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DIALOG

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-

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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 FliG, 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, FlhB was exposed to FliK 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 fraction 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 FlhB 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 FlhB 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 Karlinsey, 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-specificity 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 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 T3S4 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 long 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 injectasome 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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