Kinetic Analysis of the Growth Rate of the Flagellar Hook in *Salmonella typhimurium* by the Population Balance Method

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ABSTRACT The growth rate of flagellar hooks in *Salmonella typhimurium* was analyzed by computer-aided simulation of the length distributions of mutant hooks of uncontrolled length (polyhooks). The wild-type hook has a relatively well-controlled length, with an average of 55 nm and a standard deviation of 6 nm. Mutations in the *fliK* gene give rise to polyhooks. A histogram of the lengths of polyhooks from a *fliK* mutant shows a peak at 55 nm with a long monotonic tail extending out to 1 μm. To analyze the growth rate, we employed the population balance method. Regression analysis showed that the histogram could fit a combination of two theoretical curves. In the first phase of growth, the hook starts with a very fast growth rate (40 nm/min), and then the rate exponentially slows until the length reaches 55 nm. In the second phase of growth, where the hook length is over 55 nm, the hook grows at a constant rate of 8 nm/min. Second mutations in either the *fliK* or *flhB* genes, as found in pseudorevertants from *fliK* mutants, give rise to polyhook filaments (phf). The ratio between the numbers of hooks with and without filament was 6:4. The calculated probability of filament attachment to polyhooks was low so that the proportion of hooks that start filament growth was only 2% per minute. The lengths of polyhooks with and without filaments were measured. A histogram of hook length in phf's was the same as that for polyhooks in single-site *fliK* mutants, against the expectation that the distribution would shift to a shorter average. The role of FliK in hook length control is discussed.

GLOSSARY

a	reciprocal of the cell division cycle (1/s)
b	hook growth rate factor (1/nm)
c	arbitrary constant (−)
G	nucleation rate of the hook (number/s)
k	growth rate of hook (nm/s)
L	hook length (nm)
N	population (number)
n	population density (number/nm)
t	time (s)

Greek symbols

α	population fraction of polyhook onto which filaments are attached per unit time (1/s)
β	ratio between the numbers of polyhook without filament and the total numbers of polyhook

Subscripts

0	initial value or value at $L = 0$
1	without filaments
2	with filaments
t	total number

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INTRODUCTION

The bacterial flagellum is a complex structure consisting of several parts with a defined size and shape; the major ones are the filament, hook, and basal body (DePamphilis and Adler, 1971). In *Salmonella typhimurium*, flagellar filaments are left-handed helices and have an average length (7–10 μm) and helical geometry that seems to exert power most effectively under physiological conditions (Jones and Aizawa, 1991; Macnab, 1996). The basal body consists of four rings and a rod (DePamphilis and Adler, 1971). The inner two rings are made of subunits of a single protein, FliF (Ueno et al., 1992). A complex of the outer two rings has an extraordinarily well-defined diameter, forming two-dimensional crystal sheets in a lipid bilayer (Akiba et al., 1991). The hook connects the filament to the basal body and works as a universal joint to convey torque, generated at the base, to the filament.

Like the flagellar filament, the hook is a tubular structure consisting of a single kind of protein, hook protein (FlgE). The hook self-assembles in vitro (Kato et al., 1982). In vivo, however, a scaffolding protein FlgD is necessary for hook subunits to polymerize; without FlgD, they are secreted into the medium as monomers (Ohnishi et al., 1994). The hook length can be indefinitely long in in vitro polymerization experiments, whereas in vivo it seems to be strictly defined. Hirano et al. (1994) carried out accurate measurements of the hook lengths and obtained an average length of 55 nm ± 6 nm, showing that the regulation of the hook length is not quite as tight as had been thought (for a review, see Aizawa, 1996).

To elucidate the mechanism of hook length control, we have studied mutants that produce extraordinarily long structures called polyhooks. The mutations that give rise to polyhooks are not in the hook protein gene *flgE*, but in *fliK* (Patterson-Delafield et al., 1973). Consequently, FliK has

been assumed to measure the hook length somehow and thus regulate it. The sequences of mutant *fliK* genes revealed that even large truncations of the gene give rise to polyhooks. If FliK is a molecular ruler such as those in tobacco mosaic virus or lambda phage, truncated FliKs would give rise to short hooks rather than polyhooks (Williams et al., 1996). Moreover, structural analysis of the *fliK* gene sequence indicates that FliK is fairly hydrophilic, but there are domains that lack α-helix (Kawagishi et al., 1996). However, all known molecular rulers are composed mainly of α-helix (Williams et al., 1996). Therefore, considering all of these lines of evidence, it seems unlikely that FliK is a molecular ruler.

Rigorous analysis of pseudorevertants of *fliK* showed that second-site mutations, not only in *fliK* but also in *flhB*, could give rise to polyhooks (Hirano et al., 1994; Minamino et al., 1994). The roles of the two genes seem distinguishable: FliK measures hook length and FlhB determines the substrate specificity of export (Williams et al., 1996). The mechanism by which FliK measures hook length is still a mystery, but it seems to be a complicated phenomenon involving the flagellar protein export system (Williams et al., 1996; Kutsukake, 1997).

We have measured the hook lengths of various mutants (Hirano et al., 1994). When ~100 polyhooks from SJW107 were measured, the histogram of lengths showed a flat distribution without any well-defined peaks. We then increased the number of particles from 100 to 1000. The histogram now showed a peak at 55 nm, which is the average length of wild-type hooks. Although single mathematical equations could express the shape and the peak of the distribution of hooks, the biological meaning of the equation was not obvious. We employed the population balance method to calculate the growth rate of hooks isolated from *fliK* and *flhB* mutants. A mechanism for regulating hook length is discussed.

MATERIALS AND METHODS

Bacterial strains

All strains used in this study were derived from *Salmonella typhimurium* SJW1103 (Yamaguchi et al., 1986). SJW107 is a *fliK* polyhook mutant (Hirano et al., 1994; Williams et al., 1996). SJW3124 is a *flhB* extragenic suppressor of SJW107 (Hirano et al., 1994) and has the mutation R302F (see MY2709 in Williams et al., 1996). SJW3125 was derived from SJW3124 and has a *flhB-flgK* double mutation (Hirano et al., 1994).

Isolation of HBB

Hook basal bodies from various strains were purified according to the method of Aizawa et al. (1985), with a minor modification (Hirano et al., 1994).

Electron microscopy

Samples were negatively stained with 2% phosphotungstic acid (pH 7.0 or 4.4) and observed with a JEM-1200EXII electron microscope (JEOL, Tokyo, Japan). Micrographs were taken at an accelerating voltage of 80 kV and a magnification of ×20,000. The length of the hook was measured on negatives, using a magnifier (PEAK scale lupe 15×). Immunoelectron microscopy was carried out as previously described (Ohnishi et al., 1994).

Population balance method

We assume that the number of hooks doubles per cell division and that each hook grows at a rate $k(L)$ ($= \mathrm{d}L/\mathrm{d}t$). In a finite time Δt, the hook grows by $k(L)\Delta t$. Among the number $N(L, t)$ of hooks of lengths between L and $(L + \Delta L)$, the number that grow longer than $(L + \Delta L)$ during the following time Δt will be

$$N(L, t) \cdot k(L) \cdot \Delta t/\Delta L$$

Note that population $N(L, t)$ itself is influenced by the size of length segments (ΔL). In the same manner, the number of hooks that come in this range will be

$$N(L - \Delta L, t) \cdot k(L - \Delta L) \cdot \Delta t/\Delta L$$

Therefore, the increment of the number of hooks in this range during the time Δt can be given as follows:

$$[N(L, t + \Delta t) - N(L, t)] \tag{1}$$
$$= -[N(L - \Delta L, t) \cdot k(L - \Delta L) - N(L, t) \cdot k(L)]\Delta t/\Delta L$$

By dividing Eq. 1 by $\Delta t \Delta L$, introducing the population density $n(L, t)$ [$= N(L, t)/\Delta L$], and taking the limit as Δt and ΔL approach zero, we get the population balance equation:

$$\partial n(L, t)/\partial t = -\partial(k \cdot n(L, t))/\partial L$$

and, if k is constant,

$$\partial n(L, t)/\partial t = -k \cdot \partial n(L, t)/\partial L \tag{2}$$

The total number of hooks at $t = 0$ is expressed as

$$t = 0; \qquad N_{\mathrm{to}} = \int_0^\infty n(L, t)|_{\mathrm{t}=0}\, \mathrm{d}L \tag{3}$$

The relationship between the population density at $L = 0$ and the nucleation rate of the hook by a cell division (G) is written as

$$L = 0; \qquad n(L, t) = G/k \tag{4}$$

and as the total number of hooks (N_t) can be expressed in terms of the reciprocal of the cell division cycle (a) as

$$N_t = N_{\mathrm{to}} \cdot 2^{at} = N_{\mathrm{to}} \cdot \exp(\ln 2 \cdot at) \tag{5}$$

G is given by

$$G = \mathrm{d}\,N_t/\mathrm{d}t = \ln 2 \cdot a \cdot N_{\mathrm{to}} \cdot \exp(\ln 2 \cdot at) \tag{6}$$

Under the initial condition (Eq. 3) and the boundary condition (Eq. 4), Eq. 2 can be solved, giving

$$n(L, t) = [\ln 2 \cdot aN_{\mathrm{t0}}/k] \cdot \exp[\ln 2 \cdot a(t - L/k)] \tag{7}$$

On the other hand, if the value of k decreases exponentially with increasing value of L as follows:

$$k = k_0 \cdot \exp(-bL) \tag{8}$$

it is necessary to solve numerically.

Next we consider the case in which filaments attach to polyhooks. Because the time rate of change of the population of polyhooks without filaments can be expressed as the difference between the rate of the

nucleation by a cell division and that of decreasing by attachment of filaments, the following equations can be obtained:

$$dN_{t1}/dt = N_{t0} \ln 2 \cdot a \cdot \exp(\ln 2 \cdot at) - \alpha N_{t1} \quad (9)$$

$$dN_{t2} = N_{t0} - N_{t1} \quad (10)$$

where N_{t1} and N_{t2} are the populations of polyhooks without and with filament, and α is the population fraction of polyhooks on which filaments are attached per unit time. Integration of Eq. 9 gives the following equation with an arbitrary constant c:

$$N_{t1} = [\ln 2 \cdot a/(\ln 2 \cdot a + \alpha)] \cdot N_{t0} \cdot \exp(\ln 2 \cdot at) + c \cdot \exp(-\alpha t) \quad (11)$$

If t is sufficiently large, the ratio between the number of polyhooks without filament and the total number of polyhooks is expressed as

$$\beta = [n_{t1}(L, t)/(n_{t1}(L, t) + n_{t2}(L, t))] = [\ln 2 \cdot a/(\ln 2 \cdot a + \alpha)] \quad (12)$$

where $n_{t1}(L, t)$ and $n_{t2}(L, t)$ are the population densities of polyhook without and with filament. On the other hand, the population balance equations in this case may be written as

$$\partial n_{t1}(L, t)/\partial t = -\partial(k \cdot n_{t1}(L, t))/\partial L - k \cdot \partial n_{t1}(L, t)/\partial L \quad (13)$$

$$\partial n_{t2}(L, t)/\partial t = \alpha \cdot n_{t1}(L, t) \quad (14)$$

The initial conditions for Eqs. 13 and 14 may be written as

$$t = 0; \qquad \beta \cdot N_{t0} = \int_0^\infty n_{t1}(L, t)|_{t=0}\, dL \quad (15)$$

$$t = 0; \qquad (1 - \beta) \cdot N_{t0} = \int_0^\infty n_{t2}(L, t)|_{t=0}\, dL \quad (16)$$

and the value of $n_{t1}(L, t)$ at $L = 0$ is written as

$$L = 0; \qquad n_{t1}(L, t) = G/k \quad (17)$$

If the growth rate of the polyhook k is constant, Eqs. 13 and 14 can be solved analytically, the population densities are obtained as

$$n_{t1}(L, t) = [\ln 2 \cdot aN_{t0}/k] \cdot \exp[\ln 2 \cdot a(t - L/k\beta)] \quad (18)$$

$$n_{t2}(L, t) = [(1 - \beta) \cdot \ln 2 \cdot aN_{t0}/k\beta] \cdot \exp[\ln 2 \cdot a(t - L/k\beta)] \quad (19)$$

and the total population density $n(L, t)$ ($= n_{t1}(L, t) + n_{t2}(L, t)$) is given by

$$n(L, t) = [\ln 2 \cdot aN_{t0}/k\beta] \cdot \exp[\ln 2 \cdot a(t - L/k\beta)] \quad (20)$$

RESULTS

Histogram of hook length of a *fliK* mutant SJW107

We previously reported (Hirano et al., 1994) that the lengths of wild-type hooks were distributed in a narrow range with a peak at 55 nm and a standard deviation of 6 nm, but those of polyhooks were distributed in a wide range (from 20 nm to 1 μm) without any noticeable peaks. The number of particles measured was ~100 for each strain, and therefore the histogram of polyhook lengths was rather sparse.

To obtain a more precise distribution, we increased the number of particles measured to over 1000. In the process, we modified the purification protocol. When polyhook basal bodies were purified by CsCl density gradient centrifugation, very short hooks that had just started growing gathered at the top fraction of the gradient. To avoid this fractionation, we used simple ultracentrifugation instead of density gradient centrifugation, and all particles seen in the electron micrographs were counted and measured.

Fig. 1 shows the histogram for 1180 polyhooks isolated from a fliK mutant SJW107. The peak and shape of the distribution are evident. To our surprise, the peak was at 55 nm, which is the average length of wild-type hooks. Thus, even in polyhook mutants, hooks as short as wild-type ones are the major species. Because of the protocol used for preparation of the sample, the appearance of the peak cannot be explained as an artefact.

Was the same position (55 nm) of the peaks from the wild-type hook and polyhook distributions a coincidence? If not, how could the hook length be regulated in *fliK* mutants? Why can polyhooks continue to grow? To answer these questions, we analyzed hook growth rate by simulating the experimental curves of polyhook distributions with theoretical curves.

Mathematical analysis of hook length distributions

First we analyzed the tail part of the polyhook distribution (from 60 nm to 1000 nm in Fig. 1), where data points were abundant and reliable. For curve fitting, we carried out

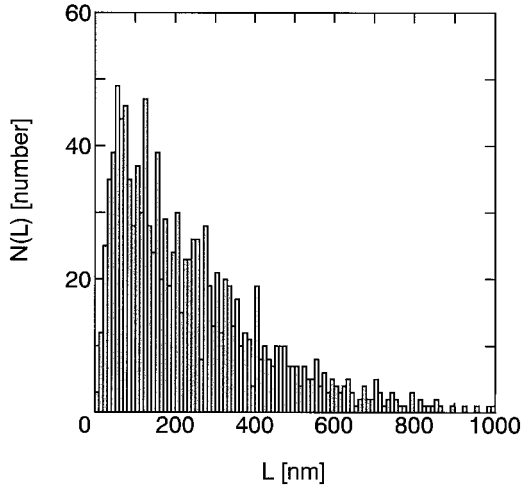

FIGURE 1 A histogram of hook lengths in a polyhook mutant SJW107. Hook lengths of 1180 particles were measured directly from negatives. The hook length (L, abscissa) was divided into 10-nm segments. The ordinate indicates the number of particles at each length, $N(L)$.

regression analysis, using a logarithmic scale for the ordinate (Fig. 2). The distribution is obviously linear and can be expressed as

$$\ln N(L) = A - BL \tag{21}$$

where A (= intercept on the ordinate) = ln 110, B (= slope) = 0.0042/nm. This equation can be rewritten in a general form as follows:

$$N(L) = A' \exp(-B'L) \tag{22}$$

Exponential relationships often appear in biological systems that involve cell growth and division. It is natural to assume that the number of hooks on a cell multiplies by a factor of 2 during each cell cycle, because the number of flagella stays approximately constant over many generations (Iino, 1974). As the cell number increases, the population of hooks concomitantly grows. Therefore, we can assume that the number of hooks constantly increases with time and each hook grows continuously. As a consequence of kinetic properties of a population, we obtain a histogram as shown above (Fig. 1).

Application of population balance method

For quantitative estimation of the hook growth rate, we employed the population balance method that has been developed for the evaluation of kinetic properties of crystal growth of inorganic salts (Penlidis et al., 1986; Marchal et al., 1988). This method sets an assumption about the behavior of each particle and describes time-dependent changes in the number of particles in a certain state. In our case, the change in the number $N(L)$ of hooks of lengths between L and ΔL per unit time is described as follows:

$\partial N(L)/\partial t$
= (the number of hooks that come into this range)
− (the number of hooks that grow out of this range)

This relationship leads us to a differential equation, which is solved by analytical computation (for details, see Materials and Methods). We obtain the following equation for population density ($n(L, t)$) as a function of length (L) and time (t):

$$n(L, t) = [\ln 2 \cdot aN_{t0}/k] \cdot \exp[\ln 2 \cdot a(t - L/k)] \tag{23}$$

We take into account not only the growth rate, but also the cell division cycle. In the calculation, the cell division cycle ($1/a$) of *S. typhimurium* is assumed to be 20 min. We rewrite Eq. 22 as follows:

$$N(L) = C \cdot \exp[D(t - L/k)] = C \cdot \exp D\,t \cdot \exp(-DL/k) \tag{24}$$

Comparing Eq. 23 with Equation 22, we obtain $A' = C \exp Dt$ and $B' = D/k$. Therefore, k is constant; that is, the growth rate is constant. The parameter A is a function of time. In our experiments, time is fixed at the point when the cells were harvested. As time passes, the number of hooks to measure and the number of longer hooks increase; in the regression analysis, the slope (B) would stay the same, but the intercept with the ordinate (A) will become large.

Constant growth phase

From the analysis shown above, we conclude that the majority of polyhooks (92%) grow at a constant rate. From the best fit, the constant growth rate (k) was determined to be 8 nm/min. Because the pitch of the basic helix of the structural lattice of the hook is 2.5 nm and two turns of the basic helix contain 11 hook subunits (Wagenknecht et al., 1982), the value 8 nm/min corresponds to ~18 subunits/min.

Initial growth phase

Now we consider the distribution below 55 nm. If the hook were to grow at a constant rate at all times and the number of hooks on a cell were to multiply by a factor of 2 after each cell division, the shorter hooks would then dominate the population. If this were the case, the distribution would show a monotonic exponential curve (Fig. 3, *curve 1*). However, hooks shorter than 30 nm were rarely seen, so that the distribution had a peak at around 55 nm (see Discussion).

The scarcity of very short hooks suggests that initially the hook may grow not at a constant rate, but at a much faster rate. The rapid increase in hook length from 0 to 55 nm can be expressed in several ways. Two simple forms of growth rate that decreases with length are as follows:

$$dL/dt = 1/(a + bL) \tag{25}$$

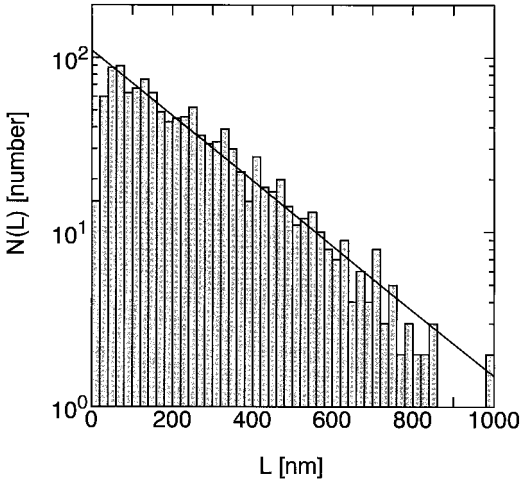

FIGURE 2 Regression curve of Fig. 1. The ordinate is expressed on a logarithmic scale. The theoretical line is straight and can be expressed as $\ln N(L) = A - BL$, where A (= intercept on the ordinate) = ln 110, B (= slope) = 0.0042/nm. The hook length was divided into 20-nm segments, that is, twice the size of those in Fig. 1.

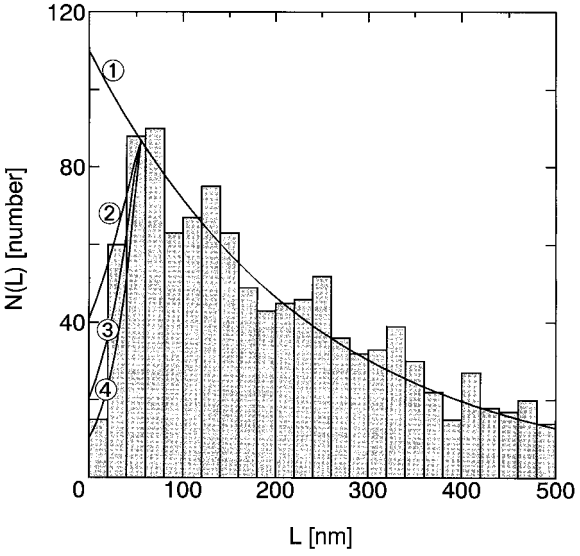

FIGURE 3 Theoretical curves calculated by the population balance method for the histogram of Fig. 1. If we assume that hooks grow at a constant rate, the theoretical curve will be exponential and does not fit below 55 nm (*curve 1*). Thus we assumed two different modes for hook growth: one below 55 nm and the other above 55 nm. The curve below 55 nm can be expressed as $dL/dt = k_0\exp(-bL)$. Because of the scarcity of data points, k_0 can be any value between 20 and 80 nm/min: (*curve 2*) 80, (*curve 3*) 40, (*curve 4*) 20 nm/min. The curve above 55 nm is expressed as $dL/dt = k$, where $k = 8$ nm/min. We chose $b = 0.03$ nm^{-1}, so that the two curves were joined at 55 nm. The hook length was divided into 20-nm segments.

$$dL/dt = k_0 \exp(-bL) \tag{26}$$

Because of the scarcity of data points in this range, they are consistent with either expression. However, the biological meaning of Eq. 24 is not clear. As the growth rate of flagellar filaments can be expressed in exponential form (Iino, 1974), we assume that Eq. 25 represents the growth rate of hooks below 55 nm. The biological meaning of this equation will be discussed later.

There is some uncertainty in the initial growth rate k_0; values between 20 and 80 nm/min are consistent with data (Fig. 3, *curves 2–4*). We tentatively chose the value to be 40 nm/min, which corresponds to ~88 subunits/min. The growth rate decreases exponentially with time ($b = 0.03$/nm) until the length reaches 55 nm. The value of the parameter b was chosen so that the curve connected continuously with the curve of the second phase. With these values, the time for growth to 55 nm is calculated to be ~3.5 min.

In conclusion, there are at least two phases in polyhook elongation. The growth rate of the hook is very large at the beginning and then decays exponentially until the length reaches 55 nm. After 55 nm, the growth rate becomes constant and continues indefinitely as long as hook subunits are supplied.

This length of 55 nm does not seem to be controlled by a molecular ruler, because even *fliK* mutants that lack FliK can control hook lengths. Because the control is transient, another factor is required to stop the growth. One possibility is that control is exerted by the amounts of hook proteins to be supplied (see Discussion).

Effects of a FlhB mutation

As well as *fliK* mutations, certain mutations in the *flhB* gene give rise to polyhooks (Hirano et al., 1994; Minamino et al., 1994), but in a somewhat different way.

We have never found any *flhB* mutants that give rise to polyhooks only; they always produce polyhook filaments. The length distribution of *flhB* polyhooks could be affected by filament formation, which permanently stops the growth of the hook. To avoid this complication, we introduced a *flgK* mutation into a *flhB* mutant. FlgK, also called HAP1, is a junction protein between the hook and filament. A *flgK-flhB* double mutant (SJW3125) retained FlgD at the tip of the hooks, as shown by immunoelectron microscopy (Fig. 4), therefore allowing the hook to grow as long as hook proteins were being supplied.

There are no extraordinarily long polyhooks in *flhB* mutants, such as are seen in *fliK* mutants (Hirano et al., 1994). The peak in the distribution of the lengths of *flhB* polyhooks is at around 55 nm, but the tail of the curve disappears before 300 nm.

The distribution of hook lengths of SJW3125 was analyzed in the same way as described above. The curve between 0 and 55 nm is discontinuous, rather like a delta function peak (Fig. 5). In fact, the shape around the peak resembles that of wild-type hooks. The tail of the curve after 55 nm is continuous, but it quickly drops to zero before 300 nm, suggesting the rate is much slower than that of *fliK* polyhooks. The constant rate is calculated to be 1.5 nm/min, which corresponds to 2 or 3 subunits/min.

In summary, the growth rate of *flhB* polyhooks in the first phase is the same as that of wild type, but in the second phase the growth rate is much smaller than that of *fliK* polyhooks, indicating that the mechanism of controlling export of hook proteins in *flhB* mutants is different from that in *fliK* mutants.

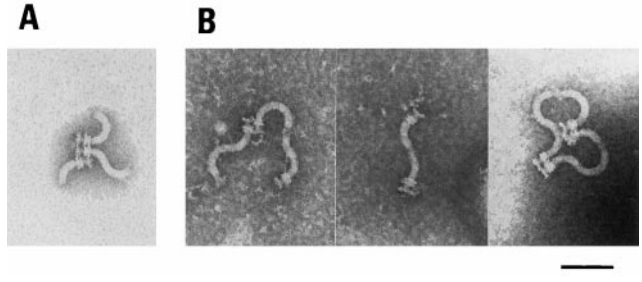

FIGURE 4 Immunoelectron microscopy of a scaffolding protein FlgD attaching at the tip of hooks. (*A*) Hook-basal bodies isolated from SJW3125, a double mutant (*flhB*, *flgK*). (*B*) The HBBs of *A* were reacted with anti-FlgD antibody. Note that particles are connected at the tip of the hook through antibody molecules. Negatively stained with phosphotungstic acid (pH 7.0). The bar represents 100 nm.

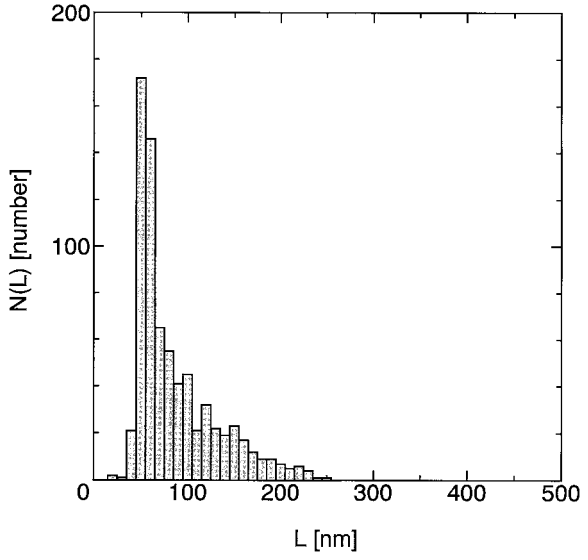

FIGURE 5 A histogram of hook lengths in a *flhB-flgK* mutant SJW3125. A total of 740 particles were counted for measurements. The hook length was divided into 10-nm segments.

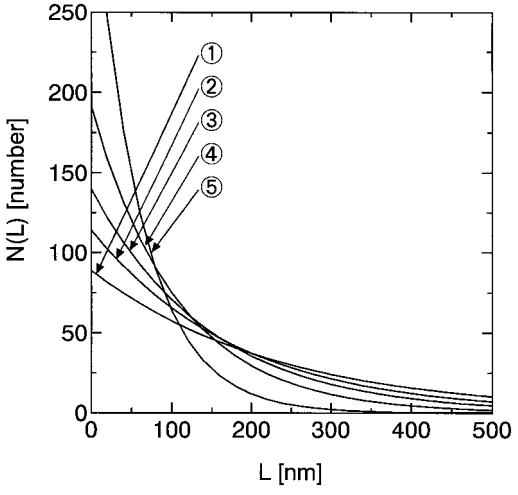

FIGURE 6 Theoretical curves for hook length when filament formation is allowed with various probabilities of attachment. The curve was expressed as $dL/dt = k$, where $k = 8$ nm/min. The parameters for each curve are (*1*) $\alpha = 0$ ($\beta = 1.0$), (*2*) $\alpha = 0.01$ ($\beta = 0.78$), (*3*) $\alpha = 0.02$ ($\beta = 0.63$), (*4*) $\alpha = 0.04$ ($\beta = 0.46$), (*5*) $\alpha = 0.1$ ($\beta = 0.26$). See Materials and Methods, Eqs. 9 and 12, for details.

Effects of filament formation

To evaluate the effects of filament formation on hook growth, a *flhB* polyhook mutant SJW3124 (retaining the same *flhB* mutation as SJW3125) was examined. We isolated all of the basal bodies attached to cells and counted the numbers of hooks with and without filaments attached. The ratio between the number of hooks alone and the number of hook-filament complexes was 60:40 (from 735 particles counted).

From this ratio and the curves of the histogram, we could calculate the probability of filament formation. Two assumptions were made. First, we assumed that hook growth would stop once filament formation had started. This assumption is logically reasonable, because the hook grows only from the tip. Second, we assumed that the probability of filament formation would be independent of hook length. This assumption was not justifiable a priori, but was validated by the results of simulation and regression analysis; if filament formation started only from long hooks, the shape of the curve would not be similar to that of polyhooks without filaments. The regression analysis yields 2% per minute as the probability of filament formation (Fig. 6).

As the probability of filament formation becomes higher in the simulations, the population of shorter polyhooks increases (Fig. 6). If the probability were as high as 10%, the curve would become sharper and the final ratio of hooks with filaments to ones without filaments would be nearly 80%. Therefore, filament formation is an important factor in maintaining an average length of hooks that is as short as possible.

DISCUSSION

Polyhooks have been thought to be hooks without length control, and almost always images of very long structures are used to represent them. However, when we measured the lengths of 1000 polyhooks, the histogram of polyhook lengths showed that hooks of wild-type length were dominant in the population. This strongly indicates that the control of hook length and the halt of hook growth are different events. Computer-aided simulation of experimental data using the population balance method suggested that there were two phases in hook growth: a quick and a slow one. In the quick phase, the hook grew very fast initially, but slowed down exponentially with time. After the length reached 55 nm, the quick phase was replaced by the slow phase, in which the hook grew at a constant rate.

Special care was taken to prepare intact polyhooks. Because hooks are much shorter than filaments and the buoyant densities of these structures are different, hooks with or without filaments focus at different positions in a CsCl density gradient. This explains why there were no hooks shorter than 30 nm in a histogram of hook length of wild type, because in that study shorter hooks were intentionally removed to measure only mature hooks with filaments (Hirano et al., 1994). For the preparation of polyhooks, CsCl density gradient centrifugation was avoided, and ultracentrifugation was used to collect all particles regardless of hook length; structures as small as the MS ring complex can be recovered by this method (Kubori et al., 1992). Measurements of polyhook lengths now revealed that hooks shorter than 30 nm do exist and contribute to a continuous curve in the histogram of polyhook lengths.

The functions of FlhB are different from those of FliK in two ways. First, mutations in FlhB give rise only to polyhook filaments. Second, hook growth rates in *flhB* mutants are smaller than those in *fliK* mutants. Third, only the MS

ring complexes, the first components in flagellar assembly, are found in *flhB* deletion mutants. These facts indicate that FlhB is more directly involved in export than FliK.

In polyhook filament mutants, polyhooks with filaments are only 40% of the population, suggesting that filament formation is impaired. Theoretical analysis shows that only 2% of the hook population is able to initiate filament formation per minute. This impairment of filament formation might reflect impairment of switching of export modes, from a mode for hook proteins to another for flagellin (see below).

Roles of FliK

Although FliK is indispensable for producing hooks of ordinary length, it is unlikely to be a molecular ruler of hook length (Kawagishi et al., 1996; Williams et al., 1996). How then can length control be achieved by FliK? It has been suggested that FliK has two domains and each domain has a different function: hook length measurement and the initiation of filament formation. Williams et al. (1996) speculated that the N-terminal domain measures hook length and that the C-terminal domain signals to FlhB the substrate specificity for export, hence initiating filament formation.

However, the fact that the peak in a population of polyhooks is at 55 nm indicates a mechanism of length control that is independent of FliK function. In *fliK* mutants, after hook length has been determined by an unknown mechanism, hook growth continues because of lack of intact FliK. Partial recovery of mutant FliK leads to the recovery of filament formation (Hirano et al., 1994; Williams et al., 1996). Therefore, we doubt that FliK has a dual function and propose a single function for it, namely, initiation of filament formation.

How does FliK initiate filament formation? FliK might interact with the C-terminal region of FlhB to export FlgM, an anti-sigma factor (Kutsukake et al., 1994). Exporting FlgM from the cell triggers the expression of late flagellar genes, including flagellin and HAPs (Kutsukake et al., 1994). Taking into account all of these data, it is likely that FliK is involved in the export of flagellar proteins necessary for filament formation such as HAP1 and HAP3.

Because neither FliK nor FlhB is directly involved in hook length determination, there seems to be no single factor to control length. It could be a combination of several factors: the amounts of hook protein available, the rate at which protein is exported, and the timing of switching of the export mode from hook to flagellin.

The rate of exporting flagellar proteins will be difficult to measure until the structure of the export apparatus is revealed. The switching mechanism of the export apparatus explains phenomena occurring in flagellation, but it is still hypothetical. Just recently, Kutsukake (1997) described a new gene, *rflH*, which could be involved in the switching machinery, resulting in hook length control along with *flhB*. The machinery itself has not yet been revealed.

The amount of hook protein available at the beginning of the process might be critical for hook length. This amount might be determined at either the translational or the transcriptional level. Direct measurements, not only in a cell but also at each flagellar base, would be desirable for measuring the pool size.

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