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An infrequent molecular ruler controls flagellar hook length in *Salmonella enterica*

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The bacterial flagellum consists of a long external filament connected to a membrane-embedded basal body at the cell surface by a short curved structure called the hook. In *Salmonella enterica*, the hook extends 55 nm from the cell surface. FliK, a secreted molecular ruler, controls hook length. Upon hook completion, FliK induces a secretion-specificity switch to filament-type substrate secretion. Here, we demonstrate that an infrequent ruler mechanism determines hook length. FliK is intermittently secreted during hook polymerization. The probability of the specificity switch is an increasing function of hook length. By uncoupling hook polymerization from FliK expression, we illustrate that FliK secretion immediately triggers the specificity switch in hooks greater than the physiological length. The experimental data display excellent agreement with a mathematical model of the infrequent ruler hypothesis. Merodiploid bacteria expressing simultaneously short and long ruler variants displayed hook-length control by the short ruler, further supporting the infrequent ruler model. Finally, the velocity of FliK secretion determines the probability of a productive FliK interaction with the secretion apparatus to change secretion substrate specificity.

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Introduction

Bacteria propel themselves through liquid environments by rotating helical flagellar filaments (Figure 1A) (Berg and Anderson, 1973). The bacterial flagellum is a motor organelle that is composed of three main structural parts: (i) a basal body that includes rotor and stator structures embedded in the cytoplasmic membrane, a rod traversing the periplasmic space and a flagellar-specific protein export system; (ii) the hook, a flexible coupling structure that functions as a universal joint between the basal body and (iii) the rigid filament serving as a propeller that extends several microns from the cell (Macnab, 2003; Chevance and Hughes, 2008). This sophisticated nanomachine is evolutionarily and structurally related to the virulence-associated injectisome or needle complex of pathogenic bacteria (Hueck, 1998; Cornelis, 2006). Common features of both the flagellum and the injectisome systems are a type III protein export machine at the base of the structures (Blocker *et al*, 2003; Cornelis, 2006) and an intrinsic control mechanism for length control of the flagellar hook or injectisome needle, respectively (Journet *et al*, 2003; Shibata *et al*, 2007). For the flagellum, rod-hook-type substrates are exported via the flagellar type III protein export system until the hook is of appropriate length (55 ± 6 nm) (Hirano *et al*, 1994) and then the type III secretion system switches substrate specificity and starts exporting filament-type substrates (Williams *et al*, 1996). For the injectisome needle system of *Yersinia enterocolitica*, the needle polymerizes to a length of 58 ± 10 nm (Journet *et al*, 2003) before substrate specificity is switched towards export of effector proteins (Sorg *et al*, 2007).

In *Salmonella enterica*, the secretion-specificity switch is thought to occur by an interaction between secreted FliK and the substrate-specificity-determining component of the flagellar secretion apparatus, FlhB (Minamino *et al*, 2006). Null mutants of *fliK* and dominant-negative alleles of *flhB* fail to switch the secretion specificity to filament-type substrates and continue uncontrolled hook polymerization (Patterson-Delafield *et al*, 1973; Hirano *et al*, 1994; Kutsukake *et al*, 1994; Minamino *et al*, 1999; Fraser *et al*, 2003). Homologous proteins of FliK and FlhB in the *Yersinia* ssp. injectisome system are YscP and YscU (Magdalena *et al*, 2002; Journet *et al*, 2003). The C-terminal domains of FliK and YscP are thought to be responsible for induction of the specificity switch within the type III secretion apparatus, presumably by interaction with FlhB or YscU, respectively (Hirano *et al*, 1994; Minamino *et al*, 2006; Sorg *et al*, 2007). Export of FliK and YscP is required for hook- and needle-length control, respectively (Minamino *et al*, 1999; Agrain *et al*, 2005). Deletions and insertions in FliK (YscP) revealed a linear correlation between length of the hook (needle) structure and the length of FliK (YscP), illustrating that these proteins determine hook (needle) length as a molecular ruler that directly measures the length of the structure (Journet *et al*, 2003; Shibata *et al*, 2007).

The fundamental problem is how, during the process of being secreted, the ruler molecule is able to transmit hook (needle)-length information beyond the cell surface back to the type III export apparatus in the inner membrane in order to flip the switch. Several models for the mechanism of how FliK (YscP) regulates hook (needle) length have been proposed. Initially, a molecular ruler model was not considered for the flagellar system because all *fliK* mutants isolated

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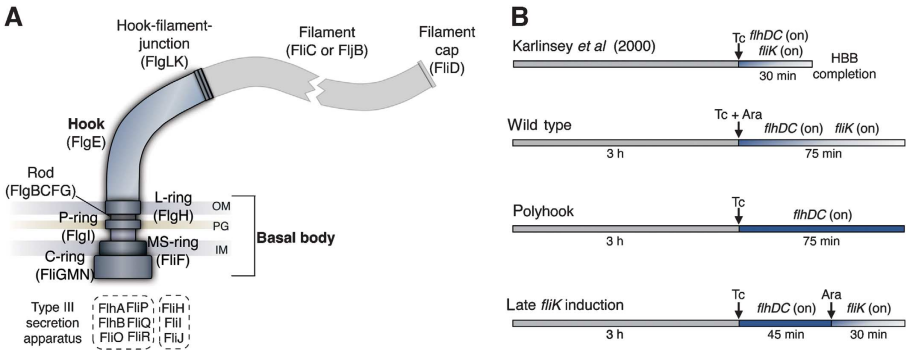


Figure 1 (**A**) Schematic of axial components of the bacterial flagellum. The structure of the bacterial flagellum can be divided into three parts: (i) the basal-body structure that harbours the flagellar-specific type III secretion apparatus at the base; (ii) the hook that functions as a flexible coupling structure between the basal body and (iii) the rigid filament. Stator elements (Mot proteins) that span the inner membrane and apply torque to the C-ring in response to transmembrane proton flow are not shown. Rod-hook length is determined by a molecular ruler, FliK, that in turn induces a switch in secretion specificity from rod-hook-type to filament-type substrates upon hook completion, presumably by interaction with FlhB, a component of the type III secretion apparatus at the base of the structure. (**B**) Schematic of experimental outline. An overnight culture of a strain expressing the flagellar master operon *flhDC* from a Tc-inducible P_{tetA} promoter is diluted into fresh LB and grown for 3 h. After 3 h growth, flagellar gene expression is induced by addition of Tc and 30 min after induction transcription of Class 3 promoters is observed, which indicates HBB completion (Karlinsey *et al*, 2000). Here, we uncoupled FliK expression from flagellar genes expression to analyse the effects of late FliK induction on switching from HBB-type secretion to filament-type secretion in a strain deleted for its native *fliK* gene and expressing *fliK* from the inducible P_{araBAD} promoter (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK*$^+$ Δ*fliK*). In the first sample ('wild-type'), flagellar gene expression and *fliK* expression are induced simultaneously by addition of Tc to induce the flagellar master regulator *flhDC* (P_{tetA}-*flhD*$^+$*C*$^+$) and Ara to induce *fliK* expression (P_{araBAD}-*fliK*$^+$) resulting in hooks of wild-type length. In the second sample ('polyhook'), only Tc is added to induce flagellar gene expression, giving rise to polyhooks because FliK is not induced. In the third sample ('late *fliK* induction'), flagellar gene expression is induced for 45 min without FliK expression. This allows for hook-length growth beyond the physiological length. Afterwards, *fliK* is induced by addition of Ara and the culture grown for an additional 30 min to allow for induction of the secretion-specificity switch and filament assembly.

resulted in longer, not shorter, hooks (Kawagishi *et al*, 1996). However, mutants in the C-ring components *fliG*, *fliM* or *fliN* were identified that resulted in short hooks (Makishima *et al*, 2001). Thus, it was proposed that the cytoplasmic rotor of the flagellum functions as a measuring cup. This C-ring cup would fill up with hook subunits that would correspond to the required number of hook molecules for the assembly of a hook of appropriate length. Upon emptying of the cup, FliK would be able to access and interact with FlhB (Makishima *et al*, 2001). Recent results show, however, that controlled hook lengths are observed in mutants missing parts or all of the C-ring (Konishi *et al*, 2009; Erhardt *et al*, 2010). Later, a static-ruler model has been proposed, where a single ruler molecule resides in the secretion channel and is attached to the growing tip of the needle (hook) structure (Journet *et al*, 2003). In this model, needle (hook) subunits must be able to pass by the retained ruler inside a secretion channel of around 2 nm in diameter (Shaikh *et al*, 2005). Finally, an alternative model was proposed, where FliK is intermittently secreted throughout hook growth and the length signal is determined via a stochastic process, where the probability of hook growth termination is an increasing function of hook length (Erhardt *et al*, 2010; Keener, 2010). In this work, we present experimental evidence in favour of this infrequent molecular ruler model and provide for the first time a mechanism for flagellar hook-length determination by FliK in which the velocity of FliK secretion dictates the probability of a productive interaction with the secretion apparatus for the specificity switch to occur.

Results

Experimental approach and motility of the model strains

The infrequent ruler model for flagellar hook-length regulation predicts that the FliK ruler can be intermittently secreted with hook (FlgE) subunits at any time during hook polymerization. During secretion, FliK takes temporal measurements of hook length. The probability of a productive interaction of the FliK C-terminus with the type III secretion apparatus, which is a prerequisite for the switch in secretion specificity, increases with hook length. Termination of hook polymerization would be unlikely for short hooks, but highly probable at longer hook lengths. A possible mechanism is that the speed of FliK secretion is facilitated, while the hook is shorter than its physiological length. This would prevent a productive interaction of the FliK C-terminus with the FlhB component of the secretion system within the cytoplasmic membrane. In hooks of the physiological length or greater, the rate of FliK secretion is slow enough to allow ample time for the C-terminus of FliK to interact with FlhB and flip the specificity switch to late substrates.

One prediction of the infrequent ruler hypothesis is that FliK triggers the secretion-specificity switch every time FliK is secreted through a hook of physiological length or greater. To this end, we envisaged an *S. enterica* model strain in which *fliK* induction (and FliK protein secretion) is uncoupled and independently controlled from hook-basal-body (HBB) assembly. In this strain, induction of flagellar gene expression is controlled by a tetracycline (Tc)-inducible promoter (Karlinsey *et al*, 2000), thereby enabling us to control and synchronize expression of the flagellar master regulator *flhDC* (P_{tetA}-*flhD*$^+$*C*$^+$) and accordingly HBB gene expression and assembly. It has been previously reported that approximately 30 min after induction of the *flhDC* operon, the secretion-specificity switch has occurred, which corresponds to HBB completion (Karlinsey *et al*, 2000). Approximately 60 min after induction of flagellar genes, external filaments of about 2 μm length are observed (Karlinsey *et al*, 2000).

In a strain that is deleted for its chromosomal *fliK* gene, induction of the flagellar master regulator will result in HBB

assembly. However, in the absence of FliK, the secretion apparatus will fail to flip the secretion-specificity switch and hook growth will continue beyond physiological lengths, resulting in a polyhook phenotype. To control FliK expression in the cell, the *fliK* gene was placed under arabinose (Ara) induction (P_{araBAD}-*fliK*$^+$). This model strain (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK*$^+$ Δ*fliK*) allows for induction of FliK at times after hook length has reached its physiological length. As shown in Supplementary Figure S1, the model strain displayed motility comparable to wild type on soft agar plates containing Tc and Ara as inducers of both FliK and flagellar gene expression.

To test the infrequent ruler model for hook-length determination, the model strain was grown under three different conditions (Figure 1B). For the 'wild-type' control, flagellar genes (P_{tetA}-*flhD*$^+$*C*$^+$) and *fliK* expression (P_{araBAD}-*fliK*) were induced simultaneously by addition of both inducers for 75 min. In case of the polyhook control, flagellar gene expression in the absence of *fliK* expression was induced by addition of only Tc for 75 min. No FliK or FliC secretion was detected under those conditions (Supplementary Figure S2). To assess the effects of late FliK secretion in a population, where the majority of the hooks have polymerized beyond the physiological length, expression of the flagellar master regulator was induced with Tc for 45 min followed by late induction of *fliK* expression by addition of Ara for an additional 30 min to allow for the switch to late-substrate secretion mode.

Switch to late-substrate secretion occurred immediately after FliK induction in hooks greater than the physiological length

In the absence of FliK, addition of Tc for 45 min ensures that hooks polymerize beyond their physiological length. It takes 30 min to grow the completed HBB structure after induction of the flagellar master operon (*flhDC*) (Karlinsey *et al*, 2000). We induced *fliK* 45 min after addition of Tc followed by an additional 10 or 30 min growth to determine if FliK could induce the secretion-specificity switch in HBBs with elongated hooks. Figure 2A shows an *in vivo* analysis of this specificity switch using detection of flagellar filaments by immunostaining as a marker for the switch to late-substrate secretion. Flagellar filaments will only form if the secretion apparatus flipped to late-secretion mode by interaction of the ruler molecule FliK with the FlhB component of the secretion system. For a quantitative analysis of the number of HBBs in a synchronized culture that flipped to late-substrate secretion, HBB complexes and filaments were immunostained using hook- and filament-specific antibodies (Figure 2A). As displayed, almost every HBB switched to late, filament-type secretion, if FliK was induced late after the hooks had polymerized beyond their physiological length. Importantly, after only 10 min of *fliK* induction, short filaments were attached to nearly every HBB, where observed (Figure 2A, 'late FliK 10 min'). This suggested that the first ruler molecule secreted into HBBs with elongated hooks immediately flipped the specificity switch, as predicted by the infrequent ruler

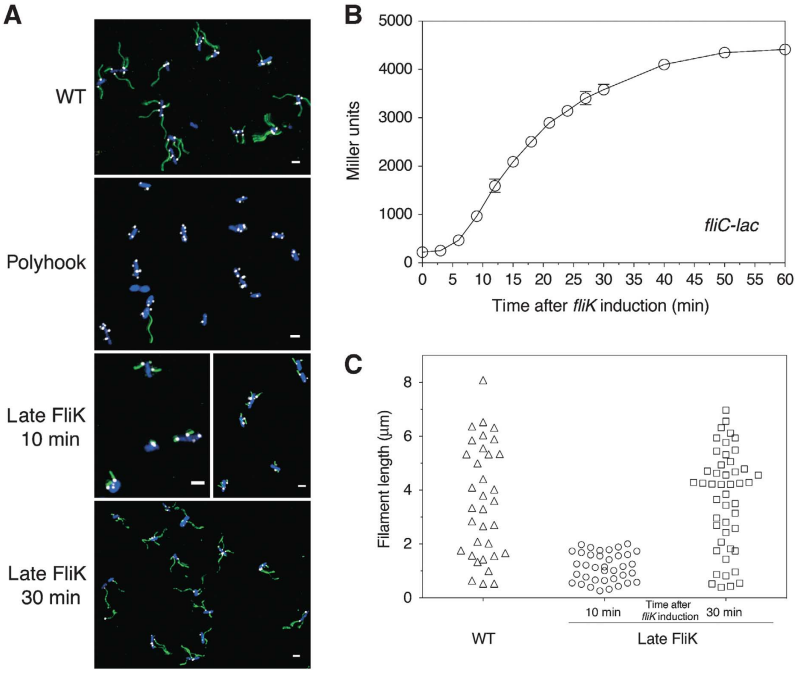


Figure 2 FliK induces secretion-specificity switch in hooks >wt length. (**A**) Immunostaining of assembled HBB complexes and filaments. 'WT': simultaneous induction of *fliK* and flagellar genes expression; 'polyhook': flagellar genes were induced without induction of *fliK*; 'late FliK': late *fliK* induction after 45 min of flagellar genes expression for 10 and 30 min, respectively. Tc was not removed prior to addition of Ara. Representative fluorescent microscopy images of strain TH16941 (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK*$^+$ Δ*fliK flgE*::*3xHA*) are shown. DNA (blue), hooks (white) and filaments (green). Scale bar = 2 μm. (**B**) Time lapse of *fliC* transcription after *fliK* induction. FliK expression in strain TH17502 harbouring a *fliC-lac* reporter was induced late after 45 min of flagellar genes expression. β-galactosidase activity was assayed according to Materials and methods. The inducer of flagellar gene expression, Tc, was not removed prior to addition of Ara. Data are presented as mean ± s.d. of three independent, biological replicates. (**C**) Filament-length distribution after 10 and 30 min of late *fliK* induction compared with the simultaneous induction of *fliK* and flagellar genes expression ('WT'). Number of filaments measured: 'WT' = 34, '10 min' = 36 and '30 min' = 47.

hypothesis. The switch allowed for the activation of late-substrate gene transcription by the secretion of an inhibitor of late gene transcription. Transcription of the filament gene *fliC* started approximately 5 min after induction of *fliK*, as shown in Figure 2B, and we could detect short filaments by immunofluorescence 10 min after *fliK* induction (Figure 2A). We observed an average filament length of 1.1 μm 10 min after late *fliK* induction (Figure 2C), corresponding to an approximate average growth rate of 0.22 μm/min, if filament gene transcription initiated 5 min after *fliK* induction (see above). We observed filaments of up to 7 μm in length 30 min after late *fliK* induction (or approximately 25 min after induction of filament gene transcription) and this translated to a maximal growth rate of up to 0.28 μm/min. Reported filament growth rates *in vivo* ranged from 0.10 to 0.55 μm/min (Stocker and Campbell, 1959; Iino, 1974).

In the control sample, where *fliK* expression was never induced, rarely a filament was observed (about 5–10% of detected HBBs) (Figure 2A, 'polyhook'). The low frequency of filaments can be explained by a combination of spontaneous switching of the type III secretion apparatus to late secretion and basal expression of the P_{araBAD}-*fliK* allele that would result in some FliK expression and secretion.

Next, we obtained the hook-length distribution of the model strain under different FliK induction conditions (Figure 3C). In the first sample, both flagellar genes and *fliK* were induced simultaneously (labelled 'WT' in the figure). In the second sample, only flagellar genes were induced (labelled 'polyhook' in the figure) and in the third sample, *fliK* expression was induced only after 45 min of flagellar gene expression (labelled 'late FliK' in the figure). In case of simultaneous expression of HBB genes and $fliK_{405}$, an average hook length of 43 ± 6 nm was observed (Figure 3C, left panel). This corresponds to the prediction of nine FliK molecules secreted per 42 nm hook under conditions, where FliK was over-expressed from the Ara promoter. The average hook length is approximately 12 nm shorter as previously observed under wild-type conditions (Hirano *et al*, 1994). However, this result can be explained by simultaneous, non-hierarchical expression of HBB genes

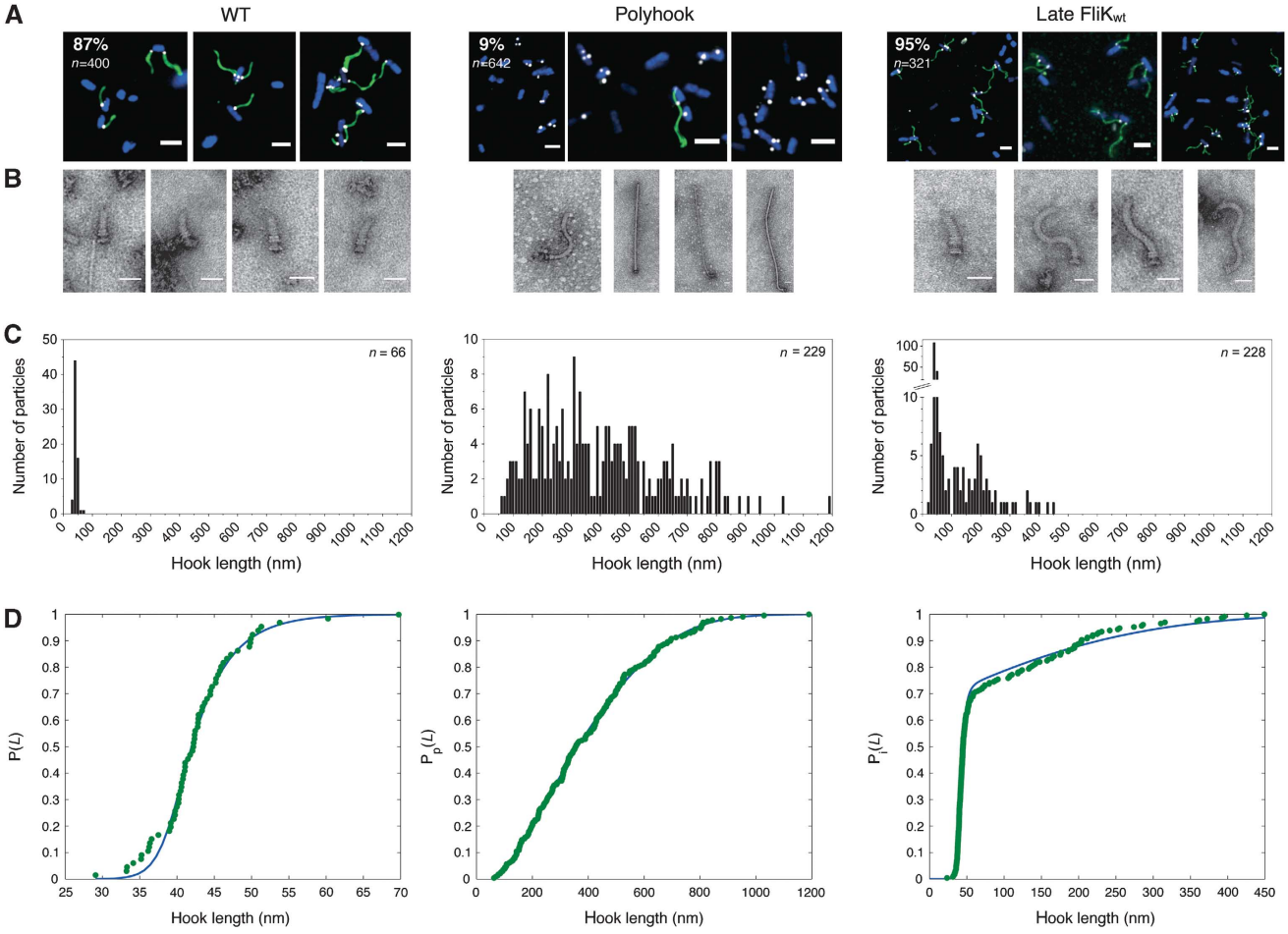


Figure 3 Late FliK secretion induces secretion-specificity switch in elongated hooks. Left panels (WT): simultaneous induction of *fliK* and flagellar genes expression. Middle panels (polyhook): flagellar genes were induced without induction of *fliK*. Right panels (late FliK): late *fliK* induction after 45 min of flagellar genes expression. (**A**) Representative fluorescent microscopy images of strain TH16941 (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK*$^+$ Δ*fliK flgE*::*3xHA*). Tc was removed prior to addition of Ara to prevent formation of nascent HBBs. Number of cells counted for the presence/absence of HBB–filament complexes: 'WT' = 400, 'polyhook' = 642, 'late FliK' = 321. Fraction of HBBs with attached filaments is given in the upper left corner. DNA (blue), hooks (white) and filaments (green). Scale bar = 2 μm. (**B**) Representative electron micrograph images of hooks isolated from strain TH16791 (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK*$^+$ Δ*fliK*). Scale bar = 50 nm. (**C**) Histogram of measured hooks of strain TH16791. Number of measured hooks: 'WT' = 66, 'polyhook' = 229, 'late FliK' = 228. (**D**) CDF of hooks measured for TH16791. Measured hook lengths shown as asterisks and $P_i(L)$ (solid curve) computed from equation 8 using $L^* = 470$ nm.

and *fliK*, contrary to what is the case under wild-type conditions, and overproduction of *fliK* expressed from the strong P_{araBAD} promoter. In fact, it has been previously reported that over-expression of *fliK* produced shorter hooks (45 ± 6 or 46 ± 7 nm) (Muramoto *et al*, 1998; Minamino *et al*, 2009). The slight differences in hook length under FliK over-expression conditions can be explained by the fact that in previous over-expression experiments, a population of wild-type hooks was already present prior to the start of the FliK over-expression. However, we started expression of FliK and flagellar HBB genes simultaneously, which would result in the presence of over-produced FliK already before the construction of any hooks.

When *fliK* was not expressed in the polyhook sample, hook-length control was completely abolished with hooks up to 1.2 μm length (Figure 3C, middle panel). This is consistent with the hook-length distribution observed in a *fliK* deletion strain (Patterson-Delafield *et al*, 1973). Importantly, in the sample, where *fliK* expression was induced late after physiological HBB completion, hook length appears to be partially controlled (Figure 3C, right panel). Hooks longer than 500 nm were not observed contrary to the polyhook sample and the histogram revealed a prominent population of 42 ± 5 nm. In this sample, *fliK* was induced for 30 min, while the inducer for HBB genes was still present due to experimental limitation that did not allow us to remove the inducer of HBB genes. Accordingly, production of nascent 'wild-type' HBBs during the 30-min time frame, where both HBB inducer and FliK were present, accounted for this prominent peak. Induction of HBB genes for 30 min is sufficient to allow formation of wild-type HBB as shown previously (Karlinsey *et al*, 2000). This explains the prominent 'wild-type' peak at 42 nm. It is important to stress, however, that the isolation procedure for HBBs requires either very long hooks (e.g. as found in a polyhook phenotype) or attached filaments and this suggested that the longer hooks up to 500 nm length indeed switched to late-substrate secretion and had a filament attached during the HBB preparation. Wild-type HBBs were re-purified from strain TH16791 (P_{araBAD}-$fliK_{405}$) and independently imaged on a different electron microscope to confirm our length measurements. We observed an average hook length of 43 ± 5 nm, the same as described above (Supplementary Figure S6).

In order to exclude that only nascent HBBs switched to filament-type secretion after late FliK induction (e.g. as seen in Figure 2), we repeated the late *fliK* induction experiment under conditions, where Tc, the inducer of flagellar genes, was removed after 45 min before the induction of *fliK* by the addition of Ara (Figure 3A). This ensured that during the following 30 min of FliK expression, no nascent HBBs were produced and FliK was secreted through old HBBs with elongated hooks. Under these conditions, approximately 95% of detected HBBs indeed switched to filament-type secretion (Figure 3A, right panel).

In the Materials and methods, we present a mathematical model of the infrequent ruler mechanism that allowed us to use wild-type hook-length data and polyhook data to predict the length distribution of hooks produced by late *fliK* induction. First, we used the experimentally obtained hook-length data from the wild-type sample (Figure 3D, left panel) to estimate the function $P_c(L)$, the probability of FliK interaction with FlhB at hook-length L. The length of the wild-type ruler was captured by the parameter $\hat{L} = 39$ nm. The cumulative distribution function (CDF) $P(L)$ for this data is shown in Figure 3D, with data points shown as asterisks. An estimate of $P_c(L)$ is shown in Supplementary Figure S3B. The second data set is polyhook data, determined from a culture in which there was no *fliK* induction. The culture was grown for 75 min. The histogram of lengths is shown in Figure 3C (middle panel) and the CDF $P_p(L)$ for this collection of polyhooks is shown in Figure 3D (middle panel). The third type of data is from a culture grown for 75 min, with induction of *fliK* at time $T_0 = 45$ min (Figure 3, right panel). Figure 3D (right panel) shows the CDF of the data (shown as asterisks) and the predicted CDF $P_i(L)$ determined from equation (8), using the functions $P_c(L)$ and $P_p(L)$.

The agreement between the curve $P_i(L)$ and the data is striking. There is some difference, however, which is possibly explained by the fact that in the derivation of equation (8), the velocity of hook growth is assumed to be constant, independent of length. A better estimate of the length distribution at the time of induction would require more detailed knowledge of the velocity of hook growth as a function of length. In spite of this caveat, however, the excellent agreement between the late *fliK* induction data and the prediction based on information from sample 1 (WT) and sample 2 (polyhook) gives strong evidence in favour of the hypothesis that hook-length determination is by an infrequent ruler mechanism with a switching probability function $P_c(L)$. This analysis was further applied to several data sets with varying induction times T_0. The results with the same agreement are shown in Figure 4 ($T_0 = 45$, 55 and 65 min).

Secretion of FliK deletion and insertion alleles in elongated hooks immediately induced the secretion-specificity switch

To further assess the ability of late FliK secretion in triggering the specificity switch in hooks greater than the physiological length, we engineered FliK deletion and insertion variants and tested their ability to control hook length after late *fliK* induction. First, a long FliK variant was generated by inserting a 164-amino-acid fragment of YscP between amino acids 140 and 141 of FliK, resulting in $FliK_{570}$. A short FliK variant was constructed by deleting amino acids 161 through 202 of FliK, resulting in $FliK_{363}$. $FliK_{570}$ (reported hook length 81.6 ± 9.5 nm) and $FliK_{363}$ (reported hook length 43.5 ± 8.0 nm) retain hook-length control if expressed from the native P_{fliF} promoter (Shibata *et al*, 2007). In order to allow for inducible expression, both FliK variants were expressed from the chromosomal P_{araBAD} promoter. The ability of the FliK variants to flip the specificity switch to late-substrate secretion was first analysed by filament immunostaining (Figure 5A; Supplementary Figure S4A). Under conditions where nascent HBBs were no longer produced after *fliK* induction, late FliK secretion switched 91% ($FliK_{570}$) and 96% ($FliK_{363}$) of the detected HBBs to filament-type secretion (Figure 5A). Next, the hook-length distribution after late *fliK* induction was determined, albeit because of experimental constrictions under conditions where production of new HBBs was still possible (Figure 5B and C, Supplementary Figure S4B and C). When flagellar genes and $FliK_{570}$ were expressed simultaneously, the hook-length histogram revealed a peak at 79 ± 6 nm (Supplementary Figure S4C, first column). Hook length was not regulated

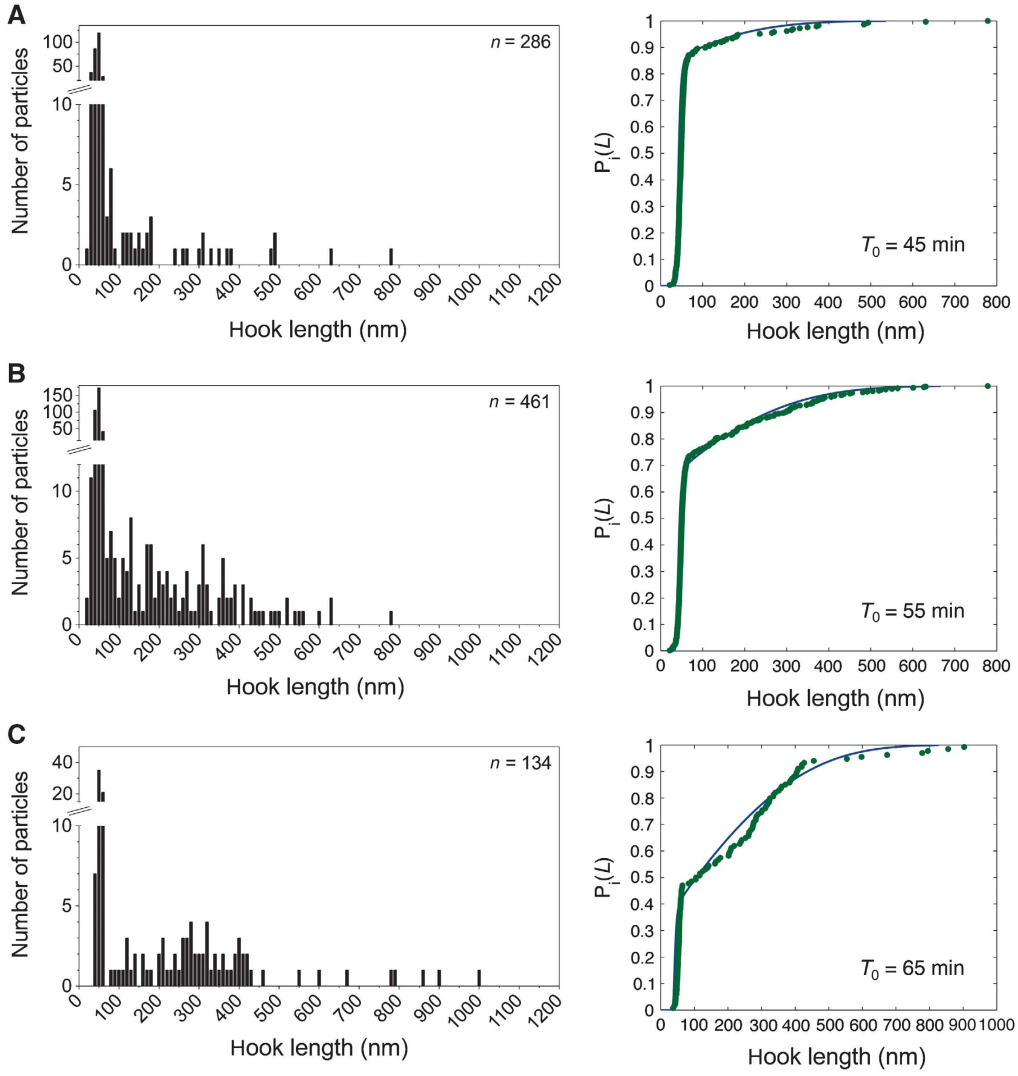


Figure 4 Late induction of *fliK* at varying times T_0. Strain TH16791 (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK*$^+$ Δ*fliK*) was grown in the presence of Tc (inducer of flagellar genes) for (**A**) 45 min, (**B**) 55 min and (**C**) 65 min. Afterwards, *fliK* expression was induced by addition of Ara for a total sample time of 80 min. Left panels: histogram of measured hooks. Number of measured hooks: '$T_0 = 45$ min' = 286, '$T_0 = 55$ min' = 461, '$T_0 = 65$ min' = 134. Right panels: CDF of measured hooks, data shown as asterisks and $P_i(L)$ (solid curve) computed from equation 8 using $L^* = 600$ nm ($T_0 = 45$ min), 440 nm ($T_0 = 55$ min) and 260 nm ($T_0 = 65$ min).

with hook lengths up to 960 nm when $FliK_{570}$ was not expressed (Supplementary Figure S4C, second column). When $fliK_{570}$ was induced late after 45 min of HBB genes expression, hooks longer than 400 nm were not observed with a peak at 78 ± 7 nm that can be attributed to nascent HBBs (Figure 5C, right panels). For the shorter $FliK_{363}$ variant, an average hook length of 38 ± 6 nm was found under conditions where flagellar genes and $FliK_{363}$ were expressed simultaneously (Supplementary Figure S4C, third column). When only flagellar genes were expressed, hook length was not controlled with lengths up to 980 nm (Supplementary Figure S4C, fourth column). Under conditions where $FliK_{363}$ was expressed late, maximal hook lengths were 380 nm with a peak corresponding to nascent HBBs at 39 ± 6 nm (Figure 5C, left panels).

The measured hook-length data of the polyhook and wild-type samples for both $FliK_{570}$ and $FliK_{363}$ were then used to predict the late $FliK_{570}$ ($FliK_{363}$) hook-length distribution $P_i(L)$ computed as described in Materials and methods. The length of the ruler variants was described by $\hat{L} = 34$ nm for $FliK_{363}$ and $\hat{L} = 75$ nm for $FliK_{570}$ with $\alpha = 0.66\ \mathrm{nm}^{-1}$, and $\rho = 3$. As it was the case for late wild-type $fliK_{405}$ induction, we found excellent agreement between the experimental data of late induction of $fliK_{570}$ and $fliK_{363}$ and the prediction of the infrequent ruler model (Figure 5D).

Velocity of FliK secretion inversely correlated with hook length

The infrequent ruler model predicts a mechanism for rapid secretion of the FliK ruler molecule in hooks shorter than the physiological length such that the C-terminus of FliK does not interact with FlhB during export. Contrary, in hooks of physiological length or longer, the rate of FliK secretion must decrease to allow time for a productive interaction of the FliK C-terminus with the secretion apparatus. It has been reported that the FliK N-terminus interacts with the hook cap FlgD and assembled hook subunits (Moriya *et al*, 2006; Minamino *et al*, 2009). More frequent interactions of FliK

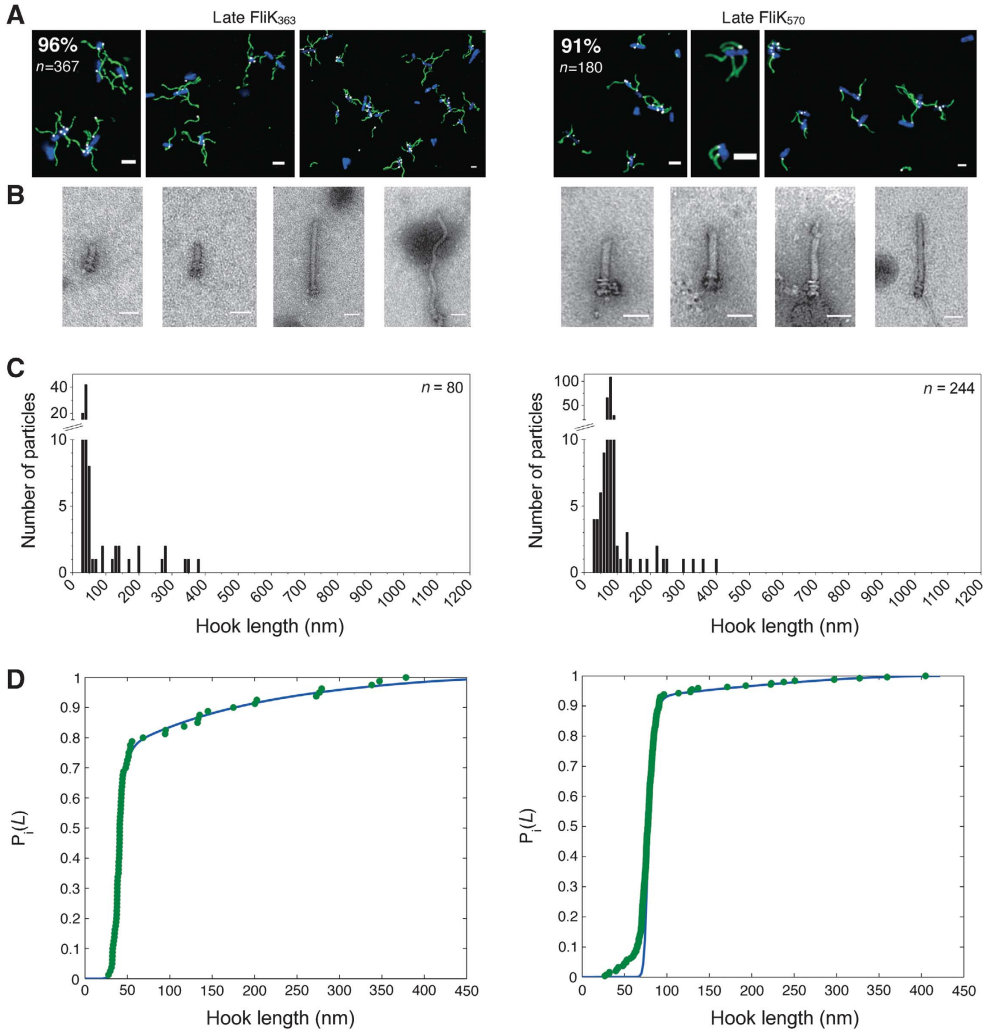


Figure 5 Late secretion of short $FliK_{363}$ and long $FliK_{570}$. $FliK_{363}$ (left panels) and $FliK_{570}$ (right panels) expression was induced late after 45 min of flagellar genes expression. (**A**) Representative fluorescent microscopy images of strain TH17012 (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliKΔ161–202 ΔfliK flgE*::*3xHA*) and TH17011 (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK(140′-yscP(217–381)-′141fliK) ΔfliK flgE*::*3xHA*). Tc was removed prior to addition of Ara to prevent formation of nascent HBBs. Number of cells counted for the presence/absence of HBB–filament complexes: 'late $FliK_{363}$' = 367, 'late $FliK_{570}$' = 180. Fraction of HBBs with attached filaments is given in the upper left corner. DNA (blue), hooks (white) and filaments (green). Scale bar = 2 μm. (**B**) Representative electron micrograph images of hooks isolated from strain TH16997 (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliKΔ161–202 ΔfliK*) and TH16996 (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK(140′-yscP(217–381)-′141fliK) ΔfliK*). Scale bar = 50 nm. (**C**) Histogram of measured hooks of strain TH16997 ($FliK_{363}$) and TH16996 ($FliK_{570}$). Number of measured hooks: 'late $FliK_{363}$' = 80, 'late $FliK_{570}$' = 244. (**D**) CDF of hooks measured for strain TH16997 and TH16996. Measured hook lengths shown as asterisks and $P_i(L)$ (solid curve) computed from equation 8 using $L^* = 440$ nm ($FliK_{363}$) and $L^* = 540$ nm ($FliK_{570}$).

with hook subunits could explain a slower FliK secretion rate in longer hooks. Additionally, it would be feasible that the nascent FliK N-terminus starts to fold as it exits the secretion channel to facilitate the rate of secretion for the C-terminus. When FliK is secreted through HBBs with a combined length that is smaller than that of the elongated FliK molecule, initial folding of the FliK N-terminus could act as a Brownian ratchet that rapidly pulls the FliK molecule past the type III secretion apparatus and through the channel (Keener, 2010). We tested the velocity of FliK secretion in the model strain, where flagellar gene expression can be synchronized and is uncoupled from FliK expression (P_{tetA}-*flhD*$^+$*C*$^+$ P_{araBAD}-*fliK*$^+$ *ΔfliK*). Flagellar genes were expressed for 45 min, giving rise to polyhooks, before Tc, the inducer of flagellar genes, was removed. Afterwards, *fliK* was induced and intra- and extracellular FliK protein levels were determined using quantitative western blot analysis (Figure 6A, upper panels). Protein levels of basal-body complexes remained constant as detected by immunoblotting against cellular FliM (C-ring protein). Cellular FliK protein was detected approximately 25 min after induction and detectable levels of secreted FliK were observed at approximately 30 min after induction. Next, the shortest possible HBB structure with defined length was analysed for the rate of FliK secretion. In a strain background that is deleted for the hook structural gene, *flgE*, flagellar basal-body assembly halts after PL-ring formation. This results in the shortest possible basal-body structure that is capable of extracellular secretion. As displayed in Figure 6A (lower panels), secreted FliK could be detected approximately 25 min after *fliK* induction. As predicted, significantly more

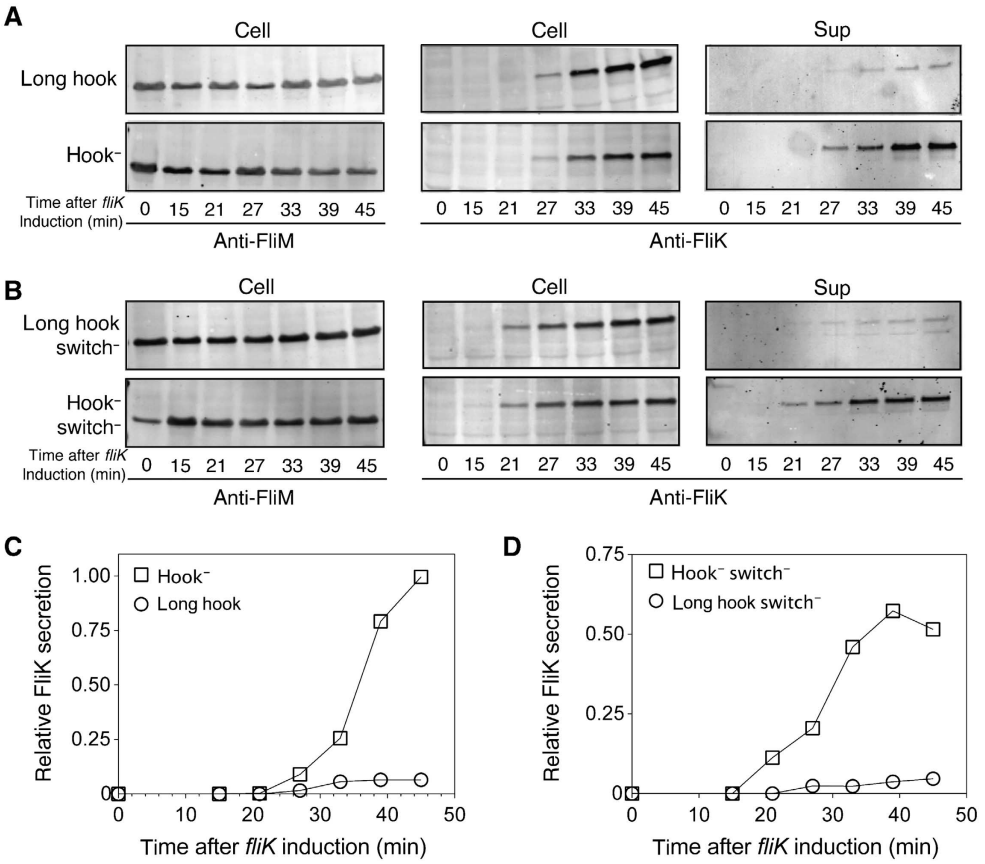


Figure 6 Velocity of FliK secretion is dependent on hook length. Cellular FliM (left panels), cellular FliK (middle panels) and extracellular FliK levels (right panels). Expression of flagellar genes and *fliK* was induced as outlined in Materials and methods. Residual Tc was washed out before induction of FliK and samples were taken at the time points indicated. Representative western blots are shown. (**A**) Top: FliK secretion in strain that produces elongated hooks (strain TH16791). Bottom: FliK secretion in the absence of the hook (strain TH17069). (**B**) Top: FliK secretion in a long hook strain that is unable to switch to late-substrate secretion mode (TH17076, FlhB autocleavage defective mutant). Bottom: FliK secretion in the switch$^-$, hook$^-$ strain TH17112. (**C**) Relative levels of secreted FliK normalized against intracellular FliM of TH16791 and TH17069 from the blot shown in (**A**). (**D**) Relative levels of secreted FliK normalized against intracellular FliM of TH17112 and TH17076 from the blot shown in (**B**).

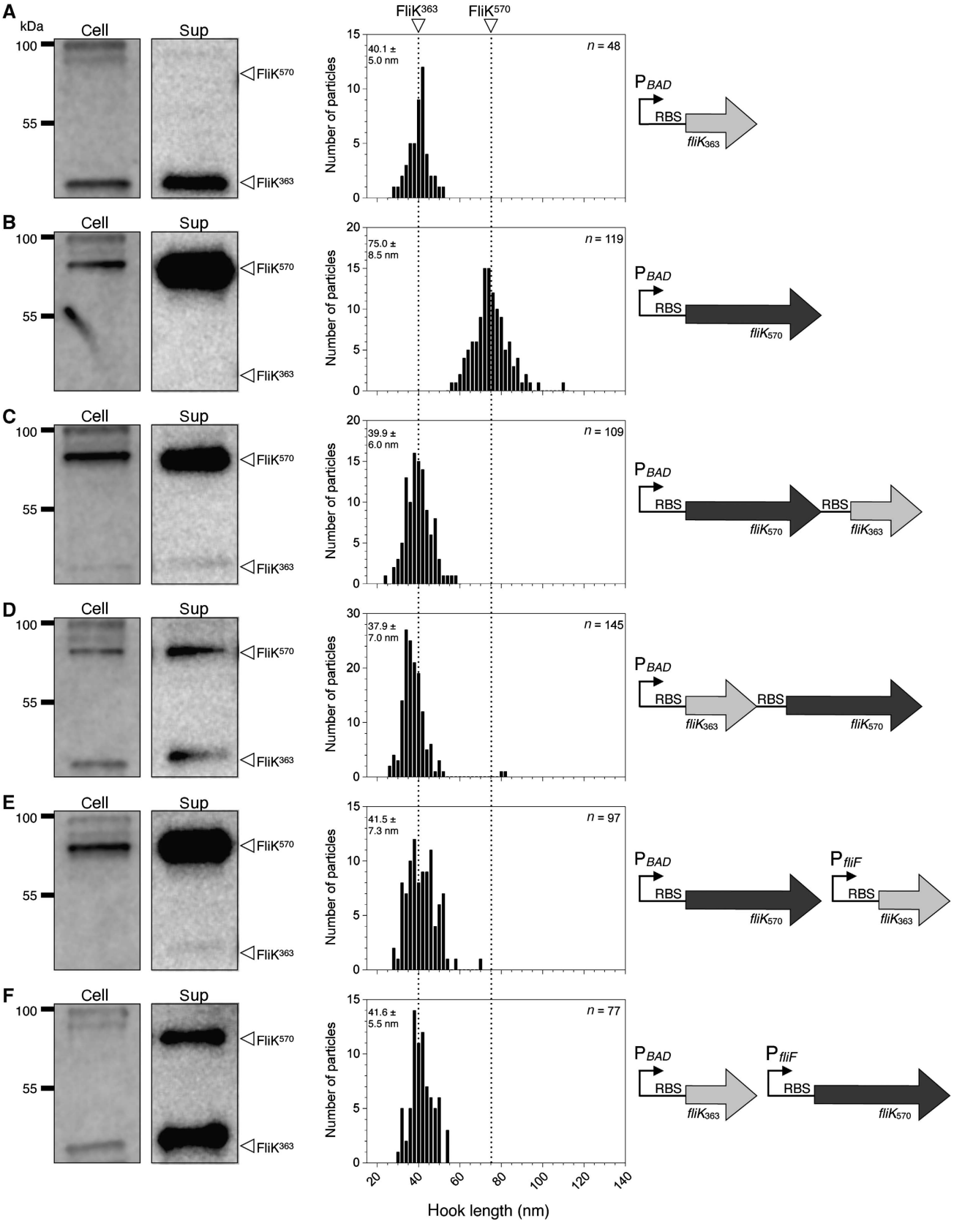
FliK was secreted in the hook deletion background as compared with the polyhook sample. Cellular FliK levels were comparable with slightly higher FliK levels in the polyhook sample presumably due to the reduced FliK secretion rate. However, one could also argue that the lower levels of secreted FliK under polyhook conditions was due to the ability of secreted FliK to flip the specificity switch, which would result in cessation of rod-hook-type secretion in the polyhook sample. A switch of secretion specificity to late substrates would eliminate secretion of FliK resulting in lower levels of secreted FliK. For this reason, we repeated the quantitative analysis of FliK secretion in a $flhB_{N269A}$ background that does not undergo autocleavage, which prevents the secretion-specificity switch (Fraser *et al*, 2003). The $flhB_{N269A}$ allele was introduced into the model strain (P_{tetA}-$flhD^+C^+$ P_{araBAD}-$fliK^+$ Δ*fliK* $flhB_{N269A}$) and secreted FliK protein was detected after late *fliK* induction. As presented in Figure 6B, intracellular and secreted FliK was detected roughly at the same time and in comparable quantities as in the $flhB^+$ background (Figure 6A). This illustrated that the capability of the secretion apparatus to secrete rod-hook-type substrates was not impaired in the $FlhB_{N269A}$ background. The late *fliK* induction experiment was repeated in the same strain background, where the *flgE* (hook) gene was deleted. Importantly, in this hook deletion mutant, the rate and levels of FliK secretion were significantly increased if compared with the polyhook sample in the same $FlhB_{N269A}$ background (Figure 6B and D).

These results demonstrate that FliK was secreted at significantly higher levels in the absence of the hook than under polyhook conditions, thus providing evidence for the mechanism suggested above, where FliK–FlgE interactions and/or N-terminal folding of the nascent FliK N-terminus determine the rate of FliK secretion.

Merodiploid Salmonella strains secreting short and long FliK variants produced only one population of short hooks that is controlled by the short FliK ruler

The data described above provides strong evidence in favour of an infrequent ruler model for flagellar hook-length determination, where the FliK ruler is intermittently secreted throughout the rod-hook-polymerization process. Recent results from the homologous needle-length control system in *Yersinia* described an opposing model, where a static, one-ruler-per-needle ruler regulates needle length (Wagner *et al*, 2010). Wagner *et al* used an elegant experimental

approach employing merodiploid bacteria expressing YscP rulers of different sizes and found that the merodiploid *Yersinia* produced a mixture of short and long needles. Accordingly, the authors concluded that a static ruler remains within the needle during needle polymerization to control needle length in *Yersinia*. These results are not in agreement with the results presented here for the flagellar system, and accordingly, we set out to test the effect of



co-production of two FliK variants on flagellar hook length. The infrequent ruler hypothesis predicts that, under conditions where both a short and a long ruler are co-produced, hook length would still be controlled by the short ruler. If both rulers are secreted throughout the rod-hook-polymerization process, then the first ruler in place when the appropriate hook length has been reached will flip the secretion-specificity switch. Under situations where multiple ruler molecules are secreted during the process of hook assembly, the switching event would most likely be catalysed by the short ruler.

As displayed in Figure 7, four different genetic constructs were engineered that co-expressed a short ($FliK_{363}$) and a long ($FliK_{570}$) ruler. First, the short *fliK*$_{363}$ variant was inserted in one chromosomal operon downstream of the long *fliK*$_{570}$ variant under the control of the P_{araBAD} promoter (Figure 7C). Here, $FliK_{570}$ was expressed and secreted in elevated levels compared with $FliK_{363}$; however, the hook-length distribution revealed only one single peak at 39.9 ± 6.0 nm. This peak corresponds to the hook length obtained when only the short ruler ($FliK_{363}$) is expressed (40.1 ± 5.0 nm; Figure 7A), whereas the long ruler ($FliK_{570}$) alone produced hooks of an average length of 75.0 ± 8.5 nm (Figure 7B). Alternatively, the long *fliK*$_{570}$ variant was inserted after the short *fliK*$_{363}$ variant in one operon under the same chromosomal P_{araBAD} promoter (Figure 7D). In this type of construct, both the expression and secretion of both ruler variants was almost identical. The hook-length distribution again revealed a single population with a peak at 37.9 ± 6.0 nm. Next, the long $FliK_{570}$ ruler was expressed from the chromosomal P_{araBAD} promoter, whereas the short *fliK*$_{363}$ ruler was inserted at its physiological chromosomal locus under control of its native P_{fliF} promoter (Figure 7E). Here, expression from the P_{araBAD} promoter was much stronger than from the native promoter, but the hook-length distribution again showed a single peak at 41.5 ± 7.3 nm. Finally, the short ruler was expressed from the P_{araBAD} promoter, whereas the long ruler was under its native control (Figure 7F). In this construct, approximately equal amounts of both rulers were secreted, but the bacteria produced only one population of short hooks peaking at 41.6 ± 5.5 nm. Taken together, these results provide convincing evidence in favour of the infrequent ruler model for the determination of flagellar hook length and refute a static-ruler hypothesis in this model system.

Discussion

Here, we present experimental evidence in support of an infrequent ruler mechanism for the determination of flagellar hook length. In this model, the molecular ruler FliK is intermittently secreted during hook polymerization (Figure 8). Hook length is measured by secretion of a FliK molecule and hook polymerization will continue until a secreted FliK molecule is in close proximity and provided with sufficient time for a productive interaction with the FlhB component of the type III secretion apparatus at the base of the flagellum to flip a switch in secretion specificity (Erhardt *et al*, 2010; Keener, 2010). The infrequent ruler model predicts a mechanism of hook-length determination in which the probability of a productive FliK interaction with the secretion apparatus is an increasing function of hook length (Keener, 2010). For small hook lengths, induction of the switch is unlikely presumably because FliK is secreted too fast for a productive interaction with FlhB. Likewise, for hooks of physiological or longer length, virtually every secreted FliK molecule flips the secretion-specificity switch. Here, we show that as predicted by the model, the switch to late-substrate secretion is induced immediately upon FliK secretion in hooks greater than the physiological length. Furthermore, the experimental data displayed excellent agreement with the predicted probability curves calculated using mathematical models of the infrequent ruler mechanism. We also provide experimental evidence for the suggested mechanism, where the velocity of FliK secretion is faster in shorter hooks than in longer ones, thus explaining the greater probability of a productive FliK-induced specificity switch in hook with increasing length. Under physiological conditions, FliK secretion would be initially fast and would gradually slow down until the hook has polymerized to a minimal length, which FliK over-expression experiments suggest is about 40 nm. FliK secretion through hooks of minimal or longer lengths would result in the passage of the C-terminal region of FliK to be slow enough to allow for induction of the specificity switch. Increased or decreased expression of the *fliK* gene would result in more or less measurements of hook length during hook polymerization and accordingly have an effect on the average hook length. If evolutionary pressures select for building flagella as quickly as possible, the level of *fliK* gene expression would be set to produce the shortest functional hook structures followed by filament polymerization.

It has to be stressed that the infrequent ruler model proposed here accounts for all published data on flagellar hook-length control. As mentioned above, the infrequent ruler model predicts that more frequent measurements of hook length by an increased rate of FliK expression would result in shorter hook lengths. More frequent measurement would increase the probability of a productive interaction of FliK with the secretion apparatus that would flip the specificity switch at a shorter hook length. Shorter hooks have indeed been observed under conditions where FliK measures hook length more frequently, for example over-expression of FliK (Muramoto *et al*, 1998; Minamino *et al*, 1999), under-expression of FlgE (Muramoto *et al*, 1999) or in a hook-

Figure 7 Hook-length distribution of merodiploid strains co-expressing short and long FliK variants. Left panels: pellet and supernatant fractions were analysed by immunoblotting using polyclonal anti-FliK antibodies. Middle panels: hook-length distribution of purified HBB complexes of the different strains used. The average hook length ± s.d. is given in the upper left corner and was obtained by non-linear regression analysis of the Gaussian distribution. The average hook lengths for $FliK_{363(art)}$ (nucleotide sequence given in Supplementary Figure S5) and $FliK_{570}$ are indicated by the dashed line. Number of measured hooks for each construct is indicated in the figure. Right panels: schematic of the various genetic constructs. (**A**) Strain TH7556 expressing the artificial $FliK_{363(art)}$ variant from P_{araBAD}. (**B**) Strain TH16996 expressing $FliK_{570}$ from P_{araBAD}. (**C**) Strain TH17555 expressing $FliK_{570}$–$FliK_{363(art)}$ from P_{araBAD}. (**D**) Strain TH17557 expressing $FliK_{363(art)}$–$FliK_{570}$ from P_{araBAD}. (**E**) Strain TH17457 expressing $FliK_{570}$ from P_{araBAD} and $FliK_{363}$ from P_{fliF}. (**F**) Strain TH17458 expressing $FliK_{363}$ from P_{araBAD} and $FliK_{570}$ from P_{fliF}.

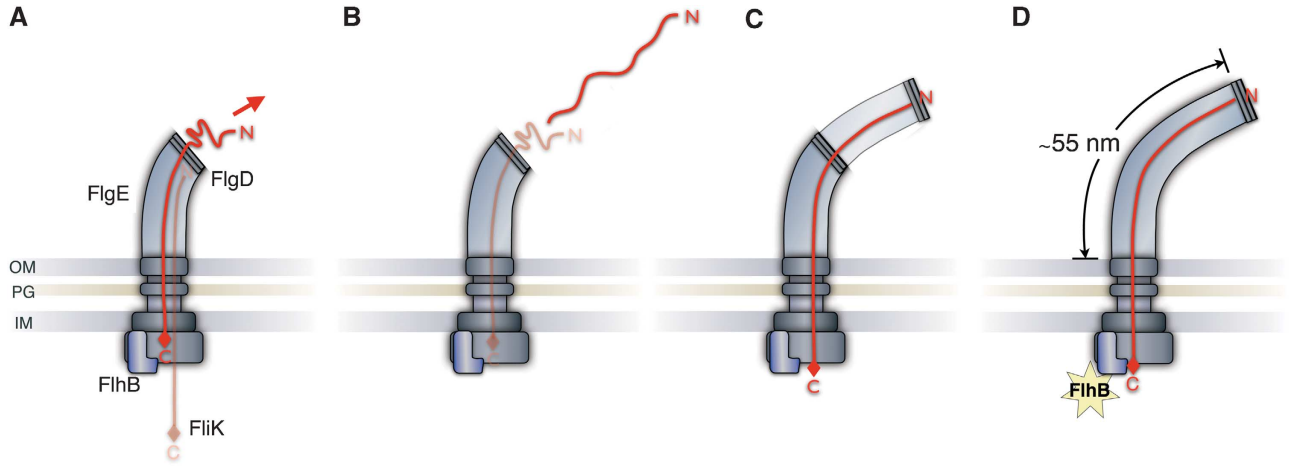


Figure 8 Model of hook-length determination by the infrequent ruler mechanism. (**A**) FliK is intermittently secreted during hook polymerization. During FliK secretion, hook polymerization temporarily halts and the N-terminus of FliK interacts with assembled FlgE and FlgD subunits during its secretion. The lack of interactions in short hooks or the folding of the secreted FliK N-terminus as it exits the secretion channel rapidly pulls the FliK molecule past FlhB through the channel without induction of the secretion-specificity switch (see text for details). (**B**) FliK is secreted outside of the cell and hook polymerization continues. (**C**) The hook has grown to the physiological (or longer) length (here: 55 nm). A new FliK molecule is secreted and this time secretion is slower because of more frequent interactions of the FliK N-terminus with assembled hook subunits. Additionally, the N-terminus of FliK is not yet secreted outside of the cell, where its fold might enhance secretion. (**D**) The C-terminus of FliK is now closely aligned to FlhB and the slower rate of FliK secretion allows for sufficient time for a productive FliK–FlhB interaction that induces the secretion-specificity switch (indicated by a yellow star in the figure).

polymerization defective mutant (Moriya *et al*, 2006). Low (0.07–0.8 molecules per cell), medium (40–80 molecules per cell) and high (2000–6000 molecules per cell) FliK levels resulted in hook lengths of 75 ± 46, 55 ± 9 and 46 ± 7 nm, respectively (Muramoto *et al*, 1998). The FliK titration data have been quantitatively analysed using a mathematical model of the infrequent ruler hypothesis in Keener (2010).

Similarly, the model predicts that over-expression of the hook subunit FlgE or under-expression of FliK would result in longer hooks because of less frequent or even only one measurement during hook assembly, and this has also been reported previously (Muramoto *et al*, 1998; Muramoto *et al*, 1999). Recently, it has been shown that wild-type and FlhB variants had similar kinetic profiles and apparent affinities suggesting that the specificity switch is more complex (Morris *et al*, 2010). An increased velocity of FliK secretion in hooks shorter than the physiological length could account for these observations. Moriya *et al* (2006) reported hook alleles that took longer to polymerize, which resulted in shorter hook lengths. This led to the proposal of a molecular clock. We can now explain these results in light of FliK secretion. Moriya *et al* showed that more FliK was secreted in the hook mutants that were slower to polymerize. We interpret this to mean that FliK is taking more measurements per hook growth and as in FliK over-expression experiments this resulted in shorter hooks.

Recent results from the homologous needle-length control system in *Yersinia* resulted in an opposing model in which a single, static ruler is attached inside the secretion channel throughout needle polymerization (Wagner *et al*, 2010). The authors employed merodiploid strains that co-expressed two YscP rulers of different sizes and found two populations of needle lengths regulated by the two rulers. It remained to be tested if the conclusions of the authors could also be applied to the hook-length control system described here. Accordingly, we duplicated the experimental setup of Wagner *et al* and co-expressed two different FliK ruler molecules in a variety of merodiploid strains. We found that in all cases, the short FliK ruler would control hook length and not the long variant. These results are in agreement with the infrequent ruler hypothesis, where the ruler molecules are intermittently secreted throughout the HBB-polymerization process. The model predicts that the first ruler that remains inside the secretion channel of a hook of appropriate length will be able to flip the specificity switch. In the merodiploid scenario, the short ruler will almost always be the first ruler that is able to terminate hook polymerization if both rulers are secreted throughout the HBB assembly process.

In summary, although closely related on a functional level, the actual mechanism of length control appears to be different in the flagellar and needle-length control systems. The data described here, together with published results, provide overwhelming evidence in favour of the infrequent ruler model for flagellar hook-length determination and refute a static-ruler option for the length control of the flagellar hook. Additionally, it has been shown for the flagellar hook-length control system that the molecular ruler FliK is secreted during hook polymerization (Minamino *et al*, 1999), thus providing additional evidence against a static-ruler mechanism for the flagellar system. Moreover, the hook channel has a diameter of around 2 nm (Shaikh *et al*, 2005) and this implies that the secretion channel is too narrow to accommodate a static FliK molecule and hook subunits that need to pass by during hook polymerization at the same time. However, we cannot rule out that the channel is dynamic as suggested for the *Yersinia* needle inner channel (Wagner *et al*, 2009). A general problem of the one-ruler-per-hook model is the prerequisite of an attachment of the ruler molecule to the capping protein of the growing structure. While the hook cap FlgD has been shown to interact with the ruler FliK and could thus provide such an attachment site, interactions with the hook subunits FlgE and FliK have also been reported (Moriya *et al*, 2006;

Minamino *et al*, 2009). This led us to propose that the sum of interactions of the ruler with both the hook subunits and the capping proteins would slow-down ruler secretion in extended hook structures in order to allow for a productive interaction of the C-terminus of the ruler with the component of the secretion apparatus responsible for the switching. In case of the injectisome needle system, to date, no capping protein has been identified.

In summary, we believe that the strong agreement between the published data on hook-length control by FliK, our experimental results and the mathematical models presented here provide convincing evidence in favour of the proposed infrequent ruler mechanism for flagellar hook-length control in *Salmonella*.

Materials and methods

Bacterial strains, construction of strains, media, β-galactosidase assays, FliK secretion assay, SDS–PAGE, western blotting and fluorescent microscopy

These procedures are described in the Supplementary data. All bacterial strains used in this study are listed in Table I.

Isolation of HBBs, electron microscopy and measurements of hook length

HBB isolation was carried out by the methods described in Aizawa *et al* (1985) with minor modifications. Flagellar samples were not collected by CsCl gradient centrifugation, but were pelleted at 60 000 *g* for 1 h using a Beckman 50.2Ti at 4°C. Flagellar filaments were de-polymerized after purification by suspension in acidic solution (pH 2.5) as described in Aizawa *et al* (1985). Purified HBB samples were negatively stained with 2% uranyl acetate on copper coated grids. Images were captured using a Hitachi H-7100 electron microscope at an acceleration voltage of 125 kV. Hook lengths were measured using NIH ImageJ 1.42q software and average hook lengths were obtained by non-linear regression analysis of the Gaussian distribution using GraphPad Prism 5.0c.

Mathematical model of the infrequent ruler hypothesis

The infrequent ruler mechanism hypothesis is that FliK is intermittently secreted during hook growth and that the probability of FliK interaction with FlhB leading to hook growth termination is an increasing function of the length of the hook at the time secretion of FliK occurs. We let $P_c(L)$ denote the probability of productive interaction of FliK leading to occurrence of the specificity switch when the hook is length L. The rate at which FliK secretion occurs is $ar(L)$, where $r(L)$ is the rate of secretion of all secretants and a is the fraction of secreted molecules that are FliK. We let P be the probability that hook growth is terminated at length less than or equal to L. Since the overall rate of productive interaction of FliK is $ar(L)\,P_c(L)$, the time rate of change of P is given by

$$\frac{dP}{dt} = ar(L)P_c(L)(1-P), \tag{1}$$

and that of L by

$$\frac{dL}{dt} = br(L)\Delta, \tag{2}$$

where b is the fraction of secreted molecules that are FlgE and Δ is the length increment from polymerization of a single FlgE molecule. It follows (dividing equation (1) by equation (2)) that

$$\frac{dP}{dL} = \beta P_c(L)(1-P), \tag{3}$$

where $\beta = a/b\Delta$.

If testing with FliK begins when the hook is length L_0, then $P(L_0) = 0$. To clarify the notation, we let $P(L|L_0)$ be the probability that a hook is terminated at length L given that testing was initiated at length L_0. Then, solving equation (3) subject to $P(L|L_0) = 0$ we find

$$P(L|L_0) = 1 - \exp(-\beta \int_{L_0}^{L} P_c(\eta)d\eta). \tag{4}$$

This equation can be used to determine $P_c(L)$ from $P(L|L_0)$ by rewriting it as

$$-\beta \int_{L_0}^{L} P_c(\eta)d\eta = -\ln(1 - P(L|L_0)). \tag{5}$$

We can use the experimentally obtained hook-length data from the wild-type sample (Figure 3D, left panel) to estimate the function $P_c(L)$. The CDF for this data is shown in Figure 3D, with data points shown as asterisks. The data shown in Figure 3D are replotted as $-\ln(1-P(L))$ versus L as shown in Supplementary Figure S3A. The solid curve in this figure is the function

$$G(L) = \frac{1}{\rho}\ln(1 - \exp(-\alpha\hat{L}) + \exp(\alpha(L - \hat{L}))), \tag{6}$$

Table I *Salmonella enterica* serovar *Typhimurium* LT2 strains used in this study

Strain number	Genotype	Reference
TH3730	$P_{flhDC5451}$::*TPOP*	Karlinsey *et al* (2000)
TH6701	Δ*araBAD925*::*tetRA*	Lab collection
TH16791	Δ*araBAD7606*::*fliK*$^+$ Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH16941	Δ*araBAD7606*::*fliK*$^+$ *flgE7742*::*3xHA* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH16996	Δ*araBAD1089*::*fliK(fliK140-yscP(217–381)-141fliK)* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH16997	Δ*araBAD1090*::*fliK*(Δ*aa161–200)* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx}*vh2*	This study
TH17011	Δ*araBAD1089*::*fliK(fliK140-yscP(217–381)-141fliK) flgE7742*::*3xHA* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx}*vh2*	This study
TH17012	Δ*araBAD1090*::*fliK*(Δ*aa161–200) flgE7742*::*3xHA* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH17069	Δ*araBAD7606*::*fliK*$^+$ Δ*flgE7599* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH17076	Δ*araBAD7606*::*fliK*$^+$ *flhB7152*(N269A) Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH17112	Δ*araBAD7606*::*fliK*$^+$ Δ*flgE7599 flhB7152*(N269A) Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH17457	Δ*araBAD1089*::*fliK(fliK140-yscP(217–381)-141fliK)* Δ*fliK6140*(Δ*aa161–202)* $P_{flhDC5451}$::*TPOP*	This study
TH17458	Δ*araBAD1090*::*fliK*(Δ*aa161–200) fliK6127(fliK140-yscP(217–381)-141fliK)* $P_{flhDC5451}$::*TPOP*	This study
TH17502	Δ*araBAD7606*::*fliK*$^+$ Δ*fliK6140* $P_{flhDC5451}$::*TPOP fliC5050*::*mudJ fljB*^{enx} *vh2*	This study
TH17555	Δ*araBAD1114*::*fliK(fliK140-yscP(217–381)-141fliK)-fliK*(Δ*aa161–200, artificial DNA sequence)* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH17556	Δ*araBAD1113*::*fliK*(Δ*aa161–200, artificial DNA sequence)* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study
TH17557	Δ*araBAD1115*::*fliK*(Δ*aa161–200, artificial DNA sequence)-fliK(fliK140-yscP(217–381)-141fliK)* Δ*fliK6140* $P_{flhDC5451}$::*TPOP fljB*^{enx} *vh2*	This study

with $\hat{L} = 39\,\text{nm}$, $\alpha = 0.66\,\text{nm}^{-1}$, and $\rho = 3$, determined by a simple visual fit. This allows us to estimate $\beta P_c(L)$ as

$$\beta P_c(L) = \frac{dG}{dL} = \frac{\alpha \exp(\alpha(L - \hat{L}))}{\rho(1 - \exp(-\alpha\hat{L}) + \exp(\alpha(L - \hat{L})))}, \qquad (7)$$

shown in Supplementary Figure S3B. This form for $P_c(L)$ was derived in Keener (2010). The solid curve shown in Figure 3D is the CDF $P(L|0)$ as determined from equation (4) using the function $P_c(L)$ given in equation (7). The second data set is polyhook data, determined from a culture in which there was no *fliK* induction. The histogram of lengths is shown in Figure 3C (middle panel) and the CDF for this collection of polyhooks is shown in Figure 3D (middle panel). To use the information provided by this data, we need a representation of the CDF, $P_p(L)$ (subscript p for 'polyhook'). We found an excellent fit of the data using a function of the form $P_p(L) = \exp(f(L))$, where $f(L)$ is a fifth order polynomial. The details of this polynomial are not informative and so are not provided here. A plot of $P_p(L)$ is shown as a curve in Figure 3D (middle panel).

The third type of data is from a culture grown for 75 min, with induction of *fliK* at time $T_0 = 45\,\text{min}$ (Figure 3, right panel). To predict this distribution from $P_c(L)$ and $P_p(L)$, we first note that the function $P_p(L)$ gives information about when hooks were initiated. That is, suppose induction of *fliK* is started at the time that polyhooks of length L^* in Figure 3 (middle panel) are just initiated. This means that at the time of *fliK* induction, $P_p(L^*)$ have yet to be initiated, and the length distribution of hooks already initiated is given by $p_p(L + L^*)$, where $p_p = (d/dL)P_p(L)$ (with a slight caveat mentioned in Results). It follows that the distribution of hooks lengths produced by late induction of *fliK* is given by

$$P_i(L) = P(L|0)P_p(L^*) + \int_0^L P(L|L_0)p_p(L_0 + L^*)dL_0. \qquad (8)$$

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: ME, JPK and KTH designed the experiments. ME, HMS and DHW performed experiments. JPK designed and performed the mathematical modelling. ME, HMS, DHW, JPK and KTH analysed data. ME, JPK and KTH wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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