Excretion of Flagellin by a Short-Flagella Mutant of 
Salmonella typhimurium

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A nonmotile mutant of Salmonella typhimurium, SJW1254, has very short flagella (<0.1 μm long) due to a mutation in the structural gene of flagellin (H2). When ammonium sulfate was added to the culture medium of SJW1254 grown to the late-log phase, a large amount of protein precipitated. Gel electrophoresis and immunodiffusion showed that more than 90% (wt/wt) of the precipitated protein was flagellin. The mutant flagellin appeared to be excreted in the monomeric form, in an amount comparable to the amount in the flagellar filaments of wild-type bacteria. No such precipitate was obtained from the medium of wild-type bacteria. The mutant flagellin had the same apparent molecular weight (55,000) and isoelectric point (5.3) as the wild-type flagellin, but differed in mobility in polyacrylamide gel electrophoresis under nondenaturing conditions. Moreover, the mutant flagellin did not polymerize in vitro under various conditions in which wild-type flagellin polymerized. These results suggested that the mutant bacteria excreted flagellin because the flagellin polymerized poorly and therefore could not be trapped at the tip of the flagellar filament. This short-flagella mutant should be useful for studying the mechanism of flagellin transport.

The flagellar filaments of Salmonella typhimurium are tubular structures with a diameter of about 20 nm and a variable length of up to 20 μm. They grow in vivo by the addition of globular protein subunits (flagellin) to the distal ends of the filaments (7, 10, 17). Thus, during the process of flagellar elongation, the flagellin molecules should be transported to the tip, possibly through the central hole of the filament (4, 15, 16, 19).

Recently, Yamaguchi has isolated a mutant of S. typhimurium which is nonmotile owing to its extremely short flagella (<0.1 μm in length). Genetic analysis has shown that this strain has a single mutation in the flagellin structural gene (H2). Being interested in how the mutation in the flagellin gene leads to the production of short flagella, we tried to examine the nature of the mutant flagellin. In the course of this study, we found that the mutant bacteria excreted a large amount of defective flagellin into the culture medium.

MATERIALS AND METHODS

Bacterial strains. The short-flagella mutant of S. typhimurium, SJW1254, is a nonmotile strain derived by spontaneous mutation from a motile strain, SJW806, which produces flagella in an H2-monophasic manner (8). The flagellar antigen type of these strains is e, n, x. Both the mutant and the parent bacteria aggregated when anti-e, n, x-flagellin antibody was added to the medium.

Preparation of flagellin. The flagellin of the parent strain was prepared by the method of Asakura et al. (2, 3). Briefly, the bacteria, grown in 1% polypeptone and 1% yeast extract, were harvested in the late-log phase. Flagellar filaments were detached from the cell bodies by shaking concentrated bacterial suspensions and purified by differential centrifugation and polymerization-depolymerization cycling. Monomeric flagellin was obtained by heating the flagellar solution at 65°C for 3 min.

The flagellin of the short-flagella mutant was isolated in the following manner. The mutant bacteria, grown in M9 medium (13), were removed in the late-log phase by centrifugation at 10,000 × g for 30 min. Solid ammonium sulfate was dissolved in this medium to 40% saturation (about 1.8 M) to precipitate the flagellin. After standing overnight at 4°C, this solution was centrifuged at 10,000 × g for 30 min, and the pellet (AS-precipitate) was dissolved in a solution containing 0.15 M NaCl and 10 mM sodium phosphate buffer (pH 7.0; standard buffer solution). After dialysis against standard buffer and centrifugation at 100,000 × g for 60 min to remove insoluble materials, the solution was subjected to column chromatography with Sephacryl S-200 superfine gel (Pharmacia Fine Chemicals, Piscataway, N.J.), and a peak fraction that eluted at a position corresponding to that of monomeric flagellin was collected as the final sample.

Isolation of short flagella. The mutant bacteria grown to late-log phase were centrifuged at 10,000 × g for 20 min, and the pellet was suspended in an equal volume of standard buffer solution. This suspension was
blended in a homogenizer (Nihon Seiki, Co., Tokyo) at about 15,000 rpm for 10 min. Cell debris was removed by centrifugation at 20,000 \( \times \) g for 15 min. The short flagella in the supernatant were pelleted at 100,000 \( \times \) g for 60 min, resuspended in standard buffer, and used as the sample of mutant flagella.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method of Davis (6), except that a slab-gel apparatus was used. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (20). Isoelectric focusing was carried out by the method of O'Farrell (14), with the destaining method of Vesterberg et al. (18).

Other methods. Anti-\( e, n, x \)-flagellin antibody was obtained by injecting purified flagella from SJW806 into a rabbit. Immunodiffusion was performed by the method of Clausen (5). Protein concentrations were determined by the modified biuret method of Gornall et al. (9) or by the method of Lowry et al. (12). A JEM 100C microscope (JEOL Co., Ltd., Tokyo) was used for electron microscopy.

RESULTS

Electron microscopy of mutant flagella. The mutant flagella attached to the cell body were difficult to observe by electron microscopy because they were very short. Thus, we observed the flagella after detaching them by treatment of cell suspensions with a blender (see above). The flagella were fairly uniform in length, and almost all of the filament seemed to be composed of the basal hook portion (Fig. 1a and b). When these flagella reacted with anti-\( e, n, x \)-flagellin antibody, a small portion at the tip of the filaments was always labeled with the antibodies (Fig. 1c). From these images, we estimated that flagellin constituted only 10 to 20 nm of the filament, whereas the hook was about 70 nm long, as in a normal flagellum (Fig. 1d). The filaments in Fig. 1 probably were not fragments of longer filaments, because almost all of the flagella had the basal hook.

Excretion of flagellin. Genetic studies have shown that the short-flagella mutant has a point mutation in the flagellin structural gene (\( H2 \)) (Yamaguchi, unpublished data). We expected that mutant and normal bacteria would synthesize equivalent amounts of flagellin. If this was true, the flagellin should be present either inside or outside the cell body. To examine whether the flagellin was excreted by the mutant, we used the method of ammonium sulfate precipitation, since we knew that Salmonella flagellin can be precipitated by ammonium sulfate at 40% saturation (1).
The mutant bacteria were grown in M9 medium and removed from the medium by centrifugation in late-log phase. When ammonium sulfate was added to this bacteria-free medium to 40% saturation, the medium became turbid, owing to production of precipitate. In contrast, almost no precipitate was produced when the parent strain was cultured and treated as above. Figure 2 shows the SDS-polyacrylamide gel electrophoresis pattern of the AS-precipitate from the medium of the mutant. There is an essentially single band with an apparent molecular weight of 55,000, which is the same molecular weight as that of the parent strain flagellin. From a densitometric scan of the pattern, we estimated that this band amounted to more than 90% of the total protein precipitated in 40%-saturated ammonium sulfate. The AS-precipitate cross-reacted with anti-e, n, x-flagellin antibody to form a single precipitin line (Fig. 3). From these results, we concluded that the short-flagella mutant excreted a large amount of flagellin into the medium.

Table 1 shows the amounts of protein obtained from the medium by various treatments. The amount of AS-precipitate from the medium of the mutant was comparable to the amount of parent bacteria flagella obtained by blender treatment (Table 1, lines 1 and 4); the small difference between them could be explained by the difference in the method used to obtain flagellin. The AS-precipitate from the medium in which the mutant was grown did not increase noticeably when ammonium sulfate was saturated to 100%. Hence, we concluded that almost all of the flagellin in the medium was recovered in the AS-precipitate. The second line of Table 1 shows that little precipitate was obtained when the medium of the mutant was centrifuged at 100,000 x g for 120 min without the addition of ammonium sulfate. This result indicated that the flagellin in the medium of the mutant existed in monomeric or oligomeric forms, since centrifugation of such an extent is sufficient to sediment flagellar fragments as small as the basal hook. In contrast to the mutant, only a minute amount of AS-precipitate was obtained from the medium of the parent strain (Table 1, line 3).

**Purification of mutant flagellin.** The mutant flagellin precipitated with ammonium sulfate was purified as described above. After the AS-precipitate was dissolved in and dialyzed against standard buffer (see above), the flagellin became unsedimentable by centrifugation at 100,000 x g for 120 min. This is in good contrast to the parent strain flagellin, which polymerizes in the presence of a high concentration (0.8 to 1.2 M) of ammonium sulfate and remains polymeric in standard buffer (1). As electron microscopy did not show ordered polymeric structures in the initial AS-precipitate, it is likely that the mutant flagellin did not polymerize into filaments in 40%-saturated (1.8 M) ammonium sulfate solution.

Figure 4 shows the elution profile of the gel filtration at the last step of the purification. On examination by SDS-polyacrylamide gel electrophoresis, the peak fraction was found to contain flagellin as an almost single component. Hence we kept this fraction as the final sample of the

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**FIG. 2.** SDS-polyacrylamide gel electrophoresis of the AS-precipitate and flagellins. Lane 1, purified flagellin of SJW806; lane 2, the AS-precipitate from the culture medium of SJW1254; lane 3, the SJW1254 flagellin sample purified as described in the text.

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**FIG. 3.** Immunodiffusion of the AS-precipitate and flagellin. Wells 1, 2, and 3 contained anti-e, n, x-flagellin antibody, purified SJW806 flagellin, and the AS-precipitate from the medium of SJW1254, respectively.
TABLE 1. Amount of proteins obtained from culture medium

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amt of protein (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJW1254</td>
<td></td>
</tr>
<tr>
<td>AS-precipitate</td>
<td>6.0</td>
</tr>
<tr>
<td>UC-precipitate</td>
<td>0.1</td>
</tr>
<tr>
<td>SJW806</td>
<td></td>
</tr>
<tr>
<td>AS-precipitate</td>
<td>0.4</td>
</tr>
<tr>
<td>Flagella(^a)</td>
<td>4.7</td>
</tr>
</tbody>
</table>

\(^a\) Protein was precipitated by centrifugation at 100,000 \(\times g\) for 120 min. UC, Ultracentrifuge.

\(^b\) Flagellar filaments were obtained by the blender treatment used for the isolation of SJW1254 flagella (see text).

mutant flagellin. The purity of flagellin in this sample was estimated from the densitometric scan of the gel to be more than 98% (Fig. 2). The final yield was 5 to 10 mg of flagellin per 1 liter of culture; this value was compatible with the yield of flagella purified from the parent strain by an ordinary method (2). In Fig. 4, the position where the mutant flagellin was eluted was the same as that of the monomeric parent flagellin (data not shown). This result indicates that the mutant flagellin assumed a monomeric form under these conditions. Since we did not use procedures tending to depolymerize flagella throughout the purification steps, it is reasonable to conclude that the mutant flagellin was excreted in the monomeric form.

Characterization of mutant flagellin. As stated, the mutant flagellin had the same apparent molecular weight as the parent flagellin on SDS-polyacrylamide gel electrophoresis. This result is consistent with the genetic evidence that the mutant carries a point mutation in the flagellin gene (\(H2\)). We found that the mutant and wild-type flagellins had the same isoelectric point (5.3; Fig. 5) but that they had different mobilities in polyacrylamide gel electrophoresis performed in the absence of SDS (Fig. 6a). The difference in mobility was not observed when 8 M urea was included in the buffer and sample solutions of

FIG. 4. Chromatography of the AS-precipitate on Sephacryl S-200 superfine. The arrow indicates the void volume. The shaded area indicates the fraction containing the flagellin. OD\(_{280}\), Optical density at 280 nm.

FIG. 5. Isoelectric focusing of the flagellins from the mutant and parent strains. Lane 1, SJW1254 flagellin; lane 2, SJW806 flagellin. The isoelectric point was 5.3.

FIG. 6. Polyacrylamide gel electrophoresis of flagellins in the absence (a) and presence (b) of 8 M urea. Lane 1, SJW806 flagellin; lane 2, SJW1254 flagellin; lane 3, mixture of SJW806 and SJW1254 flagellins.
the electrophoresis (Fig. 6b). These results suggest that the mutant flagellin is different from the wild-type flagellin in tertiary structure, charge distribution, or both.

We tried to polymerize the mutant flagellin in vitro under various conditions in which normal flagellin is polymerized, but these attempts were unsuccessful. Instead, our preliminary studies showed that the mutant flagellin had an inhibitory effect on the polymerization of wild-type flagellin: when the mutant flagellin was mixed with the wild-type flagellin in a protein ratio of 1:25, polymerization was almost completely inhibited. Studies on the mechanisms of the inhibition of polymerization are now in progress and will be reported elsewhere.

DISCUSSION

We showed above that a short-flagella mutant of *Salmonella* excreted monomeric flagellin into the medium. Of all the proteins present in the medium, the excreted flagellin was present in by far the largest amount. Since the density of the bacteria at the final stage of growth in these experiments was about $10^9$ cells per ml, the results in Table 1 suggest that the amount of flagellin obtained from one cell should be about $6 \times 10^{-15}$ g in the mutant and $5 \times 10^{-15}$ g in the parent bacteria. A 1-μm flagellar filament is constructed of 2,200 flagellin molecules, each with a molecular weight of 55,000. Thus, the flagellin excreted by a mutant cell could make a 30-μm-long flagellar filament, or three 10-μm-long filaments. From these considerations, we conclude that flagellin is produced in a normal amount in the mutant bacteria and that most of the flagellin is excreted.

Our preliminary studies suggested that the mutant flagellin polymerized very poorly. It is easy to understand why such a defective flagellin is excreted if we assume that the transportation system for flagellin is working normally in the mutant. By analogy, we may expect that some fla mutants that have defects in flagellar morphogenesis, but not in the synthesis of flagellin, will excrete flagellin. In agreement with this prediction, Yamaguchi (unpublished data) has observed that flagellin is excreted in certain fla mutants that have been shown to be normal in the synthesis of flagellin (11). Those mutants, as well as the short-flagella mutant reported here, should be useful in clarifying the mechanism of flagellin transportation, especially the energetics and the exact path of flagellin secretion.

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LITERATURE CITED


