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In vitro reconstitution of flagellar filaments onto hooks of filamentless mutants of *Salmonella typhimurium* by addition of hook-associated proteins

(flagellar assembly)

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ABSTRACT **An *in vitro* system for reconstituting flagellar filaments onto hooks of filamentless mutants of *Salmonella typhimurium* was used to investigate the role played in filament formation by the three hook-associated proteins (HAPs, products of the *flaW*, *flaU*, and *flaV* genes). These proteins—FlaW, FlaU, and FlaV—are believed to be assembled in this order at the distal end of the hook. When the recipient hooks were provided by *flaU* mutants, whose hook tips contained FlaW only, exogenous FlaU was essential for polymerization of both exogenous and endogenous flagellin, whereas exogenous FlaV inhibited such polymerization. When the recipients were *flaV*-mutant hooks, whose tips contained FlaW and FlaU but not FlaV, exogenous FlaV inhibited polymerization of exogenous flagellin. FlaV also inhibited polymerization of exogenous flagellin at the tips of filament fragments. In contrast, FlaV was essential for polymerization of endogenous flagellin onto *flaV*-mutant hooks, and onto short filaments that had been made (in the absence of FlaV) by polymerization of exogenous flagellin on the tips of *flaV*-mutant hooks. These results suggest that FlaV acts not only at the tip of the hook to initiate growth of the filament, but also at the tip of the growing filament, and that FlaV is essential for polymerization of endogenous flagellin—i.e., for the normal process of filament assembly *in vivo*.**

Morphogenesis of the bacterial flagellum starts with construction of the basal body (and possibly other components of the flagellar motor) in the cell surface layers and continues with polymerization of the component protein of the hook onto the rod structure of the basal body, to form a hook with a well-defined length of ≈60 nm. The final stage of flagellar assembly involves the polymerization of flagellin onto the distal end of the hook, to form the helical flagellar filament that propels the cell (1–3).

Many genes essential for flagellar assembly (*fla* genes) have been identified in *Salmonella typhimurium* (4, 5) and *Escherichia coli* (6, 7). Among them, *H1*, *H2*, *flaL*, *flaU*, *flaV*, and *flaW* of *S. typhimurium* appear to play essential roles in the final process of filament formation. Defects in these genes result in the production of incomplete flagella that have the hook–basal body structure but lack the filament structure. In the process of filament formation, flagellin monomer has to proceed through at least four steps: (*i*) synthesis in the cell, (*ii*) transport to the tip of the hook, (*iii*) nucleation of polymerization at the tip of the hook (initiation), and (*iv*) sequential polymerization at the tip of the growing filament (elongation).

The *H1* (or *H2*) and *flaL* genes are necessary for the synthesis of flagellin because *H1* and *H2* are (alternative) structural genes for flagellin (8) and active flagellin mRNA is not detected in *flaL* mutants (9).

flaU, *flaV*, and *flaW* are the structural genes for the three hook-associated proteins (HAPs; refs. 10 and 11). The FlaU, FlaV, and FlaW proteins are also known as HAP3, HAP2, and HAP1, respectively. The HAPs are, stoichiometrically speaking, minor components of flagellar structure that are inferred to be assembled at the distal end of the hook, in the order FlaW, FlaU, and FlaV (10). Assembly of any given component is dependent on the assembly of the prior component (a layered-structure model) (12). Although mutants defective in *flaW*, *flaU*, or *flaV* produce flagellin normally, it does not polymerize into filament and is excreted into the culture medium (13). Therefore, HAPs are thought to participate, at the tip of the hook, in the process of filament formation *in vivo*. In contrast, when purified flagellin is supplied exogenously at high concentration to a *flaV* mutant, it polymerizes onto the hook structure and the mutant becomes motile (14). Thus *flaV* is not essential for filament formation *in vitro*.

We have found (15) that the culture medium of *fla*$^+$ strains contains three kinds of HAPs as well as sheared filaments and that the culture medium of *flaW*, *flaU*, and *flaV* mutants contains the two normal HAPs as well as flagellin; thus, for example, a *flaW* mutant excretes flagellin, FlaU, and FlaV. Using these excreted HAPs together with flagellin, we have developed a system for *in vitro* reconstitution of flagellar filaments onto hooks and have used this system to investigate the function of HAPs in more detail.

MATERIALS AND METHODS

Bacterial Strains and Media. The strains used are listed in Table 1. All of the filamentless mutants are transductional derivatives of *S. typhimurium* SJW1103, which is phase-1 monophasic (expressing *i*-flagellin) and has wild-type flagellation and motility. Transduction was carried out according to the method of Kutsukake *et al.* (4). M9 medium and L broth have been described (11).

Preparation of Filament-Reconstitution Solution (FR) from Culture Medium. Bacteria were grown at 37°C to late-log phase in M9 medium containing 0.3% Casamino acids. The culture was centrifuged at 30,000 × *g* for 20 min, and the supernatant was placed in dialysis tubing, which was immersed in polyethylene glycol 20,000 (Wako Pure Chemical, Osaka, Japan). The tubing was kept at 4°C until its volume had decreased to 1/20th of the original. The resultant solution was adjusted to pH 2.4 with 1 M HCl, kept for 30 min, and

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Abbreviations: HAP, hook-associated protein; FR, filament-reconstitution solution.

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Table 1. *Salmonella* strains used

Strain	Relevant genotype	Reference or source
SJW1103	*H1*-i Δ*H2*	16
SJW1672	*H1-il506* of SJW1103	S.Y., unpublished
SJW2158	*flaU2389* of SJW1103	13, 14
SJW2172	*flaU2403* of SJW1103	13, 14
SJW2177	*flaW2408* of SJW1103	13, 14
MH103	*flaV*::Tn*10* of SJW1103	13
MH104	*flaV*::Tn*10* of SJW2158	This study
MH105	*flaV*::Tn*10* of SJW2172	This study
MH110	*flaV*::Tn*10* of SJW2177	This study
MH113	*flaV*::Tn*10* of SJW1672	This study
MH118	*flaW*::Mu dl (Ap^r*lac*$^+$) of SJW1103	K.K., unpublished
MH119	*flaV*::Tn*10* of MH118	This study

then adjusted to pH 7.2 with 1 M KOH. The solution was centrifuged at 120,000 × *g* for 1 hr. Ammonium sulfate was added to the supernatant at 0°C, to 40% saturation. After 12 hr at 4°C, the precipitate was collected by centrifugation at 20,000 × *g* for 20 min, suspended in phosphate-buffered physiological saline (PBS; 10 mM phosphate, pH 7.2/0.9% NaCl), and dialyzed against PBS. This preparation was called filament-reconstitution solution (FR) and contained any HAPs and flagellin that had been secreted into the culture medium (13, 15).

Acquisition of Motility by Filamentless Mutant Cells. Acquisition of motility was examined by monitoring polymerization of flagellin to form filaments, according to Kagawa *et al.* (17) with the following modifications. Bacteria were grown to mid-log phase in L broth, and 0.1 ml of the culture was centrifuged in an Eppendorf tube. The sedimented cells were suspended in 50 μl of FR, which had previously been heated at 70°C for 10 min to ensure that the proteins were present as monomers. After incubation at 27°C for 2 hr, the cells were observed by phase-contrast microscopy. In some experiments, 20 μl of the cell suspension was mixed with 80 μl of L broth and cultivated at 37°C for 40 min with vigorous shaking, and then the cells were observed in order to detect acquisition of motility, presumably as a result of assembly of endogenously supplied flagellin.

***In Vitro* Polymerization of Flagellin onto Filament Fragments.** A 0.7-ml aliquot of FR (5 mg of protein per ml) prepared from strain MH119 was mixed with 0.1 ml of either FR (3 mg of protein per ml) from another strain or PBS (as a control). The mixture was heated at 70°C for 10 min, transferred into a spectrophotometer cuvette, and then incubated at 27°C for 5 min. A separate, unheated sample of FR (5 mg of protein per ml) from strain MH119 was sonicated twice for 5 sec at 80 W at 0°C, in order to fragment the filaments that had grown during the ammonium sulfate precipitation step, and a 0.2-ml aliquot was added to the cuvette. The change in optical density at 350 nm was then measured at intervals.

Preparation of Hooks. Hooks from filamentless mutants were isolated and purified by DEAE-cellulose chromatography as described (10).

RESULTS

Acquisition of Motility by Filamentless Mutants via an *in Vitro* Reconstitution System. Proteins in the culture medium of various filamentless mutants and a *fla*$^+$ strain were collected and concentrated as a solution containing flagellin and HAPs, which we call filament-reconstitution solution (FR). FR was prepared from strains with each of the following genotypes: *fla*$^+$, *flaV*, *flaU*, *flaW*, *flaU flaV*, and *flaW flaV*. [Henceforth we abbreviate FR from a *flaV* mutant, for example, as FR(*flaV*).] Cells of filamentless mutants of various genotypes (*flaV*, *flaV H1 H2*, *flaU*, *flaU flaV*, *flaW*, and *flaW flaV*) were incubated in FRs and examined for motility resulting from exogenous reconstitution of filaments onto native hooks (Table 2; see also Fig. 1).

When *flaV* cells were incubated for 2 hr in FR(*flaV*), FR(*flaU flaV*), or FR(*flaW flaV*), about 90% of them became actively motile (Fig. 1*a*). Subsequent cultivation in L broth caused some deterioration in motility (Fig. 1*e*). If, instead, the cells were incubated in FR(*fla*$^+$), FR(*flaU*), or FR(*flaW*), only about 10% of them acquired motility (Fig. 1*b*). These results suggest that FR(*fla*$^+$), FR(*flaU*), and FR(*flaW*) either lacked a component required for exogenous flagellin assembly or included a component that inhibited assembly. When the resulting suspensions were diluted 5-fold in L broth and incubated for an additional 40 min to allow the cells to synthesize flagellin, about 90% of them acquired active motility (Fig. 1*f*).

To confirm that the motility acquired by growth in broth was indeed attributable to endogenously synthesized flagellin, we conducted similar tests on a *flaV H1 H2* strain with mutations in the flagellin structural genes as well as in *flaV*. When these cells were incubated in FR(*flaV*), FR(*flaW flaV*), or FR(*flaV flaU*), they acquired motility to the same level as had the *flaV* cells (Fig. 1*c*). Subsequent cultivation in L broth caused some deterioration in motility (Fig. 1*g*). However, when they were incubated in FR(*fla*$^+$), FR(*flaW*), or FR(*flaU*), they never acquired motility (Fig. 1*d*), even when subsequently cultivated in L broth (Fig. 1*h*). These results show that FR prepared from *flaV*$^+$ strains prevents *flaV* cells from gaining motility by polymerization of exogenous flagellin but enables them to construct functional filaments from endogenous flagellin. This suggests that

Table 2. Effect of various FRs on acquisition of motility

FR*	Motile fraction of mutants,† %					
	flaV	*flaV H1 H2*	*flaU*	*flaU flaV*	*flaW*	*flaW flaV*
FR(*fla*$^+$)	11 (79)	<0.2	<0.2	<0.2	<0.2	<0.2
FR(*flaV*)	91 (88)	91 (90)	0.3 (3)	89 (93)	<0.2	<0.2
FR(*flaU*)	10 (91)	<0.2	<0.2	<0.2	<0.2	<0.2
FR(*flaW*)	18 (89)	<0.2	<0.2	<0.2	<0.2	<0.2
FR(*flaU flaV*)	90 (86)	91 (89)	<0.2	<0.2	<0.2	<0.2
FR(*flaW flaV*)	86 (87)	94 (87)	0.6 (5)	89 (91)	<0.2	<0.2

*FRs were prepared from the following strains: SJW1103 (*fla*$^+$), MH103 (*flaV*), SJW2158 (*flaU*), SJW2177 (*flaW*), MH105 (*flaU flaV*), and MH110 (*flaW flaV*).

†Mutant strains were MH103 (*flaV*), MH113 (*flaV H1 H2*), SJW2158 (*flaU*), MH105 (*flaU flaV*), SJW2177 (*flaW*), and MH110 (*flaW flaV*). They were incubated for 2 hr in various FRs. The fraction of motile cells was estimated from a population of at least 500 cells, observed by phase-contrast microscopy. Cells showing swimming, tumbling, or rotation were all counted as motile. After cultivation in L broth for 40 min, cells were observed again and the motile fraction was estimated (results given in parentheses).

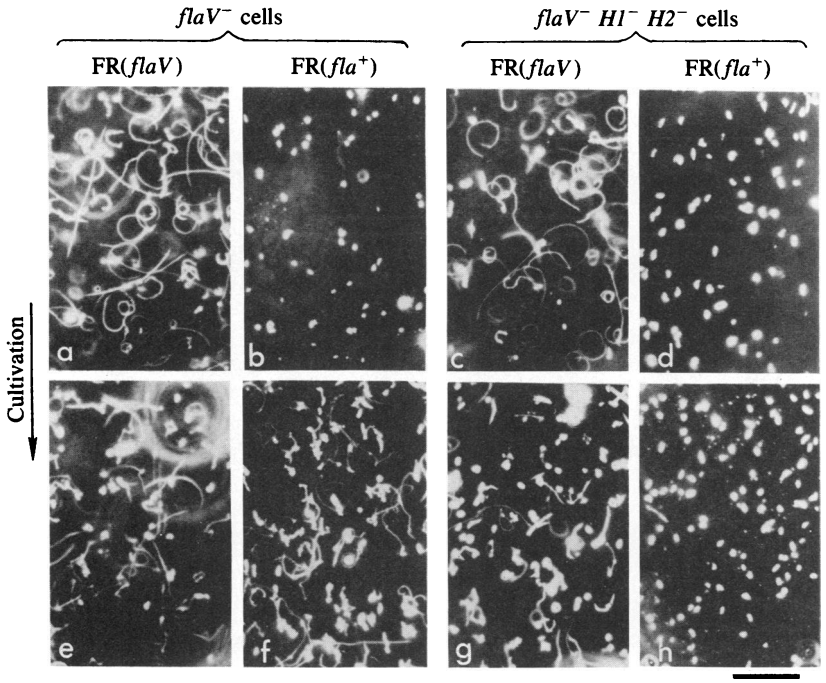

FIG. 1. Participation of FlaV in acquisition of motility. *flaV* cells (MH103) or *flaV H1 H2* (MH113) cells were incubated for 2 hr in FRs prepared from MH103 (*flaV*) or SJW1103 (*fla*⁺). Motility in FR was observed by dark-field microscopy (*a–d*) and observed again after cultivation in L broth for 40 min (*e–h*). Exposure time for motility tracks was 5 sec. (Bar = 50 μm.)

FR(*flaV*⁺) contains a component that interferes with exogenous flagellin assembly and a component that enables endogenous flagellin assembly.

When *flaU* or *flaU flaV* cells were incubated in FR(*fla*⁺), FR(*flaU*), FR(*flaW*), or FR(*flaU flaV*), they never acquired motility, even when subsequently cultivated in L broth. However, when *flaU* cells were incubated in FR(*flaV*) or FR(*flaW flaV*), motile cells were observed, although at a frequency <1%; cultivation in L broth increased the frequency to 5%. Almost all *flaU flaV* cells acquired motility when incubated in FR(*flaV*) or FR(*flaW flaV*). These results indicate that acquisition of motility by *flaU* and *flaU flaV* cells (both blocked at the stage of FlaU addition) requires FR containing FlaU but lacking FlaV.

Motility could not be restored to *flaW* or *flaW flaV* cells by incubation in FR prepared from any of the strains, even with subsequent cultivation in L broth, indicating that FlaW cannot be added exogenously under any conditions tested.

Effect of Dilution of FR on Acquisition of Motility by Filamentless Mutants. *flaU flaV* cells were incubated in FR(*flaW flaV*) that was either undiluted (3 mg of protein per ml) or diluted to various extents. At up to 2-fold dilution they acquired motility (Fig. 2*a*); this was also true if the cells were cultivated in L broth. *flaV* cells acquired motility with FR(*flaW flaV*) diluted up to 8-fold (Fig. 2*b*). On the other hand, when *flaV* cells were incubated in FR(*fla*⁺) (Fig. 2*c*) and cultivated in L broth, at 25-fold FR dilution most of the cells acquired motility, and even at 125-fold dilution 2% of them did; without the cultivation in broth, only 10% gained motility even with undiluted FR. Thus a component of FR(*flaV*⁺), even at high dilution, appears to aid endogenous flagellin assembly.

FR(*flaV*) enabled *flaV* cells to acquire motility (Table 2). However, when *flaV* cells were incubated in a mixture of 50 μl of FR(*flaV*) (3 mg of protein per ml) and 10 μl of variously diluted FR(*fla*⁺) (Fig. 2*d*), the ability to acquire motility was inhibited, with maximal inhibition occurring at 25-fold dilution of FR(*fla*⁺). Inhibition was much less pronounced if cultivation in L broth was permitted. These results further support the idea that FR(*fla*⁺) contains an inhibitor of polymerization of exogenous flagellin.

Factor Essential for Filament Elongation by Endogenous Flagellin in *flaV* Cells. *flaV* cells were mixed with FR(*flaV*) (3-fold diluted; 1 mg of protein per ml), and acquisition of

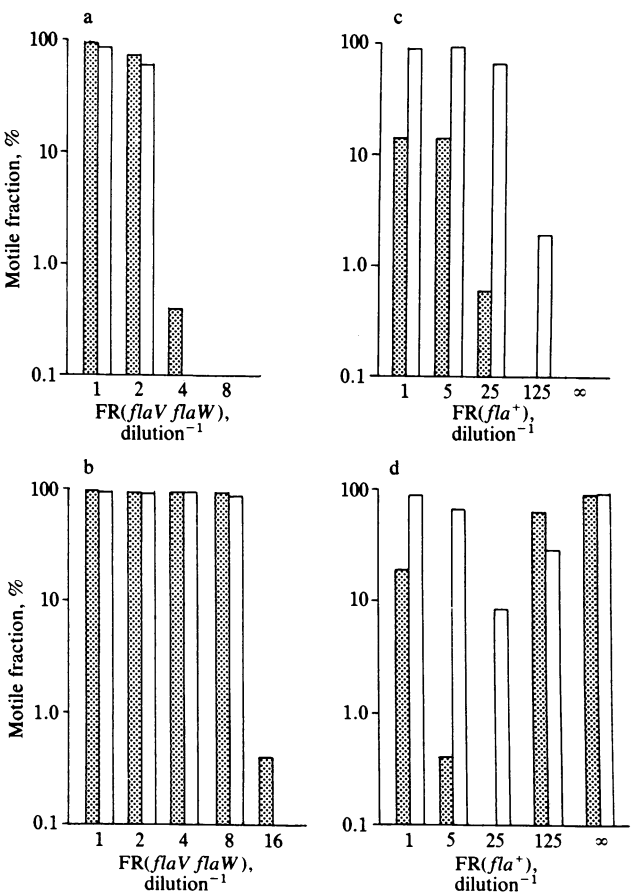

FIG. 2. Effect of FR concentration on acquisition of motility. (*a*) *flaU flaV* cells (MH105) were treated with serially 2-fold-diluted FR(*flaV flaW*) prepared from MH110. (*b–d*) *flaV* cells (MH103) were treated with serially 2-fold-diluted FR(*flaV flaW*) prepared from MH110 (*b*), serially 5-fold-diluted FR(*fla*⁺) prepared from SJW1103 (*c*), or a mixture of 50 μl of FR(*flaV*) prepared from MH103 and 10 μl of serially 5-fold-diluted FR(*fla*⁺) prepared from SJW1103 (*d*). The frequency of acquisition of motility was estimated (as for Table 2) after incubation in FR for 2 hr (stippled bars) and again after cultivation in L broth for 40 min (open bars). Blank spaces indicate that no (<0.2%) acquisition of motility was observed.

motility was observed at 10-min intervals for 80 min (Fig. 3). Motility became appreciable after a lag of 40 min. By 60 min, about 90% of the cells had acquired some motility, although most cells showed tumbling or rotating motion that would be expected if they had short flagella. Normal motility of almost all the cells was only restored after 80 min; this period may correspond to the time required to make filaments long enough for swimming.

flaV cells that had acquired partial motility showed no further improvement in motility when diluted 5-fold into L broth and cultivated for as long as 40 min in order to supply endogenous flagellin. This shows that the endogenously synthesized flagellin was unable to polymerize onto short flagella in the absence of FlaV; exogenous flagellin was presumably too dilute to assemble.

An Inhibitor of Flagellin Polymerization onto Filament Fragments. Flagellin can polymerize *in vitro* onto filament fragments, which act as nucleation structures (18). We examined the effect of FR(*fla*$^+$), FR(*flaV*), FR(*flaW*), and FR(*flaW flaV*) on such polymerization, using flagellin purified from strain MH119, which (because of a transposon insertion in *flaV* and a bacteriophage Mu-induced *flaW* mutation that is polar on *flaU*) does not synthesize any of the HAPs. To estimate the efficiency of polymerization, the turbidity (optical density at 350 nm) was measured at intervals (Fig. 4). FR(*fla*$^+$) or FR(*flaW*) completely inhibited polymerization, whereas FR(*flaV*) or FR(*flaW flaV*) had no significant effect. This suggests that FR(*fla*$^+$) and FR(*flaW*) contain an inhibitor, presumably FlaV, of the polymerization of exogenous flagellin on the tips of fragmented filaments.

DISCUSSION

FlaV Inhibits Filament Assembly from Exogenous Flagellin. Filament assembly on living cells of *S. typhimurium* does not necessarily have to proceed using endogenous flagellin. *flaV* and *flaV H1 H2* mutants have been reported to acquire motility upon addition of exogenous flagellin (14), which is able to polymerize at the tips of their hooks. Those tips contain FlaW and FlaU but lack FlaV. Consistent with this was our finding (Table 2) that *flaV* cells acquired motility after incubation in flagellin-containing FR from the culture medium of *flaV* mutants. However, when FR(*flaV*$^+$) was used, acquisition of motility was seriously impaired. Moreover, when *flaV H1 H2* cells, which do not produce flagellin, were treated with FR(*flaV*$^+$), no motility was acquired. In other words, the presence of exogenous flagellin is not sufficient to guarantee filament assembly, suggesting that FR(*flaV*$^+$) contains an inhibitor. The likely identity of this inhibitor is FlaV itself, which is known to be present in the culture medium from *flaV*$^+$ strains, including wild type (15); the mechanism for this excretion is not understood.

Thus, FlaV might act to prevent exogenous flagellin from polymerizing onto the hook. This is consistent with the evidence that exogenous flagellin is unable to polymerize on the hooks of *H1 H2* or *flaL* mutants (14); these hooks contain FlaV at their tip (12).

FlaV Is a Requirement for Filament Assembly from Endogenous Flagellin. Where *flaV* cells were permitted to synthesize protein by placing them in L broth, FR(*flaV*$^+$) enabled endogenous flagellin to assemble into filament. Likewise, *flaV* strains acquired good motility by use of endogenously supplied flagellin after they were incubated with a dissociated hook preparation (which does not contain flagellin at all and contains three kinds of HAPs and hook protein) from a *H1 H2* strain, SJW1672 (data not shown). This result indicates that FlaV that has been added exogenously to the tip of the hook enables flagellin molecules—synthesized in the cell and transported to the tip of the hook—to polymerize successfully. This occurred even when the FR was diluted 125-fold, indicating that only low concentrations of exogenous FlaV are needed to enable endogenous flagellin assembly.

Experiments that effectively titered the amount of exogenous FlaV while maintaining adequate amounts of other components produced the interesting result (Fig. 2*d*) that, at intermediate FlaV levels, reconstitution of both exogenous and endogenous flagellin was almost totally inhibited; at higher levels, endogenous flagellin could polymerize into filament. Thus, addition of endogenous flagellin appears to require a higher FlaV level than does inhibition of addition of exogenous flagellin. Possibly the number of FlaV molecules needed for endogenous addition corresponds to the number necessary to complete construction of the claw-shaped end seen on the hooks of *flaL* or *H1 H2* strains (12); the tips of their hooks contain all three HAPs and are expected to be the nucleus for polymerization of endogenous flagellin. Something less than a complete FlaV complement apparently suffices to block assembly of flagellin from the external medium.

FlaV Acts at the Tip of Growing Filaments. FlaV has been found (12) at the tip of hooks of *H1 H2* and *flaL* mutants, which completely lack filament. However, two of our results

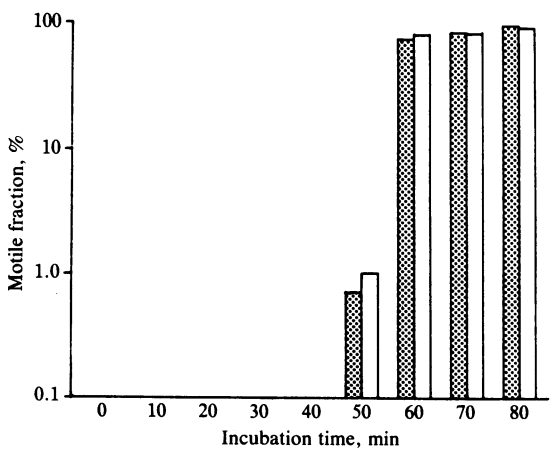

FIG. 3. Dependence of acquisition of motility on time of incubation in FR. *flaV* cells (MH103) were mixed with FR(*flaW flaV*) prepared from MH110. The frequency of acquisition of motility was estimated by observation of the cells (as described for Table 2) after incubation in FR for various times (stippled bars) and again after cultivation in L broth for 40 min (open bars). Blank spaces represent no (<0.2%) acquisition of motility.

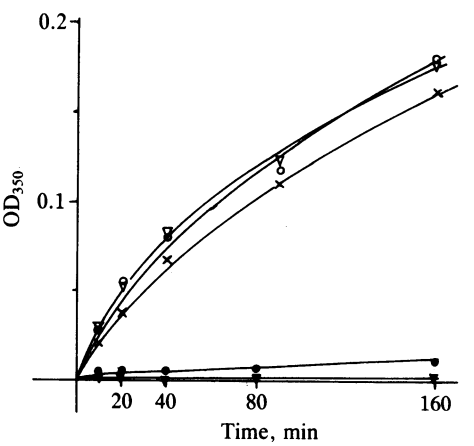

FIG. 4. *In vitro* flagellin polymerization at the tips of filaments after addition of PBS (control, ×) or of FR from SJW1103 (*fla*$^+$, ●), MH103 (*flaV*, ○), SJW2177 (*flaW*, ▼), or MH110 (*flaW flaV*, ▽). The extent of polymerization was estimated by the increase in optical density at 350 nm. (See *Materials and Methods*.)

suggest that FlaV may be located at the distal end of the filament also. First, *flaV* cells that had been incubated for a short period in FR lacking FlaV, and had acquired (by exogenous flagellin assembly) the nontranslational motion characteristic of cells with short flagella, did not improve their motility when permitted to synthesize flagellin by growth in L broth. Second, exogenous flagellin failed to assemble onto filament fragments using FR(*flaV*$^+$)—a process that proceeded when FR(*flaV*) was used. A substance having a strong inhibitory effect on the *in vitro* polymerization of flagellin has been detected in the culture medium of short-filament mutants (20). Given the present results, this substance is likely to be FlaV. We note in this regard the recent observation by Ikeda *et al.* (21) that the distal end of native filament displays a capped appearance that is absent from broken or reconstituted filament; this cap can be decorated by anti-FlaV antibody (T. Ikeda and M.H., unpublished data), lending further support to the conclusion reached above.

Role of FlaW and FlaU in Reconstitution of Filament. Inconsistencies in the literature concerning the ability of hooks from *flaU* mutants to act as seeds for *in vitro* filament assembly (14, 19) have been resolved by the present study. *flaU* or *flaU flaV* cells were able to assemble filaments from exogenous flagellin, provided that the FR used was from a *flaU*$^+$ *flaV* strain. Such an FR would convert hooks with only FlaW at their tips into hooks with both FlaW and FlaU; these, in the absence of FlaV as inhibitor, would permit exogenous flagellin to assemble.

FlaW has been shown to be at the hook-filament junction (12). However, mutants defective in *flaW* did not acquire motility under our experimental conditions, even when FlaW was supplied exogenously. It appears therefore that addition of FlaW to the hook tip can only proceed by a pathway involving direct transport of FlaW to the tip from the cytoplasm, or that FlaW is unstable in FR preparations.

A Model for Filament Formation. Based on the present studies and previous results that indicate a layered-structure model (12), we have constructed a scheme to describe the process of filament formation (Fig. 5). FlaU (HAP3) is unable to polymerize without prior assembly of FlaW (HAP1) onto the tip of the hook, and FlaV (HAP2) is unable to polymerize without prior assembly of both FlaW and FlaU. Therefore, in the normal process of filament assembly, endogenous FlaW, FlaU, and FlaV are assembled, in that sequence, at the distal end of the hook. At this point, endogenous flagellin is able to polymerize at the claw-like end composed of the three kinds of HAPs and progressively inserts itself between existing filament and FlaV, which resides at the distal end. On the other hand, exogenous flagellin is unable to polymerize at the claw-like end of a hook from *flaL* or *H1 H2* mutants, at the end of a hook to which FlaV has been added exogenously, or at the tip of native filament, because FlaV is present at the end and inhibits the exogenous polymerization. When FlaV is absent, however, exogenous flagellin at a sufficiently high concentration can polymerize on the tip of the hook of a *flaV* mutant or the tip of a fragmented filament; a similar result is obtained with *flaU flaV* mutants, provided FlaU is supplied exogenously. Endogenous flagellin cannot polymerize under these conditions, because the tip is missing FlaV; addition of exogenous FlaV to the tip of the hook of a *flaV* mutant permits endogenous flagellin assembly. When FlaU is absent from the tip of the hook, flagellin addition from either the cytoplasm or the external medium is impossible; the same is true if FlaW is missing, since FlaU addition is dependent on its presence. Thus, FlaW and FlaU are essential to the polymerization process. Although the location of FlaU has not been demonstrated directly, the HAP locations illustrated in Fig. 5 are consistent with the results obtained in this study and with the inferred mechanism of assembly. Thus, even though *in vitro* polymerization of flagellin has the rather simple character of a unidimensional crystallization process, the process *in vivo* is more elaborate, requiring the orderly participation of three distinct accessory proteins.

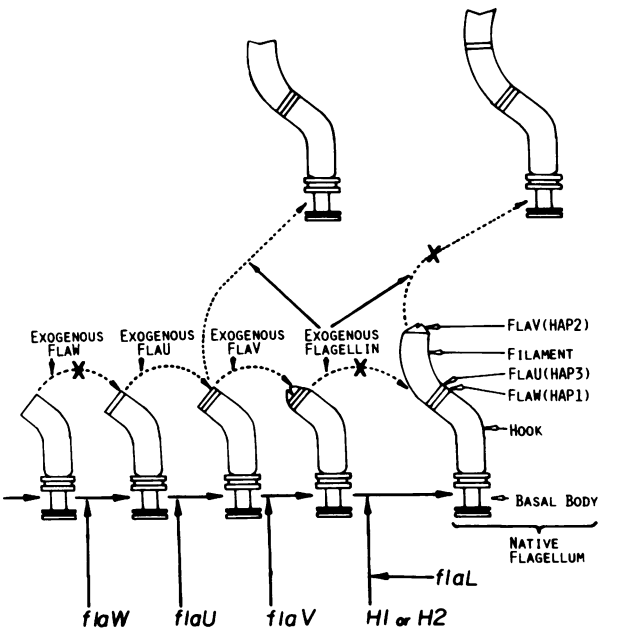

FIG. 5. Proposed process of flagellar filament formation. *H1* and *H2* are the structural genes for flagellin (8); *flaL* controls the transcription of mRNA for flagellin (9); and *flaW*, *flaU*, and *flaV* are the structural genes for FlaW, FlaU, and FlaV, respectively (11). The localizations of HAPs in the flagellar structure, as well as the assembly process, are discussed in the text.

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