secretion) is overproduced, which would enhance the secretion of flagellar proteins. The hook lengths in the C-ring mutants with overproduced FliI range from 10 nm to more than 100 nm in the presence of the intact FliK. The average length is ca. 65 nm (10). These data suggest that the C ring is necessary for tight control of the hook formation but not for determination of the hook length itself, arguing against the measuring-cup model.

THE WAITING-ROOM MODEL

On proposing a new model, I would like to make one assumption, namely, that flagellar proteins accumulate within and around the C ring prior to secretion (Fig. 3). It is likely that the flagellar proteins to be secreted are localized at the basal area of the flagellum, because each flagellum grows independently from others in peritrichously flagellated bacteria. The C ring may act as the docking station for the soluble export proteins. In addition, we remember several facts: (i) FliK$_{\Sigma}$ interacts with FlgD and FlgE (13); (ii) FliK$_{\Sigma}$ is necessary for secretion (6, 14), (iii) the C terminus of FliK is necessary for completing the whole process (6, 15), and (iv) FliK$_{C}$ interacts with FlhB (18).

When the basal area is filled with FlgD/FlgE, the N terminus of FliK (circled “N” in Fig. 3) cannot reach the secretion gate because it is held back by binding to FlgD/FlgE. The C terminus of FliK (circled “C” in Fig. 3) is anchored at FlhB in the C ring. FliK$_{\Sigma}$ is necessary for completing the whole process (6, 15), and (iv) FliK$_{C}$ interacts with FlhB (18).
covered with bound FlgE which is surging to the gate. When amounts of FlgD/FlgE decrease or are emptied from the basal area, then the N terminus of FliK has a chance to access the gate. When FliK_N enters the gate, FliK_C has a chance to bind to the FlhB of the gate. When FliK_N goes through the channel, FliK_C is pulled and detached from FlhB, which switches the substrate specificity. In this model (which I have named the waiting-room model), only one FliK molecule needs to detach from one FlhB to switch the secretion modes. Unbound FliK molecules may be secreted without affecting the state of FlhB, explaining the discrepancy in the numbers of secreted FliK molecules.

A SHORT CONCLUSION
To be honest, I am not sure about this new model. We have much more circumstantial than direct evidence; we know neither the number of FlhB subunits nor the ratio of FlgE to FliK accumulated at the base. However, I strongly believe that the hook length control is a cytoplasmic event and that the hook length is a result of the secretion of FlgE accumulated at the base. But I could be wrong in the details again. All in all, FliK may be secreted just to guarantee its efficient interaction with FlhB, which is hidden in the deepest localization in the C ring; otherwise, the probability of the interaction would be far lower.

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I thank Shigeru Yamaguchi for old stories regarding FliK, Manami Hashimoto for discussion, Shin-Ichiro Fujimoto for the cartoon figures, and David DeRosier for revising the manuscript. I also thank Kelly Hughes for being my friend and a long-time collaborator. We share data and write papers together but think differently. Kelly supports the physical ruler model, but I do not. Nevertheless, I enjoy our battle in science.

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Rebuttal: Mystery of FliK in Length Control of the Flagellar Hook

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Long history—I confess that I forgot about the 1972 Silverman and Simon Escherichia coli paper that came out before the Salmonella 1973 story (sorry Mel!) (1, 2, 4).

Length distribution. True, when hook measurements are reported as 55 ± 6 nm, it makes the reader think that they range from 49 to 61, but I have no problem with the term “tight control,” as some 95% of the rulers are within 10% of each other in length. The short hooks can be attributed to nascent structures, and the long ones we now account for as described in our recently published study (3).

FliK, the main factor in hook length control. My only comment here is that we do not know if the secretion-specificity switch prevents further hook secretion. We know it allows late substrate secretion, but it could be by extending substrate specificity and that the secretion of late substrates is preventing further hook secretion.

The physical ruler model. I would say that the physical model is popular because it is currently the only model that fits all the data. We accumulate data and then make and test models. Depending on the results of the tests, old models that no longer fit all the data are thrown out in favor of newer ones that do fit the data. It is not a popularity contest based on esthetics.

How many FliK molecules are secreted? I have to disagree with the comment “it is now shown that only one molecule is enough to determine one hook length.” This was from work with YscP and needle length control. There is no published study that shows one FliK is enough. However, I do believe that one is enough provided it is secreted after the hook is about 42 nm in length.

Problems with measuring-cup model. It is established that the C-ring and the FliHIJ ATPase complex serve to facilitate the secretion of flagellar substrates. Thus, to see a wide distribution in hook lengths is not surprising, if the affinity for FlgE and presumably also FliK is severely diminished in C-ring-defective strains.

Waiting-room model. I believe that secretion substrates accumulate at the flagellar base, and I do believe that the C-ring is a “docking station” for localizing substrates for secretion. I do not see a need to fill a base area or connect this with hook-length control. There is affinity of FliHIJ with bound substrates to the C-ring bringing substrates to the base for secretion. I am in favor of mechanisms of localized transcription or localized translation that allow substrates to accumulate locally and try to explain how, within a single cell, there are multiple flagella at different stages of assembly. How this is controlled is for me the next frontier in our studies on flagellum assembly.

Closing. I consider myself the luckiest guy in the world to have such friends and collaborators as Shin-ichi Aizawa and this great life in science. I look forward to working with Chi and our continued productivity and our differing interpretations of our data. Arguing with Chi over sushi and cold beer is always a great pleasure.

REFERENCES


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