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ASSEMBLY OF SALMONELLA FLAGELLIN IN VITRO AND IN VIVO

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Formation of a flagellar filament of Salmonella from its component protein, flagellin, is in principle a self-assembly process, which proceeds by the addition of flagellin monomers one by one to the distal end of the filament. Upon their polymerization, a conformational change of flagellin molecules occurs, and it confers polarity to the filament. For the initiation of in vitro flagellin assembly in a solution of physiological ionic strength and pH, it is essential to add fragments of flagellar filaments, which work as seeds for the polymerization of flagellin monomers. When an appropriate concentration of some anion known as a good salting-outer is added to the solution, the polymerization begins without addition of seed. Assembly of flagellins in vivo begins at the distal end of each hook. The distal ends of the hooks on the cells of a flagellin-less mutant were shown to initiate the assembly of exogenous flagellin in vitro, although the efficiency was not as high as that of the in vivo initiation. A flagellar filament elongates in vitro at a constant rate as long as a sufficient amount of flagellin is supplied, and the elongation terminates by an error occurring at the growing end of the filament. On the contrary, the rate of in vivo elongation decreases exponentially with increase of the length of the filament. Initial rate of the in vivo elongation depends on growth condition of the bacteria, while decrease of the rate per unit filament length is little affected by the growth condition. The observed limit in length of the flagellar filaments on growing bacteria is expected from the exponential decrease of their rate of elongation. The decrease of the in vivo elongation is correlated with the lowering of the transportation efficiency of flagellin monomers on their passage from the cell body through the central canal of a flagellar filament to the tip.

INTRODUCTION

The simplest form of flagellar filaments in bacteria, such as those in Salmonella, are composed of a single kind of protein, called flagellin. In a flagellar filament, flagellin molecules are aligned in eleven longitudinal rows, alternate with each other in adjacent rows, and form as a whole a tubular structure (1, 2).

The success of in vitro assembly of flagellin monomers into flagellar filaments (3–5) showed flagella to be a unique material for studies of structure formation by assembly of component macromolecules, and by appropriate use of various mutant flagellins, much information has been accumulated on the process of the assembly (6–8). The present paper is intended to summarize information obtained on Salmonella flagella and on the basic process of in vitro assembly of flagellin into flagellar filaments, and to compare the in vitro process with the process of filament formation in vivo.

Although formation of the characteristic helical structure is an important aspect of flagellin assembly, it is excluded from the present discussion because the lately published review article (9) and a report (10) cover the subject sufficiently.

BASIC PROCESS OF ASSEMBLY OF FLAGELLIN

The experimental system first attained for the in vitro assembly of Salmonella flagellin into flagellar filaments was to mix flagellin monomers obtained by dissociation of the filaments with the fragmented filaments in an appropriate salt solution (Fig. 1) (4). The conditions required for the efficient assembly are a) a concentration of flagellin higher than 5 mg/ml, b) physiological ionic strength and pH of the solution, e.g., 0.01–0.03 M phosphate buffer with 0.2–0.3 M KCl at pH 6–7, and c) temperature range of 20 to 30°. The shape of the reconstituted filaments was the same as that of the flagellar filaments from which the flagellins were prepared.

Throughout the assembly reactions, there was no growth of the filaments by joining of two or more of the fragments. Furthermore, oligomer aggregates of flagellin are not detected as an intermediate of the assembly. An additional remarkable characteristic of the reaction is that the assembly occurs only at one end of each flagellar filament in the mixture (7). As the result, filament grows unidirectionally. This was directly proven by a cross-polymerization experiment in which flagellin monomers of one antigen type were mixed with fragmented filaments of another antigen type, and the reconstituted filament portions were identified by labeling with antibody specific for either the fragment or the flagellin (Fig. 2). It was further shown that each growing end has the concave shape characteristic of the distal end of the filament attached to a bacterial body. In order to explain these phenomena, the following three steps were postulated as the basic process of flagellin assembly. a) A flagellin monomer binds to the distal end of a filament. b) The monomer is incorporated into the filament accompanied by its conformational change. c) The incorporated monomer then acts as a part of the nucleus for polymerization of the next monomer to arrive. Thus, a conformational change of flagellin molecules upon their assembly confers structural polarity to the flagellar filaments and confines the assembling site to the distal end of each filament.

In vitro assembly of flagellin at the distal end of a flagellar filament is possible even when the seed filament is attached to a bacterial cell body (11). To avoid interference of flagellin synthesized in vivo with that added in the reaction mixture, the bacteria are starved beforehand or 50 μg/ml of chloramphenicol is added to the suspension of bacteria. Efficient assembly occurs when more than 5 mg/ml of flagellin monomers are mixed with more than 10^{10} cells/ml of bacteria in 0.03 M phosphate buffer with 0.08 M K_3-citrate, pH 6.9. The addition of more than 0.08 M of K_3-citrate results in the onset of polymerization of flagellin independent of the existing filament.

The cells of straight flagella mutants are nonmotile because of the nonhelical shape

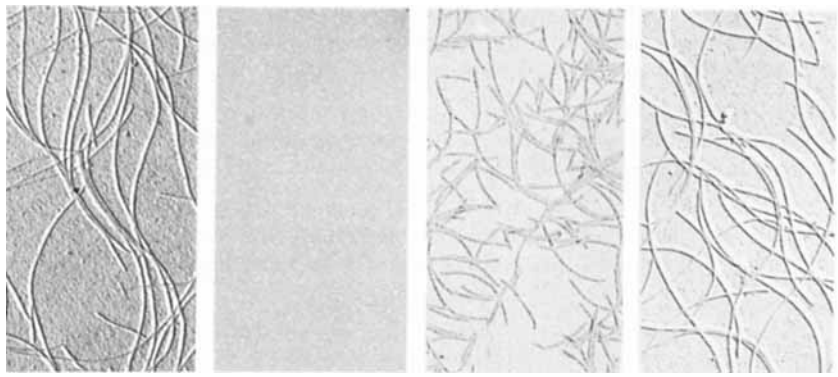

Fig. 1. In vitro assembly of Salmonella flagellin: A, flagellar filaments isolated from the cells of strain SJ25; B, solution containing 8 mg/ml flagellin, which was prepared by heating A at 57° for 30 min, in 0.03 M phosphate buffer with 0.2 M KCl (pH 6.8); C, suspension of fragmented filaments prepared by sonic vibration of A, in solution with the same salt constituents as B; and D, reconstituted filaments obtained after B and C were mixed in equal volumes and kept at 26° for 2 hours. Carbon shadowed preparations, × 13,000. Detailed procedures are in Ref. 4.

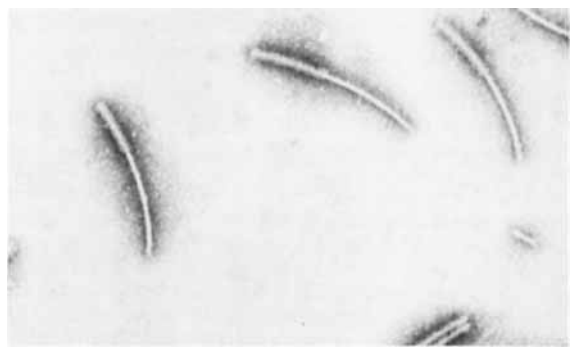

Fig. 2. Filaments reconstituted from 1.2-type flagellin with i-type seed fragments and labeled by antibody specific for i-antigen. Preparation negatively stained with 1% phosphotungstic acid, × 30,000. Detailed procedures are in Ref. 7.

of the filaments. When the cells of such a straight-flagella mutant were mixed with flagellin prepared from normal flagella, the reconstituted normal waves appeared at the tips of the straight filaments. These cells show motility when they are transferred to nutrient liquid medium (11). This result indicates that the flagellar filaments reconstituted in vitro are functionally active and they are able to confer motility on the cells carrying them.

As the in vitro assembly, the in vivo growth of a flagellar filament was found to occur by the polymerization of flagellin at the distal end of the filament. The experiment on Salmonella was based on the phenomenon that the addition of p-fluorophenylalanine

(FPA) to a culture of S. typhimurium carrying normal-shaped filaments resulted in the production of curly filaments (12). When FPA was added for a short period and the bacteria were observed, the curly waves were detected always in the distal portion of a filament (13). The growth of flagellar filaments at their distal ends was also confirmed on Bacillus subtilis by radioautography of the filaments whose flagellin was pulse-labeled with ^{3}H-leucine (14).

For flagellar filaments to grow at their distal ends, flagellin molecules synthesized in the cell body must be transported to the tips of the filaments. To test the possibility that flagellin molecules are first excreted from the cell and then used for the growth of the filaments, a normal-flagella strain and a curly-flagella strain of Salmonella were cultivated together in a liquid medium (13). Copolymerization is known to occur between normal and curly flagellin molecules and the copolymer manifests curly waves (6). Therefore, if flagellins synthesized by both strains are excreted into the medium and used for the growth of the filaments, the cells of the normal flagella strain must produce filaments with curly waves. The experimental result indicated that this is not the case: neither heteromorphous bacteria carrying both normal and curly flagella nor single flagella having both normal and curly waves were detected. Although the direct evidence is still missing, the only possible pathway through which flagellin can reach the tip of a flagellar filament is therefore considered to be the central canal of the filament.

INITIATION OF FLAGELLIN ASSEMBLY

The in vitro experiments described in the foregoing section required fragments of flagellar filaments as seeds for the assembly of flagellin. The in vitro assembly of flagellin without seeds was first demonstrated in Bacillus subtilis (1) and later in Salmonella (15). For Salmonella flagellin, the assembly without seeds requires the addition of appropriate concentration of some anion known as a good salting-outer, e.g., $(NH_4)_2SO_4$, Na-citrate, or KF at a final concentration of more than 0.5 M. The number of assembled filaments increases (and conversely their lengths decrease) as the increase of the concentration of the anion when the amount of flagellin is constant (15). These results are taken as an indication that the addition of the anion promotes spontaneous assembly of flagellin and that each polymer thus formed works as a seed for further assembly. In other words, the assembly of flagellin in vitro proceeds through two steps, namely, formation of an initial seed and elongation extending from the seed.

The initiation of in vivo filament growth is somewhat different from that of in vitro. On living bacteria, the proximal end of a filament is jointed to the distal end of a hook, and growth of the filament must begin by binding of flagellin at the distal end of the hook. Thus the distal end of each hook must play the role of a nucleus for the polymerization of flagellin in vivo.

In fact, when flagellin monomers were mixed with hooks isolated from flagellate bacteria in the presence of 0.1 M NaCl at neutral pH, filaments appeared, joined to the distal ends of the hooks (16). However, efficiency of the process was very low as compared with the reconstitution in which fragmented flagellar filaments were used as seed for flagellin assembly. Moreover, the possibility was not excluded that these hooks might contain a small amount of flagellin at their ends.

A more reliable system was invented in which flagellins were mixed with the cells of a stable flagellinless mutant derived from a normal flagella strain (17). The best condi-

tions so far found are the same as those used for the in vitro assembly of flagellin at the tips of flagellar filaments attached to cell bodies. During 3 hr at 26° after 5×10^{10} cells/ml of bacteria and 8 mg/ml of flagellin were mixed, 6.7% of the cells produced reconstituted filaments of average length 6 μm. Most of them carried single flagella, 21% of the flagellated cells had two flagella and a very few cells with three flagella were detected. When flagellin prepared from curly-mutant flagella was used, curly filaments developed, joined to the hooks.

Cells incubated under the same conditions without mixing with flagellin, or mixed with fragmented flagellar filaments, appeared entirely nonflagellate even when as many as 1000 cells were examined. When the cells of fla-AI, -B or -C mutants, which lack hooks as well as filaments (18), were used no filaments were detected on the cells.

Thus exogenous flagellin was found to assemble into a filament at the tip of a hook, although the efficiency of initiation was not high. A possible explanation for the inefficient initiation is that the flagellin molecules obtained by in vitro dissociation of flagellar filaments are different in their conformation from those synthesized in vivo, and that the former is less efficiently polymerized at the tip of a hook.

It has been noticed that polyhook (or superhook) mutants fail to produce a filament at the tip of a long hook although they carry intact structural genes for flagellin (19, 20). The polyhook mutants are presumed to lack a termination factor required for the limitation of the length of the hooks. The termination factor, if there is one, may locate at the distal end of a hook and may well play a role as a nucleus for the initiation of flagellin polymerization. If such a factor is labile and can be modified so as to be less effective as a nucleus, unless a flagellar filament is joined to it, the low efficiency of flagellin assembly at the tip of a hook is reasonably explained. During purification hooks progressively lose their activity as seed for assembly of flagellin (16). This also suggests the presence of a labile factor at the distal end of a hook.

ELONGATION OF FLAGELLAR FILAMENTS

After the initiation, assembly of flagellin continues by the process described in the first section and the filaments elongate. This process may be essentially the same, in vitro and in vivo. However, as regards the rate of elongation, there is a marked difference.

The rate of filament elongation in vitro depends on species and amount of flagellin, and on the physicochemical conditions of the environment (6, 21). However, when these are fixed, the average rate is maintained constant, regardless of the length of the existing filaments, although the distribution of the rates among filaments is nonuniform (22). This is also true even when the in vitro assembly is carried out at the tips of the filaments attached to cell bodies (Fig. 3A).

On the other hand, the average rate of in vivo elongation decreases with increase of filament length (Fig. 3B) (13). This relation is observed even among the filaments of various lengths growing on a single cell. A filament mechanically shortened by breakage also elongates at a rate depending on the length of the remaining filament. Therefore, any contribution of aging of a cell or of a flagellum-forming apparatus to the decrease in the rate of elongation is implausible.

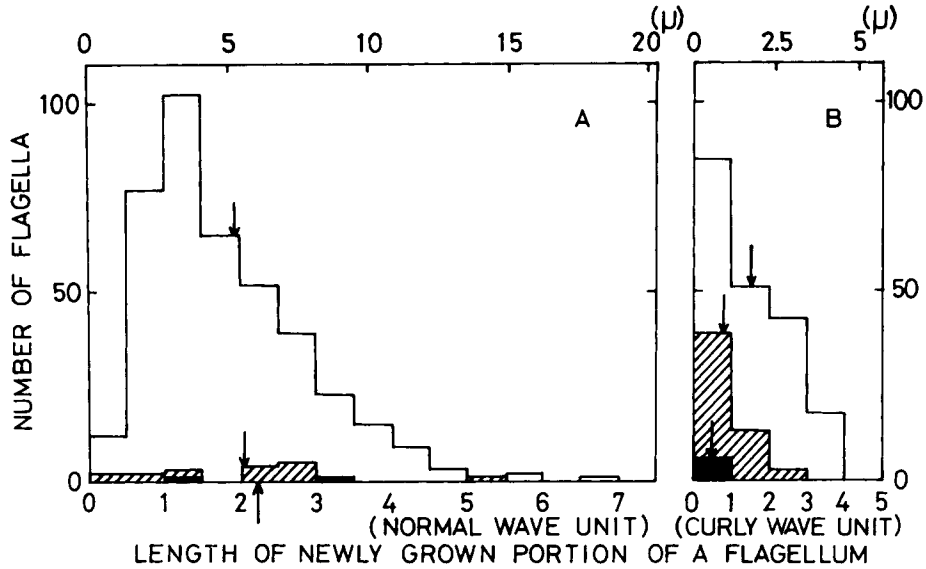

Fig. 3. Distribution of length of flagellar filaments: A, reconstituted by in vitro assembly; and B, elongated by regeneration. Notice remarkable difference in the pattern of distribution between A and B.

A. Flagellin (10 mg/ml) from normal-flagella strain TM2 of Salmonella typhimurium and cells (5×10^{10}/ml) of straight-flagella mutant SJ814 of S. typhimurium, whose flagellar filaments were shortened beforehand by mechanical shaking, were mixed in 0.03 M phosphate buffer added to 0.08 M K_3-citrate (pH 6.9). The mixture was incubated at 26° for 2 hours. The reconstituted portions were identified by normal wave form. Detailed procedures are in Ref. 13.

B. Cells of TM2 were shaken for 90 min in physiological saline and incubated in regeneration medium supplemented with 1 mg/ml p-fluorophenylalanine at 37° for 3 hours. The regenerated portions were identified by curly wave form. Detailed procedures are in Ref. 17. Length of pre-existing filament portion: $0 < \square \leqq 2$ normal wave units; and $2 < \blacksquare \leqq 3$ normal wave units. 1 normal wave unit = 2.70 μ; and 1 curly wave unit = 1.15 μ. Arrow indicates average length of newly grown portion. Observations were made on 419 flagella for A and 289 flagella for B.

The major difference between in vitro and in vivo elongation of flagellar filaments is that in the latter process flagellin monomers have to be transported from the cell body to the tip of a flagellar filament through its central canal. Therefore, the decrease in rate of elongation in the latter must be related to the decrease in the efficiency of transportation of flagellin with increase of the distance of the pathway to the tip of a filament.

LENGTH OF FLAGELLAR FILAMENTS IN GROWING BACTERIA

It is well known that the length of flagellar filaments in logarithmically growing cells of motile Salmonella is distributed, with the general pattern as shown in Fig. 4. The mode value and the maximal length differ to some extent, depending on the species of flagellin. For example, the maximal length of the filaments of phase-1 (flagellar antigen-a) cells of S. abortus-equi SJ241 is 5 normal wave units (Fig. 4A). When the structural gene for phase-1 flagellin, H1-a, is replaced by H1-gt of Salmonella strain SJ6, flagellar filaments as long as 7 normal wave units are detected (Fig. 4C). On the other hand, the introduction of a leaky flaAII$^-$ gene does not result in change in the pattern (Fig. 4B), in

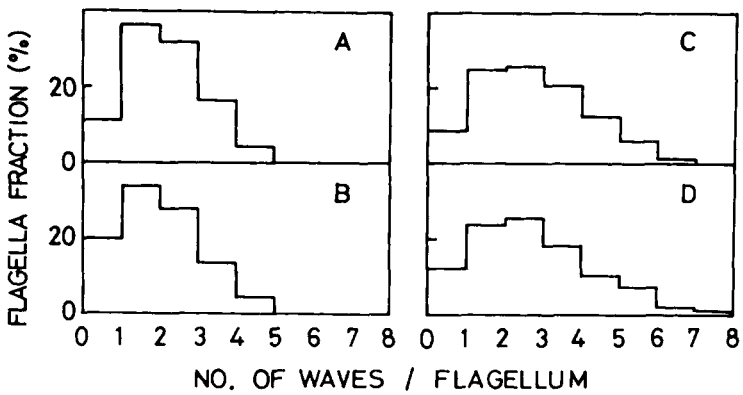

Fig. 4. Distribution of length of flagellar filaments in various Salmonella strains. A, S. abortus-equi SJ241 which is stable in phase-1 (a-antigen) and carries 2.6 flagella per bacterium; B, A pauci-flagellate (flaAII$^-$) mutant of SJ241, carrying 1.7 flagella per bacterium; C, Strain SJ925 which produces gt-type flagella in phase-1, derived by transduction of the structural gene for phase-1 flagellin from S. budapest SJ6 to SJ241; D, A paralyzed (motA$^-$) mutant of SJ925. Measurements were made on middle log phase cultures grown at 35° as described by Iino (13). Length was expressed by number of normal waves whose contour length is 2.7 μ in all the strains. Sample number was 250 for each strain.

spite of the decrease of the number of flagella per bacterium to 65% of the flaAII$^+$ cells. The introduction of motA$^-$ gene, which results in the paralysis of the cell, also does not cause a significant change in the pattern of the length distribution, but a minor fraction of flagella with 7 to 8 normal wave units is detected on the cells carrying both H1-gt and motA$^-$ (Fig. 4D). The overall distribution does not significantly change even when the temperature of cultivation is changed in the range of 25° to 40°.

The growth curve of phase-1 cells of S. typhimurium TM2 (flagellar antigen-i) cultivated in "Penassay" broth at 35°is shown in Fig. 5. In this culture, distributions of number and length of flagellar filaments in growing bacteria show almost identical patterns throughout middle log to early stationary phases (Fig. 6A–D). This means that both number of flagella per bacterium and length of filament increase are coupled with the cell cycle of the bacteria. When bacteria are in late stationary phase, the number of flagella per bacterium increases and the length distribution of the filaments shifts to longer side (Fig. 6E). This shift may be due to the decrease in the rate of formation of new flagella.

Taking flagella number in the cell population at middle log phase as 100 (Figs. 5A and 6A), the net increase of flagellar number after one hour (Fig. 5B and 6B) was calculated, and the distributions of the filament length among the flagella in the two stages were plotted on Fig. 7. On the assumption that the order of relative length among the filaments does not change throughout their elongation, the average extents of elongation in one hour of existing flagellar filaments of different initial lengths are estimated from the curve in Fig. 8, which records the difference between two lines in Fig. 7. The corresponding curve obtained on the samples of B and C in Fig. 5 are shown together in Fig. 8. These three curves are similar and conform with the hypothesis that rate of flagellar elongation decreases with increase in length.

From Fig. 8, the growth curves of a flagellar filament under the three different conditions were constructed (Fig. 9). By measuring tangents at the representative points

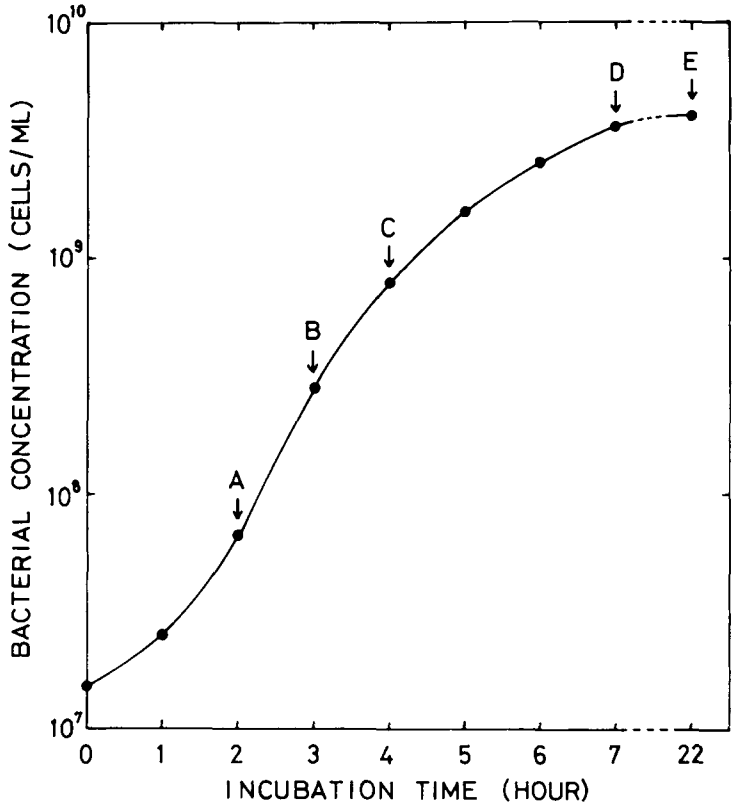

Fig. 5. Growth curve of Salmonella typhimurium TM2. Bacterial suspension from single colony grown on a nutrient agar plate was inoculated to 10 ml Penassay broth at 0 hour and cultivated at 35°. Bacterial number was counted by a bacterial counting chamber. Flagellation of bacteria observed at A, B, C, D, and E is shown in Fig. 6.

on each growth curve, rates of elongation were obtained and plotted against filament length. Among the scales tried, a logarithmic scale for rate of elongation was found to give parallel straight lines, as shown in Fig. 10. The rate measured on bacteria grown in the presence of FPA (13) is also plotted as a straight line on Fig. 10. These results indicate that the rate of elongation decreases exponentially with increase of flagellar filament length. This relation is formulated:

$$V = V_0 e^{-KL} \qquad \text{(I)}$$

where V denotes the rate of elongation of the filament at length L, V_0 the initial rate at L = 0, and K the constant characterizing degree of decrease in rate per unit length. From Eq. I, the following differential equation is derived:

$$\frac{dV}{dt} = -KV^2 \qquad \text{(II)}$$

As discussed previously, the decrease of the elongation rate is attributed to the decrease of the efficiency of transportation of flagellin through the central canal of the

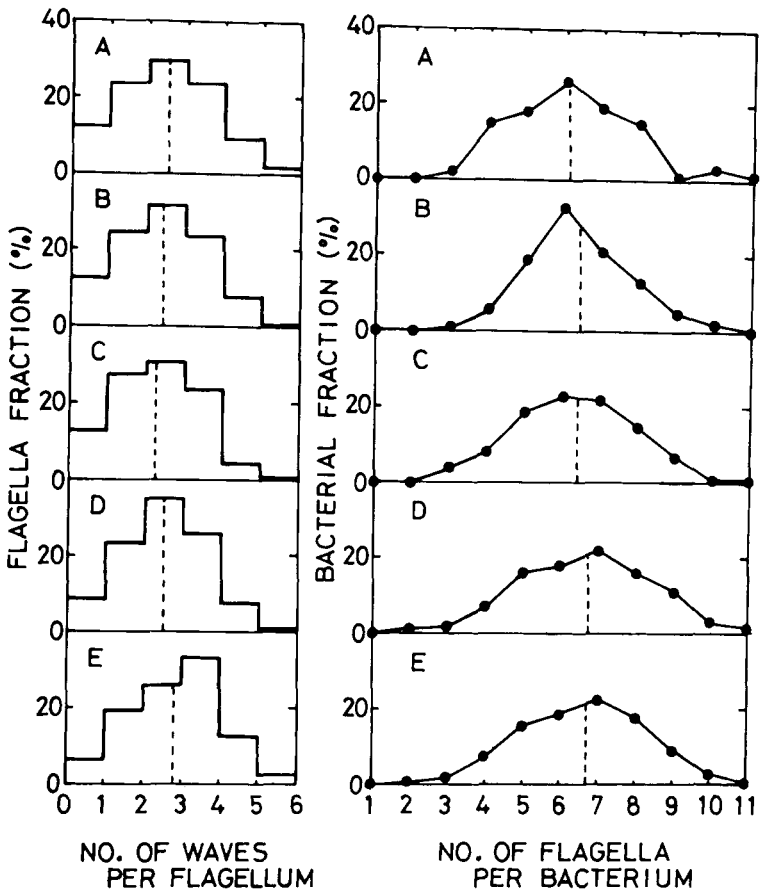

Fig. 6. Distributions of number and length of flagellar filaments at A–E of Fig. 5. Observations were made on 200 cells of each sample. Contour length of one wave = 2.70 μ.

filament. Therefore Eq. I may mean that the efficiency of transportation decreases exponentially with the increase in length of the filament. For the present, the mechanism which causes the decrease of the transportation efficiency is unknown. A possibility inferred from Eq. II is that the resistance to transportation of flagellin determines the square of the rate of elongation throughout the pathway along the central canal of a filament. A remarkable point observed in Fig. 10 is that K values are not significantly different among the four lines. This suggests that the resistance force is the same among the four samples and the differences in rate among them are attributed to the differences in the initial rate.

By extrapolating the lines in Fig. 10 to L = 0, V_0 values are obtained (Table I). The time interval, τ_0, from the incorporation of one flagellin molecule to that of the next flagellin molecule at the start of filament elongation is calculated from V_0 and the number of flagellin molecules per unit length of a filament (Table I). When V_0 and τ_0 estimated here are compared with the corresponding values observed in vitro, it is noticed that the largest V_0 in vivo is 3.4 times the observed maximal V_0 in vitro (22). The smallest τ_0 in vivo is about half of the estimated average time required for a flagellin molecule to bind,

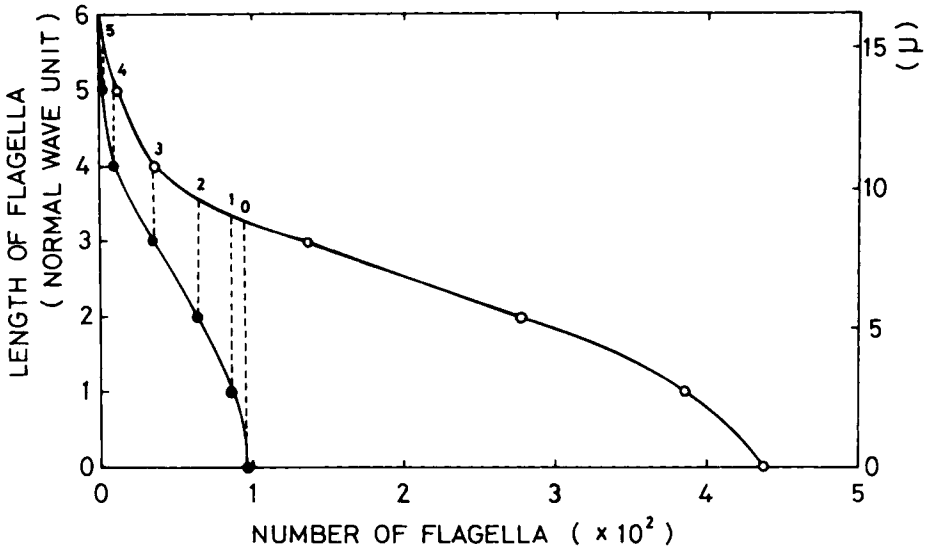

Fig. 7. Increase of number and length of flagellar filaments in culture of Fig. 5 during one hour at middle log phase, i.e., between A (●) and B (○) of Fig. 6. Number of flagella at time A was taken as 100. Dotted lines, 0, 1, 2, 3, 4, and 5, correspond to average lengths of elongation of the filaments with indicated lengths at A.

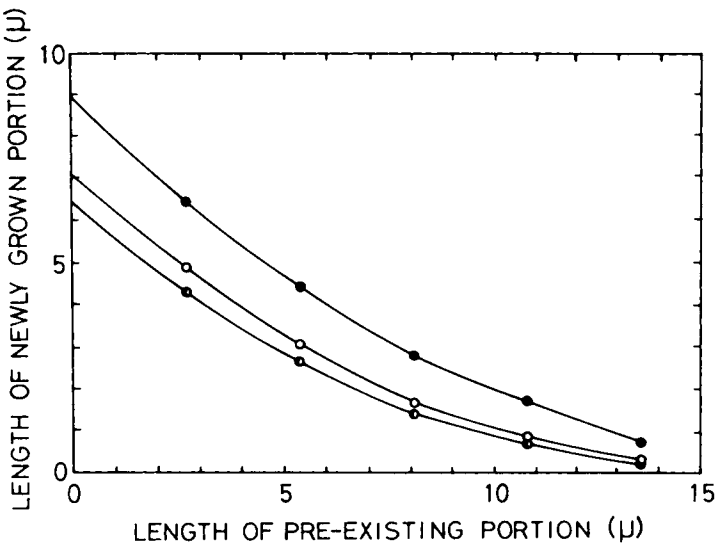

Fig. 8. Average extents of flagellar filament elongation in one hour: (●) from time A (stage A); (○) from time B (stage B); and (◐) from time C (stage C) of Fig. 5.

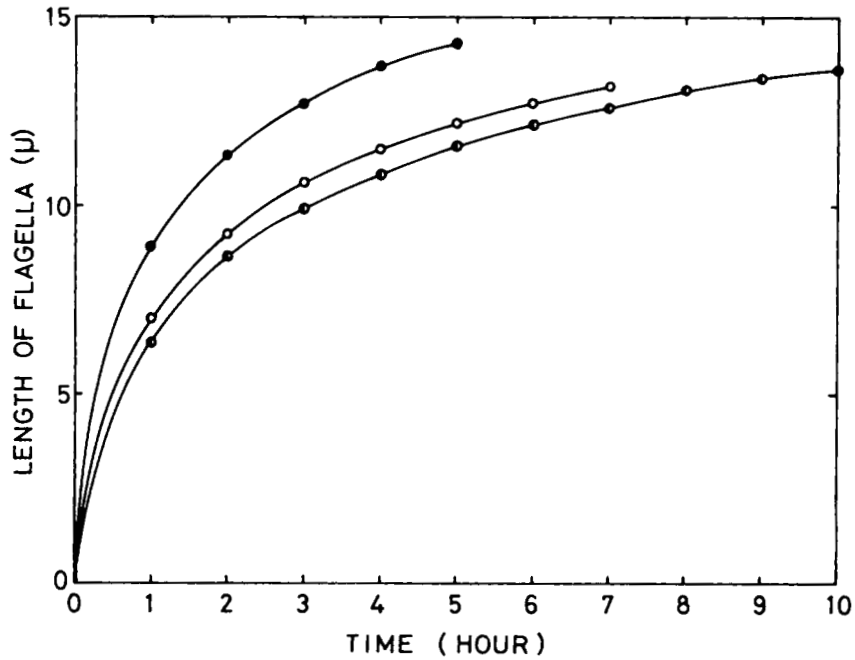

Fig. 9. Growth curves of flagellar filaments under the growth condition in stages: (●) A; (○) B; and (◐) C, derived from integration of the curves in Fig. 8.

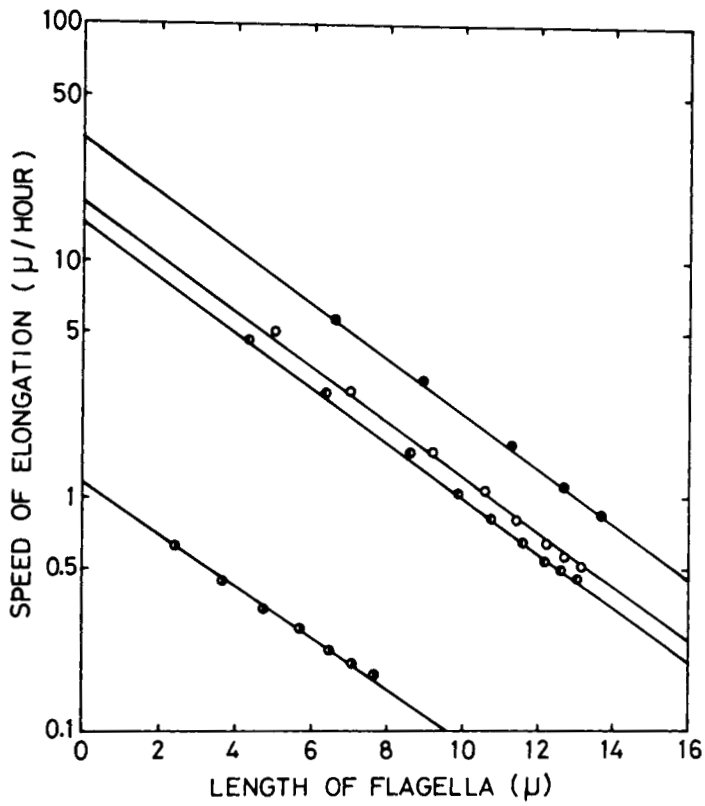

Fig. 10. Rates of elongation of flagellar filaments under the growth condition in stages: (●) A; (○) B; and (◐) C in Fig. 8, and (◐) in regeneration medium supplemented with 1 mg/ml p-fluorophenylalanine (derived from Fig. 8 of Ref. 13). Rates were plotted on logarithmic scale.

TABLE I. Parameters of Elongation of Flagellar Filaments

Bacterial Sample*	Cell Generation Time (min)	V_0 (μ/min)	K	τ_0 (sec)
A	29	0.55	0.27	0.05
B	44	0.30	0.27	0.09
C	56	0.25	0.27	0.11
FPA	–	0.02	0.26	1.36

*Corresponding to the four samples in Fig. 10.

and alter its conformation change, so as to be incorporated into a filament in vitro (21). Thus, under the optimal growth condition in vivo, flagellin molecules seem to be very efficiently incorporated into the filaments at the initial stage of filament elongation and immediately after the incorporation of a flagellin molecule the next molecule binds to the preceding molecule. The hitherto reported rates of filament elongation in vivo range from 0.05 to 0.01 μ/min (23). These values are in the range of the speeds of elongation of the middle-sized filaments in the present estimation.

TERMINATION OF FILAMENT ELONGATION

When flagellin was assembled in vitro, filaments longer than 50 μ could be detected. The elongation in vitro is, however, in practice not infinite. Even when enough flagellin is supplied in the reaction mixture, filaments inert as seeds for polymerization of flagellin appear, and their number increases exponentially as elongation proceeds (22). As a consequence, the lengths of the reconstituted filaments show a broad Γ-distribution after their growth-saturation. From these observations, Hotani and Asakura (22) assumed that an error randomly occurring at the distal end of a filament terminates further elongation of the filament. The frequency of termination depends on the sample of flagellin used for the in vitro assembly. Therefore the error was presumed to be caused by the binding of a partially denatured flagellin, or of an impurity other than flagellin present in the reaction mixture, to the growing end of a filament.

In contrast to flagellar filaments reconstituted in vitro, the observed maximal length of flagellar filaments grown on bacterial cells is in the range of 15 μ to 22 μ under ordinary cultural conditions (Fig. 4). The value differs in this range depending on the strains and cultural conditions. When a flagellum on a bacterium reaches the maximal length and detectable elongation ceases, other shorter flagella on the same bacterial body continue to elongate, reaching the maximal length. Thus the termination of the elongation of flagellar filaments on growing bacterial cells affects individual flagella. The possibility is not excluded that a fraction of the flagella stop growing before reaching the maximal length by the mechanism described for in vitro elongation. However, such a fraction may be minor, at least for cells which regularly duplicate the number and amount of flagella per bacterium in every cell cycle, as in the case of a log-phase culture of Salmonella typhimurium TM2, analyzed in the foregoing section.

As discussed, the rate of elongation of a flagellar filament decreases exponentially with increase of its length. According to this model, the filament must continue to elongate as long as flagellin molecules are supplied. Now, from Eqs. I and II, the relation

of L and the time, T, required for elongation from 0 to L is given by

$$e^{KL} = 1 + KV_0T \qquad \text{(III)}$$

From this equation, T corresponding to L = 16.2 μ, i.e., 6 normal waves, in flagellar filaments of sample A of Table I is calculated to be 9 hours. When the cells divide every half hour, the offspring of a single cell grow to 2.6×10^5 cells during this period. Thus, the cells having flagellar filament longer than 6 normal waves are estimated to constitute an exceedingly small fraction in the culture, so they will not be detected by the ordinary scale of sampling. When the generation time is extended, the flagellin supply may be regulated so that V_0 becomes smaller (Table I), and the distribution of lengths is maintained without remarkable change (Fig. 6). Flagellar filaments longer than 6 normal waves, as observed on an H1-gt *Salmonella* strain (Fig. 4C), may be caused by the smaller value of K, which depends on the species of flagellin.

Considering the above argument, it is possible to explain the observed maximal length of flagellar filaments in vivo by the same mechanism as that regulating the rate of their elongation. However, this does not exclude the possibility of the presence of a termination factor against the filament elongation in vivo. A crucial experiment may be carried out when the experimental condition is established under which elongation of flagellar filaments proceeds normally, but without cell multiplication.

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