Molecular ruler determines needle length for the Salmonella Spi-1 injectisome

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The type-III secretion (T3S) systems of bacteria are part of self-assembling nanomachines: the bacterial flagellum that enables cells to propel themselves through liquid and across hydrated surfaces, and the injectisome that delivers pathogenic effector proteins into eukaryotic host cells. Although the flagellum and injectisome serve different purposes, they are evolutionarily related and share many structural similarities. Core features to these T3S systems are intrinsic length control mechanisms for external cellular projections: the hook of the flagellum and the injectisome needle. We present evidence that the Spi-1 injectisome, like the Salmonella flagellar hook, uses a secreted molecular ruler, InvJ, to determine needle length. This result supports a universal length control mechanism using molecular rulers for T3S systems.

length control mechanisms of macromolecular structures are used throughout biology for maintaining cellular functions (1–6). Mechanisms of length control have evolved independently and range from the kinetically controlled length of the metaphase spindle involved in eukaryotic cell division, to the scaffolds controlling bacteriophage tail-lengths (2, 5). One of the best-studied and controversial mechanisms of length control is that of bacterial Type III secretion (T3S) system, which includes the virulence-associated injectisome of Salmonella pathogenicity island 1 (Spi-1), and the bacterial motility organelle, the flagellum.

The injectisome is a hypodermic needle-like organelle used by pathogenic Gram-negative bacteria to inject effector proteins into eukaryotic host cells (7). This complex organelle is embedded in the membranes and cell wall of Gram-negative bacteria. The basal structure of the injectisome includes a rod-like component that spans the periplasmic space between the inner and outer membranes, and an injectisome-specific T3S apparatus within the cytoplasmic membrane (7–9). The T3S system associated with the injectisome secretes proteins required for its structure and assembly, as well as effector proteins into host cells to facilitate pathogenesis. Extending from the injectisome basal-body at the cell surface is a rigid needle-like structure, which grows to defined lengths for different bacterial species, and serves as a conduit between the bacterium and the host cell (10–12). At the tip of the needle is the translocon, which allows the needle to dock to the host membrane (13–15) (Fig. 1A).

Like the injectisome, the flagellum contains a membrane and cell wall spanning basal-body, which also functions as a rotary motor in motility, and includes an integral membrane-associated, flagellar-specific T3S apparatus (16, 17) (Fig. 1B). From the basal-body at the cell surface extends a flexible tube, called the hook, which also grows to defined lengths and serves as a universal-joint to transmit torque generated by the basal-body to a long (up to 20 μm) helical propeller-like structure called the flagellar filament (18, 19). A defining characteristic of T3S systems is an intrinsic length control mechanism for hook and needle length that is associated with a switch in the type of substrates that are recognized and exported by the secretion apparatus (3, 4, 20, 21). For the flagellum of Salmonella Typhimurium, assembly of the basal-body is followed by the secretion of ~130 FlgE protein subunits that polymerize to form the hook (19). When the hook reaches a terminal length of ≥ 40 nm, the T3S apparatus switches secretion specificity to filament or late-type secretion substrates to complete flagellum assembly (20, 22). Here, the length of the hook and induction of the secretion specificity switch is determined by a secreted, molecular ruler called FliK (23–25).

Assembly of the injectisome proceeds in a similar fashion to the flagellum; however, the ring structures that are in the outer membrane form independent to the inner membrane-associated components of the basal-body (10, 26, 27). Subunits that make up an inner-rod structure that spans the cell wall and periplasmic space and subunits that assembly into the external needle are part of the early class of secreted substrates (8, 12). The injectisome is completed when the needle reaches an optimal length after which, the T3S system switches specificity for late secreted substrates including the translocon and effector proteins (10, 21, 28).

Many mechanisms have been proposed for length determination for the hook of the flagellar system and the needle of the injectisome system. Length control of the flagellar hook and injectisome needle by a molecular ruler was first proposed based on work on the Yersinia spp. injectisome system (3). For the injectisome system of Yersinia enterocolitica, the needles grow to a length of 58 ± 10 nm, whereas Yersinia pestis needles grow to a length of about 41 nm (3). It was shown that the N-terminal length of YscP, a nonstructural, early secretion substrate and functional homolog of FliK, determined needle lengths of the Yersinia injectisomes (10, 29, 30). An important difference between the YscP molecules from Y. enterocolitica and Y. pestis is that Y. pestis YscP is 60 amino acid residues shorter in length, which corresponded to shorter needles. Increasing or decreasing the lengths of the N-terminal domain of YscP or FliK resulted in a linear correlation between the length of the needle and the

Significance

Type-III secretion systems facilitate the virulence capability of Gram-negative bacterial pathogens. They are part of complex nanomachines that include the bacterial flagellum, which confers motility, and the injectisome, which secretes proteins from the infecting bacterial cytoplasm into eukaryotic host cells. Here we examine the mechanisms of length control for the main component of the injectisome and flagellar basal structures, the needle and the hook, respectively. The defined lengths of the hook and needle are important for their function. We find that both systems use a secreted, molecular ruler to effect length determination at the nanometer-scale. Understanding the mechanisms of length control in these systems provides insight into bacterial pathogens and a biological model for the controlled construction of nanomaterials.

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length of YscP, or the length of the hook and FliK (3). Additionally, the C-terminal domain of YscP and FliK is believed to interact with the component of the T3S apparatus that governs secretion specificity, known as YscU in *Yersinia* spp. and FlhB in the flagellar system. Loss of YscP/FliK, or dominant mutant alleles in the T3S switch component yscU/flhB, will give rise to a poly-needle and poly-hook phenotype, respectively, where the needles or hooks polymerize indefinitely and do not switch secretion specificity modes (21, 31).

Although both the flagellum of *S. Typhimurium* and injectisome of *Yersinia* spp. use molecular rulers for controlling length, the mechanisms for how measurements are achieved are proposed to work in different ways (22, 28, 32). In *Yersinia* injectisomes, a static-ruler model has been proposed (32). Here, the ruler molecule remains anchored by its N terminus to the tip of the needle in the secretion channel as the needle substrates are secreted and polymerize into the growing needle. The anchored YscP is pulled along with the growing needle until its C terminus is in proximity to the secretion apparatus to induce the secretion specificity switch (Fig. 1D). Alternatively, in the flagellar system, FliK has been shown to act as an infrequent ruler, with multiple FliK molecules secreted through the growing structure until a minimal hook-length is achieved (22, 33). In this model, the probability of inducing the secretion specificity switch is a function of the velocity of FliK secretion, which is inversely correlated to hook length. FliK enters the secretion channel and proceeds to the hook-tip and exits the cell from its N terminus (FliKα) followed by the C terminus (FliKβ). Once FliKα exits the cell, FliKα continues through the channel at an increased rate of secretion that is too fast to allow FliKβ to interact with FlhB (22). It is only when the hook has reached a sufficient length where FliKβ can interact with FlhB prior to the exit of FliKα from the cell does the secretion specificity switch at FlhB occur (Fig. 1C). Interestingly, injectisomes in *S. enterica* were proposed to measure needle lengths by a mechanism that is different from those proposed for FliK and YscP.

*S. enterica* encodes two injectisomes: the *Salmonella* pathogenicity island 1 (Spi-1) and *Salmonella* pathogenicity island 2 (Spi-2) injectisomes, which are required to cause gastroenteritis in humans (34). The Spi-1 needle is composed of PrgI subunits that polymerize to a length of ~25 nm before undergoing the secretion specificity switch. An early secretion substrate, InvJ, was believed to be the molecular ruler analog in Spi-1 injectisomes, because its absence results in a poly-needle phenotype; however, these injectisomes were reported to be missing their inner-rod structure (10, 28). Additionally, overexpression of the inner-rod subunit protein PrgJ was found to result in slightly shorter needle lengths, giving rise to an inner-rod length-control model for the Spi-1 needle (28). In this model, needle length is determined by the rate of inner-rod assembly, where the complete inner-rod structure presumably interacts with the T3S apparatus to induce the secretion specificity switch, thus terminating needle assembly (Fig. 1E). A recent study supporting the inner-rod model reports the isolation of mutant PrgJ-alleles that give rise to abnormally long needles that still undergo the secretion specificity switch and assemble the inner-rod (35). It is reasoned that these mutant alleles are defective in PrgJ–PrgJ interactions causing slower inner-rod assembly and, therefore longer needles. Still, it remained unclear as to what role InvJ plays in Spi-1 injectisome needle length control. In this study, we address the hypothesis that InvJ, like its functional homologs FliK and YscP, acts as a molecular ruler in determining needle length for the Spi-1 injectisome. Our work demonstrates that InvJ acts as a molecular ruler and supports a universal molecular ruler model for length control in T3S systems.

Results

**InvJ Acts as a Molecular Ruler to Determine Needle Length.** Hydrophobic cluster analysis of T3S system molecular rulers, including InvJ, FliK, and YscP, has revealed a conserved C-terminal domain that interacts with the T3S apparatus causing the secretion specificity switch and subsequent termination of needle or hook

**Fig. 1.** (A) Schematic of axial components in a cross-section of the Spi-1 injectisome. (B) Schematic of axial components of the bacterial flagellum. (C) Model of hook-length determination by the infrequent ruler mechanism: (i) The secreted N terminus of FliK (FliKα), shown in red halts hook polymerization and is slow to diffuse. (ii) The lack of interactions in short hooks or the folding of the secreted FliKα as it exits the secretion channel rapidly pulls the FliK molecule past FlhB (shown in orange) through the channel without induction of the secretion-specificity switch. (iii) FliK is secreted outside of the cell and hook polymerization continues. (iv) The hook has grown to the physiological length and a new FliK molecule is secreted, again halting hook growth. (v) The C terminus of FliK (FliKβ) is now closely aligned to FlhB, the slower rate of FliK secretion provides sufficient time for a productive FliK–FlhB interaction that induces the secretion-specificity switch. (D) The static ruler model for length control of the *Yersinia* injectisome: (i) A single YscP (shown in red) molecule enters the secretion channel and remains there during needle polymerization. The N terminus of YscP interacts with the needle or tip-complex and is pulled through as the needle continues to polymerize, bringing the C terminus of YscP (YscPC) near the secretion apparatus (YscU – shown in orange). (ii) When YscPC and YscU are close enough to interact, a secretion specificity switch occurs, halting needle polymerization. (E) The inner-rod model of length control of the Spi-1 injectisome: Here, both the inner-rod (shown in green) and needle (shown in blue) are assembled simultaneously. (i) Assembly of the inner-rod is facilitated by InvJ (shown in red). (ii) The completion of the inner-rod leads to substrate switching and the interruption of secretion of the inner-rod and needle proteins, thus determining the length of the needle substructure.
polymerization (29). Previous studies provide evidence to suggest that the N terminus of InvJ (InvJN) is the ruler domain for determining needle length, and its C terminus (InvJC) is responsible for inducing the secretion specificity switch by interacting with the FlhB/YscU homolog in the Spi-1 T3S apparatus, SpaS, to induce the secretion specificity switch, as is the case for FliK and YscP (29, 36, 37). Increasing or decreasing the length of the ruler molecule in either the flagellar system (FliK), or the injectisome system of Yersinia spp. (YscP) results in a linear correlation in hook or needle length, respectively (3, 4).

Because Spi-1 injectisome needle-length is reportedly controlled by PrgJ copolymerization with InvJ inner-rod subunits, we expected that, unlike FliK and YscP, varying InvJ lengths should have no effect on needle length. Conversely, if InvJ acted like FliK and YscP as a molecular ruler, then amino acid insertions that lengthen the N-terminal region of InvJ should result in needles with length increases corresponding to the number of amino acids inserted.

Segments of varying lengths from the ruler domain of YscP were used to extend the length of InvJ. These fragments were inserted into various positions before amino acid 220 in InvJ to avoid possible disruption of the C-terminal region (InvJC). The Spi-1 master-regulatory gene, hilD, was expressed from an arabinose promoter (paraBAD-hilD<sup>+</sup>) to control Spi-1 induction. In addition, cells were cultured in Spi-1 inducing media (38). Cells were harvested, and intact injectisomes were isolated, negatively stained, and imaged by transmission electron microscopy. Needle lengths were measured and distributed along a histogram as shown in Fig. 2A.

The wild-type needle length of the Spi-1 injectisome was 20.8 ± 4 nm. This length is similar to the wild-type peak lengths reported in Marlovits et al. (28). We next measured needle lengths for the InvJ length-variants. All InvJ length-variants produced needles of controlled lengths that increased with increased InvJ lengths. This conclusion was supported by the Gaussian distribution for needle lengths plotted on a histogram (Fig. 3A). When the mean needle-lengths of each InvJ length-variant were plotted against their corresponding InvJ lengths, a linear correlation of ~0.2 nm per inserted amino acid residue was observed (Fig. 2B). This result is similar to the 0.17 and 0.19 nm per amino acid residue correlations that were reported for Hook–FliK and Needle–YscP lengths, respectively (3, 4). The spacing between amino acids is 0.17 nm in an alpha-helix and 0.4 nm in an extended peptide structure (39). Thus, the change in needle length we observed with amino acid additions in InvJ closely associates with the distance between amino acid residues in an alpha-helix, which is the predicted secondary structure of the inserted fragments of YscP (30). We also verified that all InvJ length variants were secreted (Fig. S1). This finding supports the model that InvJ acts as a secreted molecular ruler. There was no effect of the inserted YscP sequences on the amount of InvJ that was produced or secreted, further supporting that the observed needle length variation was due solely to InvJ length variation.

Strains deleted for the C terminus of InvJ (InvJ<sub>ΔC</sub>) produced the poly-needle phenotype of an invJ null mutant (Fig. 4). These mutants did not induce the secretion specificity switch, and secreted InvJ<sub>ΔC</sub> at wild-type levels (Figs. S2 and S3). This finding further demonstrates similarities between InvJ and the well characterized YscP and FliK molecular ruler systems. This result also supports the requirement of InvJ<sub>ΔC</sub> to induce the secretion specificity switch and suggests that length control is universal in all T3S systems.

**Secretion Competition Between Early T3S Substrates Affects Needle Length.** The hypothesis that injectisome needle-lengths were controlled by copolymerization with the inner-rod structure was in part an interpretation of the effect of prgJ overexpression on needle length. Marlovits et al. (28) reported that wild-type

![Fig. 2.](https://www.pnas.org/cgi/doi/10.1073/pnas.1423492112) Wee and Hughes

*S. enterica* produced injectisomes with needle-lengths varying between 7 and 57 nm and a peak length around 25 nm. When the inner-rod protein, PrgJ, was overproduced, they observed injectisomes with needle-lengths varying between 7 and 47 nm and a peak length of about 25 nm. Furthermore, cryo-EM analysis of poly-needles from a Δ<sup>invJ</sup> mutant revealed that they lacked the inner-rod structure. The 10% reduction in average needle length by PrgJ overexpression and lack of an inner-rod structure in the Δ<sup>invJ</sup> mutant led Marlovits et al. to propose the needle length control model based on the rate of PrgJ polymerization. This model purports that InvJ facilitates inner-rod assembly and copolymerizes with the needle in the periplasm. Completion of the inner-rod structure induces the secretion specificity switch and terminates needle assembly. Therefore, overexpression of
PrgJ resulted in a faster completion of the inner-rod, and thereby shorter needle structures.

Our findings suggest that the simplest interpretation of their data would be that needle length is shortened due to overexpression of a secretion competitor. Excess PrgJ could reduce the secretion rate of PrgI resulting in an increase in the frequency of InvJ measurements during needle elongation. Alternatively, overproduction of PrgJ could somehow facilitate InvJ secretion. Either possibility could affect the frequency of InvJ ruler measurements and needle length and predict that overproduction of PrgJ would lower InvJ concentrations outside of the cell. Furthermore, PrgI overexpression, which produces elongated needles, should also show reduced InvJ secretion.

InvJ was proposed to interact with PrgJ for inner-rod assembly. We tested whether InvJ is secreted in the absence of PrgJ. If InvJ secretion was independent of PrgJ, we expected to detect InvJ secreted into the periplasm. The periplasmic fraction from the ΔprgJ strain was isolated following osmotic shock and examined for the presence of InvJ by Western blot analysis. As expected, in the wild-type background InvJ was present in the cellular, periplasmic, and supernatant fractions, whereas, in the prgJ mutant, InvJ was not secreted to the supernatant, but was secreted to the periplasm (Fig. 3A). This finding indicates that PrgJ is not necessary for InvJ secretion.

We then investigated how excess levels of PrgJ or PrgI affected InvJ secretion, and thereby needle length. A copy of prgJ, prgI, and invJ coding regions were cloned into plasmid pTrc99A containing an IPTG-inducible promoter (pTrc), to produce an excess of PrgJ, PrgI or InvJ secretion substrates, respectively (40). The amount of InvJ secreted in the overproduction strains along with a wild-type control was determined by Western blot analysis of the extracellular fractions.

Overexpression of PrgJ resulted in ~40% reduction in InvJ secreted levels compared with the control with PrgJ expressed only from its native Spi-1 locus (Fig. 3C). Both strains had comparable levels of cellular InvJ to wild type (Fig. 3B). We also observed the same reduction in the InvJ secreted level when PrgJ was overproduced. Overproduction of either PrgJ or PrgI resulted in a reduction of the InvJ secreted level by about one third. Thus, the overexpression of one Spi-1 secretion substrate results in reduced secreted levels of another Spi-1 secretion substrate.

Mutant Alleles of prgJ Reported to Affect Needle Length Control Show Wild-Type Needle Lengths When Expressed from the Chromosome. A recent study by Lefebre and Galán (35) identified mutations in the inner-rod protein, PrgJ, which gave rise to aberrantly long needles that were competent in inducing the secretion specificity switch. Lefebre and Galán argued that these results suggested a defect in PrgJ–PrgI interactions that caused inner-rod to assembly at a slower rate, allowing the needle to polymerize to longer lengths before the inner-rod was able to induce the secretion specificity switch. However, the Lefebre and Galán study cloned all PrgJ mutants together with a wild-type copy of the needle subunit, PrgI, into a low-copy plasmid expression system (pWSK29) to maintain a wild-type PrgJ:PrgI ratio (35). We reasoned that the abnormally long needles produced by mutant PrgJ alleles was simply an artifact of PrgI overexpression, because previous studies have shown aberrantly long needle lengths greater than 92 nm were obtained when PrgI was expressed from the same low-copy plasmid (28).

We investigated whether the prgJ needle-length control mutations would produce the extended needle structures when expressed at wild-type levels from the chromosome and not subject to potential overexpression artifacts. We chose three mutations in prgJ (S75A, V81A, and M83A) that were reported to give rise to long needles and introduced these mutations into the chromosome prgJ locus (35). None of the three chromosomal prgJ mutants

Fig. 3. (A) Western blot analysis of cultured supernatant (S), periplasmic (P), and cellular (C) fractions for the presence of InvJ (Upper) and DnaK (Lower) in wild-type, ΔinvJ and ΔprgJ backgrounds. (B) Western blot analysis of cultured supernatants (Upper) and cellular (Lower) fractions for the presence of InvJ in wild-type (WT), invJ (invJ+), prgJ (prgJ+), and prgJ (prgJ++) overexpression backgrounds. (C) Relative levels of InvJ secretion from blots show in B to wild-type (WT = 1).

Fig. 4. Electron micrographs of negatively stained whole cells from chromosomal-∆prgJ/ΔprgJ (Top Left), chromosomal-prgJ/ΔprgJ (Top Right), invJΔC (Bottom Left), prgJ S75A. Arrows indicate needle structures. Electron micrograph of purified injectisome from chromosomal-prgJ/V81A (Middle Left) and prgJ-prgI overexpression strain (Right) clearly display the short and elongated needles produced from these strains, respectively.
gave rise poly-needles; the needles were similar to those observed with wild-type (prgJ) allele (Fig. 4).

We also examined whether overexpression of prgI and prgJ at wild-type ratios would result in elongated needles. We cloned the coding region of prgJ–prgI into the plasmid pTrc99a to produce excess amounts of PrgJ and PrgI subunits at wild-type ratios. Consistent with our data, we found that needles grow beyond wild-type lengths (40). It is possible that the elongated needles from the prgJ mutant strains reported by Lefebre and Galán (35) were due to the overexpression of prgJ (Fig. 4). Although the pWSK29 plasmid used in their study is a lower copy number plasmid than the pTrc99a backbone used in this study, Plasmid copy number differences could account for the differences observed between these two studies. We would not expect plasmid copy number to affect needle length according to the inner-rod model, provided that prgJ and prgI coexpression does not change. Our findings provide further evidence that needle length is not determined by the rate of inner rod versus needle polymerization, but rather support a molecular ruler model where the frequency of measurements relative to the rate of needle polymerization determines needle length.

Discussion

In 1972, Silverman and Simon reported hook-length control mutants for the flagellum of Escherichia coli, and the following year hook-length control mutants were reported for Salmonella Typhimurium (41). Loss-of-function alleles of a gene, later named fliK, resulted in the uncontrolled hook-lengths in these two species. More than two decades later, the laboratories of Shin-Ichi Aizawa, Kazuhiro Kutsukake, and Robert Macnab began the study of FliK-dependent hook-length control in the Salmonella flagellar system (23, 42, 43). Hook growth is terminated through an interaction between the C terminus of FliK (FliKc) and an integral cytoplasmic membrane component of the flagellar T3S (fT3S) system, FlhB, which is conserved in all T3S systems (25, 44). The conformation of FlhB determines whether the fT3S system is specific for the rod-hook-type or early class of secretion substrates or for the filament-type of late class of secretion substrates (45–47). Before the N terminus of FliK exiting the secretion channel, the secretion rate is slow, but in structures with short or no hooks, the C terminus of FliK is not in the vicinity of FliK when the N terminus is exiting the channel to catalyze the secretion-specificity switch. Once hooks reach a length where the entire molecule of FliK remains within the extended hook channel, FliK is passing through the channel at a slow rate when FliKc is in the vicinity of FlhB to induce the secretion-specificity switch (22).

Similarly, for the virulence-associated type III secretion (vT3S) system of the Salmonella pathogenicity island 1 (SPI-1) injectisome, loss of the FliK functional homolog, InvJ, resulted in injectosome structures with needles of uncontrolled lengths (10). An interaction between the C terminus of InvJ and the SPI-1 FliK homolog, SpaS, is thought to produce a conformational change in SpaS that results in the cessation of needle secretion and the initiation of late substrate secretion, which includes virulence in SpaS that results in the cessation of needle secretion and the homolog, SpaS, is thought to produce a conformational change interaction between the C terminus of InvJ and the SPI-1 FliK homolog, InvJ, resulted in in-vivo infections systems (25, 44). The conformation of FlhB determines whether the fT3S system is specific for the rod-hook-type or early class of secretion substrates or for the filament-type of late class of secretion substrates (45–47). Before the N terminus of FliK exiting the secretion channel, the secretion rate is slow, but in structures with short or no hooks, the C terminus of FliK is not in the vicinity of FliK when the N terminus is exiting the channel to catalyze the secretion-specificity switch. Once hooks reach a length where the entire molecule of FliK remains within the extended hook channel, FliK is passing through the channel at a slow rate when FliKc is in the vicinity of FlhB to induce the secretion-specificity switch (22).

The idea that both FliK and InvJ could act as molecular rulers came from work in the laboratory of Guy Cornelis (3). Like Salmonella Typhimurium, Yersinia pestis and Y. enterocolitica possess vT3S systems with a FliK functional homolog, YscP, and an FlhB homolog, YscU (31). Loss-of-function mutants in the yscP gene of either species resulted in needles of uncontrolled lengths, whereas lengthening or shortening YscP produced injectisomes with longer or shorter needles corresponding to 0.19 nm per amino acid inserted or deleted.

Following the work of Cornelis and his colleagues, it was shown that the FliK protein also acted as a molecular ruler. Lengthening or shortening FliK produced flagellar structures with longer or shorter hooks corresponding to 0.17 nm per amino acid inserted or deleted (4). Further studies revealed that FliK acted to measure a minimal length of about 40 nm (22). Additionally, the YscP/FliK homolog of Shigella flexneri’s injectisome, Spa32, has also been demonstrated to increase needle length when additional amino acids are introduced (48). We decided to test whether InvJ could act as a molecular ruler in the SPI-1 system, regardless of the presence of additional length control by inner-rod completion. Just like YscP and FliK, increasing the length of InvJ corresponded to increased needle lengths of ~0.2 nm per amino acid inserted, confirming that InvJ acts as a molecular ruler to control SPI-1 needle length. Furthermore, like the molecular rulers YscP and FliK, the C terminus of InvJ proved to be essential for inducing the SPI-1 secretion specificity switch.

The effect of secretion substrate competition on SPI-1 needle-length control explains the shorter needle lengths when PrgJ was overproduced in Marlowsits et al. (28). The reduction in needle length by prgJ overexpression was a key factor leading to the inner-rod model of length control. As was previously shown with FliK in the flagellar system, increasing the frequency a molecular ruler enters the secretion channel during assembly of the structure being measured will lead to a more accurate measurement of the needle and produce shorter needle lengths on average (49–51). Alternatively, increasing the rate of hook or needle polymerization would have the added effect of decreasing the frequency a molecular ruler enters the secretion channel to make a measurement and further contribute to the longer hook or needle lengths observed. In Lefebre and Galán (35), they report mutants in prgJ that correspond to increased needle lengths. However, in that study, prgJ was coexpressed with the prgI mutants under an inducible promoter in a plasmid-based expression system, and not from the chromosome (35). Based on previous reports, we believe that having prgJ coexpressed with prgI from a plasmid led to the overproduction of needle subunits and an increase in the rate of needle assembly (28). Our own findings show normal needle length when prgJ length control mutants were expressed from their native locus. Our study induces SPI-1 by expression of hilD from the arabinine promoter, which could also account for some of the differences between the two studies. However, the data in Fig. 2 demonstrating a direct correlation between InvJ and needle lengths is consistent with a molecular ruler model and not with an inner-rod model.

The question for us is not how PrgJ measures needle length, but how PrgJ affects InvJ and PrgJ secretion. The experimental results we report here show experimental evidence in support for a conserved molecular ruler mechanism of length control across all T3S systems. Additionally, we show that secretion competition between early SPI-1 substrates can affect needle lengths by altering frequency of measurements with respect to the rate of needle polymerization.

Materials and Methods

Bacterial Strains and Culture Conditions. Cells were cultured in lysogenic broth (LB) at 0.3 M NaCl. Arabinose (0.2% wt/vol) was supplemented to cultures as needed. The generalized transducing phage of S. Typhimurium P22 HT105/1 int=201 was used in all transduction crosses (52). InvJ variants were constructed using the Lambda-RED method (53).

Injectisome Isolation, Electron Microscopy, and Needle Measurements. Needle purification was carried out by the methods described in Marlowsits et al. (28). Purified injectisomes were negatively stained with 2% (wt/vol) uranyl acetate or 2% (wt/vol) PTA on carbon-formvar coated copper grids. Images were captured using a Hitachi H-7100 electron microscope at an acceleration voltage of 125 kV. Needle lengths from intact injectisomes were measured using NIH Image 1.42q software, and the average needle length was determined by nonlinear regression analysis of the Gaussian distribution using GraphPad Prism 5.0c software.
Protein Secretion Assay, Osmotic Shock, SDS/PAGE, and Western Blotting. Protein secretion was analyzed by Western blot. Overnight cultures were diluted 1:100 in 0.3 M NaCl LB media supplemented with 0.2% arabinose and grown until the cultures reached an optical density at 600 nm of ~0.8–1.0. Cell cultures were then centrifuged for 10 min, and the supernatant was collected and filtered through a low-protein-binding 0.45-μm cellulose acetate filter (Tisch Scientific syringe filter) to remove all cells from the supernatant fraction. Aliquots (2 ml) of the filtered supernatant were then mixed with 10% TCA (final concentration) to precipitate the secreted proteins, and washed with ice-cold acetone before further analysis. Isolation of the periplasmic fraction was performed by osmotic shock. Harvested cells were resuspended in 20% Sucrose, 1 mM EDTA, 30 mM Tris-HCl solution at pH 8.0. Cultures were incubated for 10 min at room temperature, then centrifuged at 13,000 g for 10 min at 4 °C. The resulting supernatant was removed and the pelleted cells were rapidly resuspended in ice-cold water. Cultures were incubated at 4 °C for 10 min, then centrifuged at 13,000 × g for 10 min at 4 °C. The resulting supernatant, which contains the periplasmic fraction, was collected and mixed with 10% TCA (final concentration).

Precipitated proteins from the supernatant and periplasmic fractions were dissolved in Laemmli sample buffer and adjusted to the sample with the lowest OD unit (~200 OD units per μl). Fifteen microliters of each sample (equivalents of ~3,000 OD units) were loaded onto a 12% SDS/PAGE gel. Proteins were analyzed by immunoblotting using anti-InvJ antibodies (rabbit) and visualized using secondary anti-rabbit-700 antibodies (LI-COR). Protein density was obtained using NIH Image 1.42q software.

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6. Table 1. List of abbreviations used.

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Fig. S1. Western blot analysis of cultured supernatants from wild-type, InvJ-YscP length variants, and InvJ_{ΔC} strains for the presence of InvJ in the extracellular fraction.

Fig. S2. Western blot analysis of cultured supernatants for the detection of late secretion substrate SptP::6xHis in wild-type, ΔinvJ mutant, and InvJ_{ΔC} backgrounds.

Fig. S3. (A) Western blots from all InvJ secretion assays that were used in Fig. 3C. (B) Western blot analysis of InvJ levels along with BSA as a loading control. BSA was added to each sample to a concentration of 40 ng/μL before loading. The Western blot from B was included in the analysis for Fig. 3C.