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Flagellar Filament Structure and Cell Motility of *Salmonella typhimurium* Mutants Lacking Part of the Outer Domain of Flagellin

KYOKO YOSHIOKA,[1] SHIN-ICHI AIZAWA,[2]* AND SHIGERU YAMAGUCHI[3]

National Institute of Bioscience and Human-Technology, A.I.S.T., M.I.T.I., Tsukuba, Ibaraki 305,[1] Department of Biosciences, Teikyo University, 1-1 Toyosatodai, Utsunomiya 320,[2] and Izumi Campus, Meiji University, 1-9-1 Eifuku, Suginami, Tokyo 168,[3] Japan

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We have isolated spontaneous mutants of *Salmonella typhimurium* which can swim in the presence of antifilament antibodies. The molecular masses of flagellins isolated from these mutants were smaller than that (52 kDa) of wild-type flagellin. Two mutants which produced the smallest flagellins (42 and 41 kDa) were selected, and the domain structures of the flagellins were analyzed by trypsin digestion and then subjected to amino acid sequencing. The two flagellins have deletions at Ala-204 to Lys-292 and Thr-183 to Lys-279, respectively. These deleted parts belong to the outer domain (D3) of flagellin, which is believed to be at the surface of the filament. These mutant filaments aggregated side by side in the presence of salt, resulting in disordered motility.

Many species of bacteria swim in liquid by means of flagella (7, 16). The motor at the base of the flagellum rotates and the resulting torque is transferred to the helical filament (2). When a peritrichously flagellated cell of an enterobacter swims, the filaments gather into a bundle which drives the cell through the liquid. The *Salmonella* filament is a left-handed helix under physiological conditions, but it can adopt other distinct helical forms under various conditions, a phenomenon called polymorphism (10). During the tumbling motion of a cell, the transformation of filaments occurs discontinuously from the left-handed form to right-handed form to unwind a twisted bundle (17).

The filament is composed of a single kind of protein called flagellin. The existence of multiple domains in flagellin was indicated by circular dichroism (25), calorimetry (3, 27), nuclear magnetic resonance (1), and image analysis of electron micrographs (23). On the basis of the electron density map of the flagellar filament obtained by X-ray fiber diffraction, Namba et al. (18) proposed three domains in flagellin, D1 to D3, from the center of the filament axis outwards in the radial direction. The core domain (D1) is responsible for filament assembly and polymorphism. The middle domain (D2) may be related to the stability of the filament shape. The outer domains (D3) of adjacent subunits in a filament are not connected to each other. There are several lines of evidence showing that the exposed D3 domain contains the major epitopes of the flagellar antigen (H antigen) (4, 12, 20). Deletions of various sizes have been introduced into the epitope region (13). Also, the region has been substituted by peptides of foreign origin (19). These deletions and substitutions seldom permit filament assembly (13). In this study, we have isolated mutants which are motile in the presence of antibodies against the filament and found that a part of the D3 domain was lacking in these mutant flagellins. The relationship between the domain structure of mutant filaments and swimming behavior is discussed.

All strains used in this study were derived from *Salmonella typhimurium* SJW1103, a phase-1 stable strain having flagella of antigen type *i* (28). Flagellar filaments from mutants were purified by a method previously described (11). Tryptic digestion of mutant flagellins was performed in phosphate-buffered saline at 37°C. L-1-Tosylamide-2-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) was added at a trypsin-to-flagellin ratio of 1:300 (wt/wt). The reaction was stopped by the addition of a 3- to 5-fold molar excess of soybean trypsin inhibitor (Sigma) (26). Semistable fragments after tryptic digestion were separated by high-pressure liquid chromatography with an anion-exchange column (MonoQ, Pharmacia). Tryptic peptides obtained by complete digestion of semistable fragments were separated with reverse-phase columns (Bensil 5-C4, Bentech Co.; C-18, Applied Biosystems), and each peak was fractionated and analyzed with a gas-phase protein sequencer (Applied Biosystems model 477A) equipped with a PTH analyzer (model 130A) (11).

Isolation of mutants which are not aggregated by anti-flagellin antibodies. *S. typhimurium* SJW1103 cells carry *i*-type flagellar filaments (8, 28) and hence cannot swim in the presence of anti-*i*-flagellin antibody (21). We have isolated four spontaneous mutants that can swim out from cell aggregates in the presence of antibody. SJW1103 cells were inoculated on semisolid plates containing anti-*i*-flagellin antibody. (The antibody was mixed at an appropriate concentration with warm agar solution just before being poured into plates.) Mutants were isolated as swarms arising from tight colonies. Isolated mutant cells did not show cell agglutination by antibody, despite the fact that they still retained flagella. Their mutations were mapped to *fliC*, the gene coding for flagellin (6).

Suspecting that the mutant flagellins might have been significantly altered, we isolated filaments from mutant cells and analyzed them by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. All of the mutant flagellins showed molecular masses smaller than that (52 kDa) of wild-type flagellin. Among such mutants, the two (SJW46 and SJW61) that produced the smallest flagellins (42 and 41 kDa, respectively [Fig. 1]) were selected for further analysis.

Swimming behavior of mutant cells. Although SJW46 and SJW61 mutant cells can swim in liquid, their swarm sizes on

* Corresponding author. Fax: 81-286-27-7211. Electronic mail address: aizawa@generic.ics.teikyo-u.ac.jp.

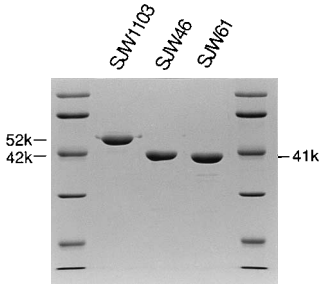

FIG. 1. SDS-polyacrylamide gel patterns of flagellins from SJW1103, SJW46, and SJW61. Their apparent molecular masses are 52, 42, and 41 kDa, respectively. In the lanes on both sides are standard size marker proteins (in kilodaltons [k]). SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (14). Polyacrylamide concentration was 12.5%. Gels were stained with Coomassie brilliant blue R.

semisolid plates (19 and 18 mm, respectively) were smaller than that (38 mm) of the wild type. Phase-contrast microscopy showed that mutant cells were as vigorously motile as the wild type in liquid media but considerably less so in viscous media (Luria broth containing 1% polyethylene glycol or 4% gelatin).

Dark-field microscopy revealed that, in viscous media, filaments on a cell associate side by side so strongly that the flagellar bundle no longer rotates smoothly. After prolonged incubation of the culture, detached bundles were often observed in the media. Furthermore, in the case of SJW61, cells also interacted through their bundles to form large filamentous clusters. Among the detached bundles, not only the normal-type waveform but also the curly-type and coiled-type waveforms were found. These observations may account for the poor motility of these mutants in viscous media.

Features of mutant flagellar filaments. When the culture of SJW46 was incubated overnight without shaking, the cells had long filaments and swam smoothly. However, when they were grown with vigorous shaking, the cells retained only short flagella and swam in a jiggly manner. By electron microscopy (JEM-1200EXII electron microscope; JEOL, Tokyo, Japan), it was shown that there were many fragmented filaments released in the medium (Fig. 2). The lengths of these filaments were typically about 0.5 μm, and their thicknesses appeared to be similar to that of wild-type filament (about 20 nm), despite the large deletion within the molecule. A dark line runs down the middle of the filament (Fig. 2, insert), suggesting penetration of stain into the central channel, a phenomenon which is seldom observed with wild-type filament. It may also suggest that the mutant filaments are less well-packed structures and therefore are more fragile.

The mutant flagellins were purified and filaments were reconstituted in vitro as described previously (11). The yield of purified flagellin was very low (about 10 mg from 1 liter of overnight culture compared with 100 mg for the wild type), probably because of the instability of the filaments. However, the reconstituted filaments were as long as 10 μm (data not shown), indicating that the ability to polymerize was not impaired by the deletion.

Limited proteolysis of mutant flagellins. Tryptic digestion of wild-type flagellin gives rise to a semistable fragment of 40 kDa and a stable one of 27 kDa (26) (Fig. 3A). Analysis of these fragments by various methods (1, 25, 27) indicated that the 27-kDa stable fragment corresponds to D3, which exists at the outer surface of the filament.

When mutant flagellins were subjected to limited proteolysis, they also gave rise to semistable fragments and stable

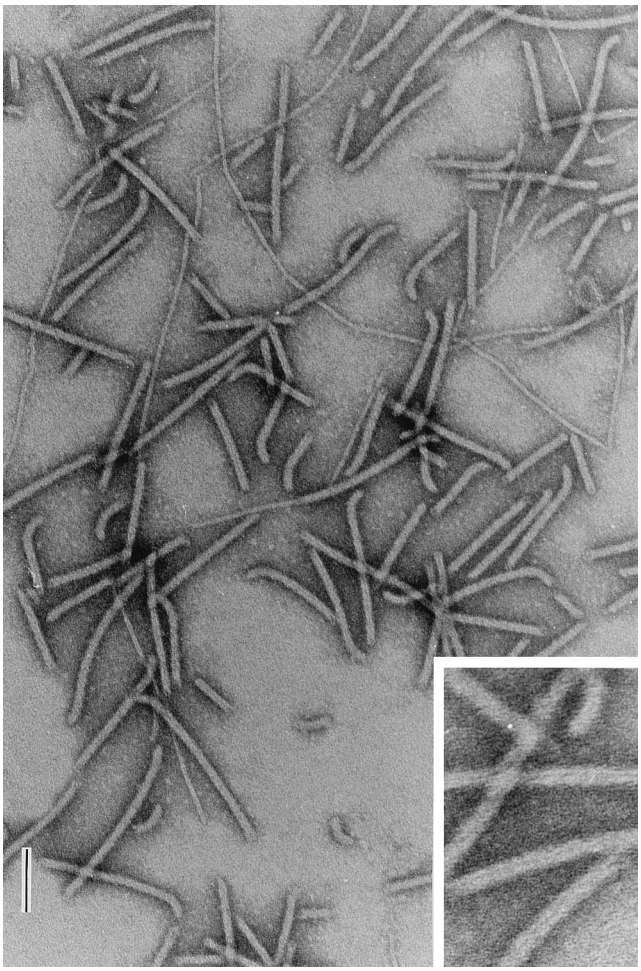

FIG. 2. Electron micrographs of SJW46 flagellar filaments released into medium during incubation with vigorous shaking. Most filaments were broken into short fragments. The middles of the filaments often show a dark line (insert). Samples were negatively stained with 2% phosphotungstic acid (pH 7.0). Bar, 200 nm.

fragments, corresponding to the 40- and 27-kDa fragments of wild-type flagellin. SJW46 flagellin was digested to a 31-kDa semistable fragment and then a 18-kDa stable one (Fig. 3B). Similarly, SJW61 flagellin gave rise to 30- and 17-kDa fragments (Fig. 3C). Judging from the difference of molecular masses between the fragments from mutants and the corresponding ones from the wild type, it is likely that the deleted regions in the mutant flagellins are included in both the larger initial fragments and the smaller (18- and 17-kDa) fragments.

In contrast to the stability of the 27-kDa fragment from wild-type flagellin, neither the 18-kDa nor the 17-kDa fragment from the mutant flagellins was stable against further treatment with trypsin. The 18-kDa fragment was almost completely digested in 2 h, and the 17-kDa fragment was digested in 3 h.

Amino acid sequencing of mutant flagellins. Since the 18- and 17-kDa fragments were too long to be analyzed in one run, they were further digested with trypsin to smaller fragments. The tryptic peptides were separated by reverse-phase chromatography and analyzed by a protein sequencer. Among the tryptic peptides obtained from the 18-kDa fragment of SJW46, we found all peptides except those corresponding to the sequence from Ala-204 to Lys-292 (89 amino acids) of the wild-

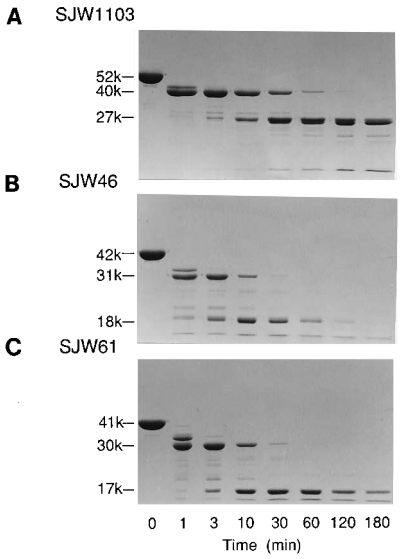

FIG. 3. Time course of proteolysis of flagellins from SJW1103, SJW46, and SJW61. The flagellin-to-trypsin ratio was 100:1 (wt/wt), and incubation was at 25°C. At the times indicated, a small portion of the reaction mixture was taken, immediately mixed with SDS sample buffer, and boiled to stop the reaction. k, kilodalton.

type sequence, thus determining the deletion site (Fig. 4). Among the tryptic peptides from the 17-kDa fragment from SJW61, we found a peptide having a sequence (VSDNVQVA NADLTEAK) indicating that the deletion site was from Thr-183 to Lys-279 (97 amino acids [Fig. 4]). SJW46 flagellin therefore contains 405 amino acids, and SJW61 flagellin contains 397 amino acids. Wild-type flagellin has 494 amino acids. These numbers agree well with the apparent molecular masses

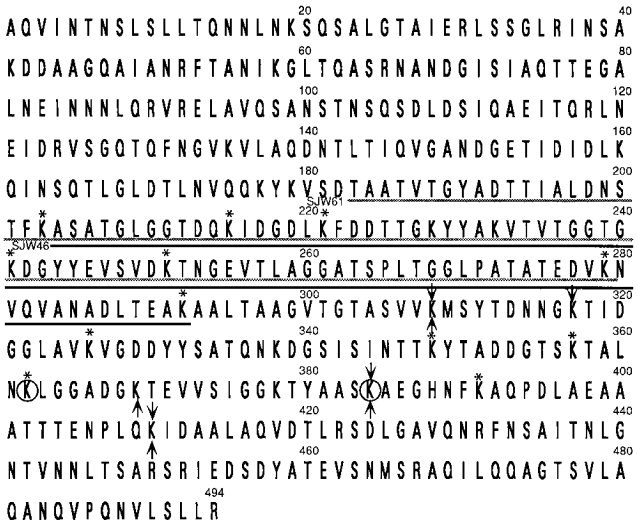

FIG. 4. Amino acid sequence of SJW1103 flagellin, determined by Kanto et al. (11). The deleted sequences of the mutant flagellins are underlined (a solid line for SJW46 and a cross-hatched line for SJW61). Methylated lysyl residues in wild-type flagellin are indicated by asterisks. Lysyl residues that are methylated in mutant flagellin(s) but not in wild-type flagellin are indicated by arrows: downward arrows for SJW46 and upward arrows for SJW61. The Lys-362 residue (circled) is not methylated in both mutant flagellins. The Lys-384 residue takes both methylated and nonmethylated forms in both mutant flagellins.

of 42 and 41 kDa, respectively, for the mutants and 52 kDa for the wild type.

The deleted parts of the mutant flagellins correspond to the outer domain (D3) of flagellin. Taking into account these data, it is reasonable to assume that the deleted parts contain the epitopes for the flagellin antigen. The newly exposed domains of the mutant flagellins in fact confer new serotypes.

Methyl lysines. It is known that lysyl residues at certain positions in flagellin are methylated (22, 24). Amino acid sequencing revealed that some lysyl residues of the mutant flagellins were also methylated, but their positions were different from those in wild-type flagellin.

In wild-type flagellin, there are 12 methylated lysyl residues at positions 203, 215, 221, 241, 251, 279, 292, 326, 348, 357, 362, and 391 (11). In SJW46 flagellin, nine lysyl residues are methylated as indicated in Fig. 4. In the case of Lys-384 both methylated and nonmethylated species are present. Lys-308, Lys-317, and Lys-410 are methylated, whereas they are not methylated in wild-type flagellin. On the other hand, Lys-362, which is methylated in wild-type flagellin, is not methylated in SJW46 flagellin. In SJW61 flagellin, nine lysyl residues are methylated. Lys-384 was found to take both forms, with the ratio of methylated to nonmethylated species being about 1:2 as judged from the peak heights from chromatography. Lys-308, Lys-369, and Lys-410 are methylated, but Lys-317 is not.

The distributions of methylated lysine residues in the mutant flagellins indicate that the tertiary structure of the domain has been altered so that a surface formerly hidden inside the molecule is now exposed. The role of the methylation is unclear, because *Escherichia coli* flagellin is not methylated and because even *Salmonella* mutants which lack the methylation enzyme (22) have flagella that function normally so that cells swim as actively as the wild type.

Polymorphic transition of mutant filaments. Filament shape changes discretely from one form to another under various conditions (10). For wild-type filaments, the normal type is typically converted into the curly type at pH values lower than 4 in the presence of 0.1 M NaCl (9).

We examined polymorphic transitions of mutant filaments by the method of Kamiya and Asakura (9). The predominant forms of the filaments as a function of pH and NaCl concentration are observed with a high-intensity dark-field microscope as previously described (5, 15).

SJW46 filaments were converted from normal to curly even at neutral pH in the presence of 0.1 to 0.3 M NaCl. At lower pHs, the curly filaments appeared over a wide range of NaCl concentrations. In the case of SJW61, the diagram was almost the same as that of SJW46 at pHs lower than 8. At pH 9, the coiled form appeared in the presence of 0.3 M NaCl. At pHs higher than 11, most of the filaments were converted into this form.

These data indicate that mutant filaments are less stable than the wild type and more easily converted into other forms over a wide range of pH values and salt concentrations.

As a result of the deletion, the net charge of the flagellin molecule was decreased; in wild-type flagellin, there are 54 negatively charged amino acid residues and 42 positively charged ones, whereas there are 41 and 34, respectively, in the mutant flagellins. The decrease of net charge of the mutant flagellin molecule will weaken the electrostatic repulsive force between the subunits in the filament and therefore affect the polymorphic nature of the filaments. Since the amino acid sequences in domains D1 and D2 are identical between the mutant and wild-type flagellin, the structural alteration in D3 induced by the deletion might be transmitted to the inner domains.

Bundle formation of mutant filaments. As described above, mutant filaments often aggregated side by side into bundles under conditions in which wild-type filaments did not. Filaments from both mutants formed bundles in the presence of more than 0.1 M NaCl (data not shown). The thickness of the bundles increased with salt concentration. Bundles attached to cells seemed to be tightly packed, since they were seldom rotating. However, cells could often still swim, using flagella that had not become part of the bundle.

The decrease of net charge of the mutant flagellins will also affect the bundle formation of the filaments. In the presence of salt, the mutant filaments easily aggregate into bundles, whereas wild-type filaments remain separated under the conditions examined. This property of wild-type filaments seems to be essential for favorable swimming of a cell, because the filaments of the swimming cell have to slip past one another to achieve the synchronized rotation of a bundle (2).

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