Length Control of the Flagellar Hook in a Temperature-Sensitive flgE Mutant of *Salmonella enterica* Serovar Typhimurium

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The flagellar hook is a short, curved, extracellular structure located between the basal body and the filament. The hook is composed of the FlgE protein. In this study, we analyzed flagellum assembly in a temperature-sensitive flgE mutant of *Salmonella enterica* serovar Typhimurium. When the mutant cells were grown at 30°C, they produced flagella of a normal length (71% of the population) and short hooks without filaments (26%). At 37°C, 70% of the basal bodies lacked hooks, and intact flagella made up only 6% of the population. Mutant cells secreted monomeric FlgE in abundance at 37°C, suggesting that the mutant FlgE protein might be defective in polymerization at higher temperatures. The average length of the hooks in intact filaments was 55 nm, whereas after acid treatment, it was 45 nm. SDS-PAGE analysis of the hook-basal body showed that HAP1 was missing in the mutant but not in the wild type. We concluded that hook length in the mutant is controlled in the same way as in the wild type, but the hook appeared short after acid treatment due to the lack of HAP1. We also learned that the true length of the hook is possibly 45 nm, not 55 nm, as has been believed.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *Salmonella enterica* serovar Typhimurium SJW1103 and SJW2219 were used in this study. SJW2219 has a point mutation in FlgE (T149N). Cells were cultured in LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl).

**Hook length measurement and histogram.** To measure hook lengths, intact flagella and hook basal bodies (HBB) were isolated according to the conventional method reported previously (6). The measured hook lengths were grouped in 5-nm increments. For the length distribution histogram of recalculated hook lengths shown in Fig. 5B, hook lengths in the intact flagella were reduced by 10 nm, and numbers of particles in different length groups were added to the particle numbers for the hook lengths of immature HBB in corresponding length groups.

**SDS-PAGE.** Proteins secreted into the medium were analyzed by SDS-PAGE as previously reported (7). Briefly, cells were grown at 30°C or 37°C and harvested until the optical density at 600 nm (OD600) reached 1.0. When cells were incubated at 30°C, the cell pellet and the culture supernatant were fractionated by differential centrifugation. The proteins secreted into the culture medium were precipitated with 7% trichloroacetic acid (TCA). Proteins were dissolved in SDS sample buffer and boiled for 3 min. Fractions were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The amounts of protein samples applied to SDS gels were normalized according to the cell density, which was measured by using the OD600.

**Western blot analysis.** The samples were subjected to SDS-PAGE and then transferred to PVDF membranes. Polyclonal anti-FliK or anti-FliC antibody (1:2,000 dilution) was used for immunoblotting.

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RESULTS

The \textit{flgE} mutant strain SJW2219 is temperature sensitive. The TS properties of the \textit{flgE} mutant strain SJW2219 were characterized in this study. As expected for TS mutants, mutant colonies exhibited motility rings on semisolid agar plates at the permissive temperature (30°C) but not at the nonpermissive temperature (37°C) (Fig. 1A). We also confirmed, by optical and electron microscopy, that SJW2219 cells grown at 30°C produced wild-type flagella and actively swam. On the other hand, when the mutant cells were grown at 37°C, they were mostly nonflagellated, only a few cells moved in liquid medium, and the mutant colonies exhibited no motility rings on soft agar plates (Fig. 1B).

Because the mutation occurs in the hook protein, it is likely that flagellar basal bodies (BB) can still be formed. To confirm the formation of BB in cells grown at 37°C, we attempted the isolation of cell surface structures from SJW2219 cells by using a previously reported method for purification of HBB (6). By electron microscopy, we found many partly assembled basal structures in the membrane preparation. Most were basal bodies with short hooks; only a few intact flagella were observed (Fig. 1C). The proportions of cells with basal structures without a hook, cells with a hook, and cells with intact flagella were 70%, 24%, and 6%, respectively (Table 1). Intact flagella were seldom found, and at most, one flagellum per cell was observed (Fig. 1B). This situation accounts for the motility defect observed.

On the other hand, it should be noted that mutant cells grown at 30°C looked similar to wild-type cells on soft agar but also produced immature basal bodies as well as intact flagella. In these cells, the proportions of basal structures without a hook, those with a hook, and those with intact flagella were 3%, 26%, and 71%, respectively. This is different from what was observed in cultures grown at 37°C (Table 1). Instead, it shows a defect in hook formation even at 30°C. Our data indicate that the TS mutation causes gradual deterioration of the assembly process, and therefore an all-or-none phenotype of hook formation is not observed.

\textit{SJW2219 cells secrete the hook protein abundantly at 37°C}. Because most of the hooks in SJW2219 cells grown at 37°C were short, we measured the amount of FlgE secreted from the cells. We collected the secreted proteins from cell-free medium by TCA precipitation and examined them by SDS-PAGE. Wild-type strain SJW1103 grown in LB medium or in tryptone yeast (TY) medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract) secreted both flagellar proteins and virulence factors, as previously reported (7). However, when SJW1103 cells were grown in low-salt TY medium, they secreted predominantly flagellar proteins and reduced amounts of the following virulence factors (8): SipA (89 kDa), FlgK (or HAP1; 60 kDa), FliC (58 kDa), FliD (56 kDa), SipC/FlgE (42 kDa), InvJ (40 kDa), and FlgL (or HAP3; 35 kDa). Wild-type cells secreted more FliC at 37°C than at 30°C and, accordingly, produced more flagella at the higher temperature (Fig. 2A, lanes 1 and 3).

The SDS-PAGE pattern of proteins secreted from SJW2219 cells grown at 30°C looks similar to that of wild-type cells, specif-

![FIG 1](A) Swarm plates of wild-type (WT) and SJW2219 strains. Strains were inoculated onto 0.3% soft agar plates and incubated at 30°C for 11 h (top) or at 37°C for 7 h (bottom). (B) Electron micrographs of SJW2219 cells. Cells grown in LB medium at 30°C or 37°C were stained with 1% phosphotungstic acid (pH 7). (C) Electron micrographs of immature basal structures isolated from SJW2219 cells grown at 37°C. The micrographs were negatively stained with 2% phosphotungstic acid (pH 7).

**TABLE 1** Intermediate flagellar structures in strain SJW2219

<table>
<thead>
<tr>
<th>Flagellar structure</th>
<th>No. (%) of particles in cells grown at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td>Basal body</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Hook basal body</td>
<td>38 (26)</td>
</tr>
<tr>
<td>Intact flagella</td>
<td>103 (71)</td>
</tr>
</tbody>
</table>
ically in the higher-molecular-mass region, but the mutant cells secreted increased amounts of the mutant hook protein (FlgE-T149N; 41 kDa) as well as low-molecular-mass proteins such as the rod components (FlgF [32 kDa] and FlgG [30 kDa]) and the hook cap protein FlgD (28 kDa) (Fig. 2A, lanes 2 and 4) (see reference 7 for the assignment of protein bands). Wild-type FlgE comigrates with SipC, whereas the FlgE-T149N mutant is slightly faster than the wild-type FlgE protein (41 kDa versus 42 kDa), and this band is clearly divided in two for the mutant strain.

By comparing the amounts of FlgE and FlgE-T149N, we observed that the mutant cells secreted a great amount of unpolymerized FlgE-T149N at both temperatures (Fig. 2A, lanes 2 and 4), whereas the amount of FlgE secreted from the wild-type strain into the medium was negligible (lane 1). We asked why there was excess FlgE in the culture supernatant of the mutant cells compared to that of wild-type cells. It is conceivable that mutant FlgE monomers were inefficiently incorporated into the growing hooks and therefore accumulated in the medium. Nevertheless, mutant TS cells grown at 30°C also produced excess FlgE in the medium. In fact, as mentioned above, in the preparation of intact flagella by acid treatment to prepare HBB and measure the lengths of this protein in the cytoplasm.

**FIG 2** (A) SDS-PAGE pattern of proteins secreted into the culture medium from wild-type and SJW2219 cells. Cells were grown in TY medium at either 30°C or 37°C. Secreted proteins were recovered from cell-free spent culture medium by TCA precipitation. The gel was stained with CBB; the acrylamide concentration of the gel was 12.5%. Lanes: 1, wild-type cells incubated at 30°C; 2, SJW2219 cells incubated at 30°C; 3, wild-type cells incubated at 37°C; 4, SJW2219 cells incubated at 37°C. (B) Western blot analysis of culture supernatant (Sup) and cellular (Cell) fractions of wild-type and SJW2219 cells, using anti-FliC polyclonal antibodies at a 1:2,000 dilution. Cells were grown at 30°C and 37°C to an OD600 of 1.0. Samples in lanes are the same as in panel A. (C) Detection of intracellular and extracellular FliK by using polyclonal anti-FliK antibodies (1:2,000 dilution). Samples in lanes are the same as in panel A.

Wild-type and SJW2219 cells were grown at 30°C or 37°C. We collected the secreted proteins from cell-free supernatants by TCA precipitation and analyzed them by immunoblotting using an anti-FliC polyclonal antibody (Fig. 2B). SJW2219 cells grown at 30°C produced and secreted FliC in amounts similar to those in wild-type cells (Fig. 2A and B, lanes 1 and 2). On the other hand, SJW2219 cells grown at 37°C secreted much less FliC than the wild type or the TS mutant grown at 30°C (Fig. 2A and B, lanes 3 and 4). Accordingly, in whole SJW2219 cells, FliC was not detected at 37°C (Fig. 2B, lane 4), suggesting that the fliC gene was not expressed in SJW2219 cells grown at 37°C (see Discussion).

**SJW2219 cells secrete FliK abundantly at 37°C.** SJW2219 cells grown at 37°C secreted recognizable amounts of FliK as well as rod proteins (FlgF and FlgG), as detected by SDS-PAGE (Fig. 2A, lane 4). To determine how much of the intracellular FliK protein was secreted, we analyzed the amounts of secreted and intracellular FliK. Wild-type and SJW2219 cells were grown at 30°C or 37°C. We collected secreted proteins from cell-free supernatants as mentioned above and analyzed them by immunoblotting using a polyclonal anti-FliK antibody (Fig. 2C). Production and secretion of FliK in wild-type cells were at the same levels at both temperatures. Secretion of FliK in SJW2219 cells was more than that in wild-type cells at both temperatures, as seen in the CBB-stained gel and in Western blots (Fig. 2A and C). Consequently, the cellular level of FliK in SJW2219 cells was higher than that in the wild type (Fig. 2C, lower panel). The high cellular level of FliK was most likely due to many more structures that remained in rod-hook secretion mode, resulting in more FliK secretion.

**FliK in strain SJW2219 controls hook length.** Electron microscopy showed no apparent differences in the flagellar structure of strain SJW2219 grown at 30°C and that of the wild-type strain. We attempted to measure hook lengths under various conditions, because SJW2219’s hook length has been reported to be shorter than that of the wild type (4). In a conventional method for measurement of hook length, we depolymerize filaments of intact flagella by acid treatment to prepare HBB and measure the lengths of...
hooks straightened by low pH/low temperature (9). In this study, hook length was 48 ± 5 nm (n = 383) (Fig. 3, upper panel). We also attempted to measure the lengths of hooks in intact flagella and found that the hook length was not different from that in wild-type cells (56 ± 7 nm; n = 100) (Fig. 3, lower panel). We suspected that hook length differences could be due to the acid treatment used for depolymerization of the filament. The acid treatment also could have depolymerized SJW2219 hooks. Therefore, we analyzed the protein components of purified HBB by SDS-PAGE.

Intact flagella isolated from wild-type and SJW2219 cells were incubated in acidic glycine buffer (pH 2.5) for 1 h, and the remaining structures were isolated by ultracentrifugation. The wild-type HBB showed the typical band pattern after acid treatment, with bands for FliF (65 kDa), FlgK (HAP1; 60 kDa), FlgE (42 kDa), and rod proteins (Fig. 4, lanes 1 and 2). In contrast, the band pattern of SJW2219 HBB showed that the band corresponding to HAP1 had disappeared (lane 3), indicating that acid treatment removed HAP1 from the HBB. There was no further effect on the hook after 12 h of incubation (data not shown). Therefore, we concluded that the interaction between the hook and HAP1 in the TS mutant is weaker than that in the wild type, as revealed by acid treatment, and that once the hook is formed it remains intact.

The length of the hook without the attached HAP structure is 45 nm. At this point, we knew that there were at least two reasons for the presence of short hooks in the SJW2219 strain: (i) there were many short hooks in the preparation, and (ii) HAP1 was removed from the tip of the hook following acid treatment. We then measured the lengths of short hooks (38 ± 12 nm; n = 130) and drew a diagram of the hook length distribution together with that of hook lengths for the intact flagella (Fig. 5A). The number of hooks on the BB decreased as the hook grew longer to become an

**FIG 3** (A) Distribution of hook lengths in HBB from SJW2219 (n = 383). On the right are electron micrographic images of HBB. (B) Distribution of hook lengths in intact flagella from SJW2219 (n = 100). On the right are electron micrographic images of the hook region in intact flagella.

**FIG 4** SDS-PAGE pattern of HBB isolated from wild-type and SJW2219 cells. The gel was stained with silver as described in Materials and Methods; the acrylamide concentration was 12.5%. Lanes: 1, molecular size markers (proteins secreted into culture medium from the wild-type strain); 2, wild-type HBB after incubation in acidic glycine buffer (pH 2.5) for 1 h; 3, SJW2219 HBB after incubation in acidic glycine buffer (pH 2.5) for 1 h.
intact flagellum. The shortest hooks in intact flagella were about 35 nm, suggesting that filament formation can begin only after the hook has reached a certain length. We do not know whether a minimum hook length for filament formation exists or not. We also learned that the wild-type hook includes the HAP region (HAP1 and HAP3), whereas the mutant hook does not. Stoichiometric analysis of the HBB showed that the number of HAP1 subunits is about 12, which corresponds to two turns of the helical structure, a basic helix of approximately 5.5 subunits per turn (10). Because the pitch of the basic helix is about 2.5 nm, the HAP1 region is 5 nm long. Although we do not have such a stoichiometric number for HAP3 (FlgL), we can assume that the amount of FlgL is almost the same as that of FlgK, judging from the band density of FlgL in Fig. 2A. Altogether, the HAP region extends for 10 nm between the hook and filament. We recalculated the number of hooks by adding up the numbers of immaturely short hooks and shortened hooks after acid treatment (see Materials and Methods). We redrew the diagram in consideration of the ratio of each hook preparation (Fig. 5B), and it looks similar to the length distribution of hooks treated by acid (Fig. 3A).

In conclusion, the TS flgE mutant strain SJW2219 polymerizes fewer hooks but retains the ability to control hook length. A defect in FlgE gives rise to the absence of the HAP region from the mutant hook following acid treatment. Hence, we learned that the hook length is measurably shorter in the absence of attached HAP structures (Fig. 6).

FIG 5 (A) Hook length distribution of intact flagella isolated from SJW2219 cells grown at 30°C (empty bars) and of immature HBB (filled bars). (B) Hook length distribution after recalculation of hook length by correcting for the loss of the HAP region and contamination by immature hooks.

FIG 6 Schematic representation of wild-type and mutant HBB. The hook consists of FlgE, and the HAP region contains two proteins, FlgK and FlgL. (Left) The HAP region (dotted area) remains attached to the tip of the hook of the wild-type structure. (Right) In strain SJW2219, the HAP region is missing.
We studied flagellar assembly in a TS flgE mutant. We decided to reexamine the properties and phenotype of this mutant. We confirmed previous observations, i.e., that SJW2219 cells produce shorter hook structures than those of wild-type cells due to inefficient polymerization of the hook. We also found, by electron microscopy, that the mutant hooks produced at 30°C had a wild-type average length (55 nm) in intact flagella, whereas they were shorter (45 nm) in the HBB obtained by acid treatment of the intact flagella. We showed by SDS-PAGE that the HBB preparation did not contain HAP1, which is supposedly attached to the distal end of the wild-type hook, indicating that the interaction between the mutant hook and HAP1 could be weaker than that of the wild type. Our results suggest that the FlgE-T149N mutant in SJW2219 is defective both in hook polymerization and in the stability of the FlgE-HAP1 interaction in intact full-length hook structures.

In negatively stained images of the hook-HAP-filament region, the filament appears as a plain uniform filament, but the HAP region looks blurred. When we attempt to measure the length of the hook attached to the filament, we tend to measure the length between the proximal end of the filament and the proximal end of the hook (or the upper side of the L ring). Thus, the hook length normally includes the HAP region. Wild-type HBB contain HAP proteins; therefore, we consistently measured the lengths of the hooks with HAP1 and HAP3 still attached. It should be noted that “HAP-less” hook structures have never been observed. In HAP-deficient mutants, the hook tips are covered with FlgD, the hook cap protein (11).

Why was the fliC gene not expressed in SJW2219 cells grown at 37°C? The expression of fliC is dependent on sigma 28, which is encoded by fliA. Before the HBB is complete, the function of FliA is suppressed by FlgM (anti-sigma 28). Upon completion of the HBB, FlgM is secreted and releases FliA, which can now function (12). In SJW2219 cells, most of the HBB is incomplete, and thus it is very likely that fliC expression is suppressed by FlgM which cannot be exported.

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REFERENCES