Functional analysis of the flagellar genes in the *fliD* operon of *Salmonella typhimurium*

Tatsuki Yokoseki,¹ Kazuhiro Kutsukake,¹ Kouhei Ohnishi²† and Tetsuo Iino²

Author for correspondence: Kazuhiro Kutsukake. Tel: +81 824 24 7924. Fax: +81 824 22 7067.

¹ Faculty of Applied Biological Science, Hiroshima University, Kagamiyama 1-4-4, Higashi-Hiroshima, Hiroshima 739, Japan

² School of Human Sciences, Waseda University, Tokorozawa, Saitama 359, Japan

The *fliD* genes of *Salmonella typhimurium* and *Escherichia coli* encode the filament-cap protein of the flagellar apparatus, which facilitates the polymerization of endogenous flagellin at the tips of the growing filaments. Previous sequence analysis of this operon in both organisms has revealed that the *fliD* gene constitutes an operon together with two additional genes, *fliS* and *fliT*. Based on the gene-disruption experiment in *E. coli*, both the *fliS* and *fliT* genes have been postulated to be necessary for flagellation. In the present study, we constructed *S. typhimurium* mutants in which either *fliS* or *fliT* on the chromosome was specifically disrupted. Both mutants were found to produce functional flagella, indicating that these genes are dispensable for motility development in *S. typhimurium*. However, flagellar filaments produced by the *fliS* mutant were much shorter than those produced by the wild-type strain. This indicates that the *fliS* mutation affects the elongation step of filament assembly. The excretion efficiency of flagellin was examined in the *fliD*-mutant background, where the exported flagellin molecules cannot assemble onto the hooks, resulting in their excretion into the culture media. We found that the amount of flagellin excreted was much reduced by the *fliS* mutation. Based on these results, we conclude that FliS facilitates the export of flagellin through the flagellum-specific export pathway.

Keywords: *Salmonella*, flagellar gene, filament elongation, gene disruption, flagellum-specific export pathway

INTRODUCTION

The flagellum of *Salmonella typhimurium* and *Escherichia coli* is a locomotive organelle composed of three structural parts: a basal body, a hook and a filament (Macnab, 1992). The filament extends into the extracellular space and is connected by the hook to the basal body embedded in the cell membrane. Flagellar assembly proceeds from the cell-proximal structure to the cell-distal structure. Assembly of the extracellular structures (filament and hook) is believed to involve transport of the component proteins through the flagellum-specific transport pathway, which is supposed to reside within the flagellar structure. The filament consists of a single kind of protein, flagellin. Polymerization of flagellin onto the tips of the hook requires three hook-associated proteins, FlgK, FlgL and

FliD (Homma *et al.*, 1984b, 1986). FlgK and FlgL exist at the tip of the hook constituting the hook–filament junction layer, which acts as a polymerization nucleus for flagellin monomers (Homma & Iino, 1985; Ikeda *et al.*, 1987, 1989). FliD acts as a capping protein of the filament, and is essential for polymerization of newly exported flagellin monomers at the tips of the growing filaments (Ikeda *et al.*, 1985, 1993; Homma *et al.*, 1986).

So far, nearly 50 genes have been shown to be involved in flagellar formation and function (Macnab, 1992). Almost all of them are clustered in four regions on the chromosome, called regions I, II, IIIa and IIIb. The *flgK* and *flgL* genes together constitute an operon in region I, while the *fliD* gene and the flagellin gene, *fliC*, form independent operons in region IIIa (Kutsukake *et al.*, 1988). On the basis of the sequence analysis of the *fliD* operons from *E. coli* and *S. typhimurium*, Kawagishi *et al.* (1992) showed that the *fliD* gene constitutes an operon together with two additional genes, *fliS* and *fliT*, which encodes 15 kDa and 14 kDa proteins, respectively. They constructed an *E. coli*

† Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114, USA.

mutant, IK23, in which the chromosomal *fliDST* region was deleted. Based on the complementation pattern of IK23 with the recombinant plasmids carrying various parts of the *fliD* operon, they concluded that the *fliS* and *fliT* genes are both indispensable for flagellation in *E. coli*. Recently, Chen & Helmann (1994) identified *Bacillus subtilis* genes homologous to *fliD*, *fliS* and *fliT* which are also likely to constitute an operon. They showed that integration of a plasmid into the chromosomal *fliS* gene led to a Fla⁻ phenotype, and concluded that at least the *fliS* gene is indispensable for flagellation in *B. subtilis*.

This work was initiated to elucidate the function of the genes in the *fliD* operon of *S. typhimurium* in flagellar morphogenesis. For understanding the function of specific genes, it is important to isolate defined mutants defective in the genes. Using the gene-replacement method developed by Yamada *et al.* (1993), we constructed mutants in which the chromosomal *fliS* and *fliT* genes were specifically disrupted. To our surprise, both *fliS* and *fliT* mutants were found to produce functional flagella, indicating that these genes are dispensable for flagellation. However, the *fliS* mutant produced flagella with short filaments, suggesting that the *fliS* product is required for efficient elongation of the filament. The amount of flagellin excreted from the cells was found to be much reduced by the *fliS* mutation in the *fliD*-mutant background. Therefore, we conclude that FliS facilitates the export of flagellin through the flagellum-specific export pathway.

METHODS

Bacterial strains, plasmids and media. Strains used in the present study are all derivatives of an *S. typhimurium* wild-type strain, KK1004 (Kutsukake *et al.*, 1988). KK2601 and KK2604 carry *fliD*::Tn10 and *fliC*::Tn10 mutations, respectively (Kutsukake *et al.*, 1988). The procedure for construction of gene-disruption mutants in the *fliD* operon is described below. Plasmids used were pKKD2 and pKKD4, both of which have been derived from a plasmid vector, pHSG398 (Takeshita *et al.*, 1987). pKKD2 carries a 5.9 kb *Sal*I fragment of the *S. typhimurium* chromosome inserted into the *Sal*I site of pHSG398. This fragment contains both the *fliC* and *fliD* operons. pKKD4 carries a 1.9 kb *Pst*I–*Hind*III fragment of the *S. typhimurium* chromosome inserted into the *Pst*I–*Hind*III site of pHSG398. This fragment contains only the *fliD* gene. Plasmid pUC4K, carrying a *kan* gene cassette, was obtained from Pharmacia. Ordinary culture media including L broth, L-broth agar and motility agar plates were made as described previously (Kutsukake *et al.*, 1988). Minimal medium for assay of excretion of flagellin was made as described previously (Kutsukake, 1994). Antibiotics were used at a final concentration of 25 µg ml⁻¹.

Motility assay. Motile phenotype of cells was detected as formation of spreading colonies (swarms) on motility agar plates at 37 °C.

DNA manipulation. Restriction enzymes and DNA-modifying enzymes were purchased from Toyobo and Nippon Gene. Procedures for DNA manipulation and transformation were as described previously (Kutsukake *et al.*, 1985). DNA sequencing was performed by the dideoxy chain-termination method

Fig. 1. Structure of the chromosomal region containing the *fliD* operon in the wild-type and mutant strains of *S. typhimurium*. Procedures for construction of the mutant strains are described in the text. Restriction sites were adopted from the nucleotide sequence data reported by Kawagishi *et al.* (1992) and are shown using the following abbreviations: B, *Bsu*36I; RI, *Eco*RI; RV, *Eco*RV; H, *Hind*III; Hp, *Hpa*I; M, *Mlu*I; N, *Nae*I; P, *Pst*I; S, *Sal*I. Arrows indicate the coding regions for the individual genes. *kan* indicates the *kan* gene cassette. The extent of the probe DNA used in Southern blot analysis (Fig. 2) is indicated by the hatched bar. The length of the DNA fragment which should be detected by Southern blot analysis is shown in bp under the chromosomal structure of each strain.

(Sanger *et al.*, 1977) with a Sequenase Version 2.0 sequencing kit (USB). Restriction fragments were purified from agarose gels with a GeneClean II kit (Bio101). Electroporation of *S. typhimurium* cells was performed using a Gene Pulsar system (Bio-Rad). Southern hybridization analysis of chromosomal DNA was carried out with a DIG DNA labelling and detection kit (Boehringer Mannheim) according to the manufacturer's recommendation.

Plasmid construction. The structure of the *fliD* operon and its adjacent region on the *S. typhimurium* chromosome is summarized in Fig. 1. The *fliD*, *fliS* and *fliT* genes encode proteins of 467, 135 and 122 amino acids, respectively. Plasmid pKKD2 has unique *Bsu*36I and *Nae*I sites at the 248th codon of the *fliD* gene and at the 72nd codon of the *fliS* gene, respectively. This plasmid was digested with *Bsu*36I, blunt-ended with T4 DNA polymerase, and ligated with the *kan* gene cassette which had been excised from pUC4K with *Sal*I and blunt-ended with T4 DNA polymerase. The resulting hybrid plasmid was designated pKKD2D. The same *kan* gene cassette was inserted into a *Nae*I-

digested pKKD2 to obtain pKKD2S. pKKD2 has a unique *Mlu*I site at the 105th codon of the *fliT* gene. This plasmid was digested with *Mlu*I and treated with exonuclease III. After treatment with mung bean nuclease and Klenow enzyme, the plasmid DNA was ligated with the *kan* gene cassette which had been excised from pUC4K with *Eco*RI and blunt-ended. Sequence analysis revealed that in the resulting plasmid the entire coding region of *fliT* had been deleted and replaced with the *kan* gene cassette but the *fliS* gene remained intact. This plasmid was designated pKKD2T. pKKD2, which contains two *Hpa*I sites within the *fliD* gene, was digested with *Hpa*I and self-ligated to obtain pKKD2H. The *fliD* gene on this plasmid encodes a FliD protein lacking 175 C-terminal amino acids. The *kan* gene cassette was inserted into pKKD2H at the *Nae*I site to obtain pKKD2HDS. pKKD2 was digested with *Nae*I and *Mlu*I or *Bsu*36I and *Mlu*I, blunt-ended with T4 DNA polymerase, and ligated with the *kan* gene cassettes which had been excised from pUC4K with *Pst*I and blunt-ended with T4 DNA polymerase. The resulting hybrid plasmids were designated pKKD2ST or pKKD2DST, respectively. In pKKD2ST, 64 C-terminal codons of the *fliS* gene and 104 N-terminal codons of the *fliT* gene have been removed. In pKKD2DST, 225 C-terminal codons of the *fliD* gene, an entire *fliS* gene and 104 N-terminal codons of the *fliT* gene have been removed.

Gene disruption. The genes on the chromosome were replaced with artificially disrupted genes on the plasmids according to the preligation method developed by Yamada *et al.* (1993). The detailed procedure is described in the Results section.

Preparation of flagellin monomers and *in vitro* reconstruction of filaments. Monomeric flagellin was purified from the wild-type and *fliS*-mutant strains by the method described by Asakura *et al.* (1964). For reconstruction of flagellar filaments onto the hooks of *fliD*-mutant cells, the method described by Kagawa *et al.* (1981, 1983) was adopted. The purified flagellin monomers were added to cells suspended in PBS (0.145 M NaCl; 0.15 M sodium phosphate) and incubated at 26 °C for 12 h. Formation of filaments was examined by electron microscopy.

Electron microscopic observation of flagella. In order to examine the flagellar structures attached to the cell bodies, cells grown in L broth were negatively stained with 1% (w/v) phosphotungstic acid (pH 7) and observed with a JEM120EX electron microscope (JEOL). In order to examine the flagellar basal structures, flagella including the hook-basal-body structures were fractionated from the cells by the method of Suzuki *et al.* (1978). The fractionated materials were negatively stained and observed by electron microscopy.

Electrophoretic analysis of flagellin. For the analysis of flagellin excreted into the culture medium, the cells were grown at 37 °C in the minimal medium and aliquots of culture containing a constant number of cells were clarified by centrifugation. Proteins in the resulting supernatants were precipitated by 10% (v/v) TCA and suspended in sample loading buffer (50 mM Tris/HCl, pH 6.8, 100 mM dithiothreitol, 2%, w/v, SDS, 0.1% bromophenol blue, 10%, v/v, glycerol) containing saturated Tris base. Samples were heated at 100 °C for 3 min and separated on polyacrylamide gels. The gels were stained with Coomassie brilliant blue R250. For the analysis of total flagellin, aliquots of culture containing a constant number of cells were mixed with the sample loading buffer and heated as above. After SDS-PAGE, the proteins on the gel were transferred onto a nitrocellulose membrane and incubated with a polyclonal rabbit antibody against flagellin. The blot was developed with a Smilight Western blotting kit (Sumitomo).

RESULTS

Construction of gene-disruption mutants in the *fliD* operon

Plasmids pKKD2D, pKKD2S and pKKD2T carry the *kan* gene cassettes inserted into the *fliD*, *fliS* and *fliT* genes, respectively. From these plasmids, the fragments which contain the disrupted genes but not the replication machinery were excised with *Pvu*II and self-ligated. The resulting circular molecules were introduced into a wild-type strain, KK1004, by electroporation, and kanamycin-resistant transformants were selected. The chromosomal DNAs were isolated from these transformants and digested with *Eco*RI and *Eco*RV simultaneously. The digested samples were separated by agarose-gel electrophoresis and analysed by Southern blotting using as a probe the 2574 bp *Eco*RI-*Sal*I fragment from pKKD2 (Figs 1 and 2). The 1830 bp and 3427 bp fragments should be detected in the chromosomal DNA from KK1004. On the other hand, in the chromosomal DNAs whose *fliD*, *fliS* or *fliT* genes were replaced with the disrupted ones from the plasmids, the 1830 bp fragment should be replaced by fragments of various lengths (Fig. 1). Transformants which showed the expected chromosomal structures were saved and used for further experiments. The strains in which the *fliD*, *fliS* and *fliT* genes on the chromosome were specifically disrupted were designated KK1391, KK1392 and KK1393, respectively. According to the same procedure, we constructed strains in which the chromosomal *fliD* operons were replaced with the corresponding DNAs from pKKD2DS, pKKD2ST and pKKD2DST. Their chromosomal structures were also confirmed by Southern blotting (Fig. 2). They were designated KK1394, KK1395 and KK1396, respectively.

Motility of the gene-disruption mutants

Kawagishi *et al.* (1992) reported that all the genes in the *fliD* operons are indispensable for flagellation in *E. coli*. If this was also the case in *S. typhimurium*, we could expect

Fig. 2. Southern blot analysis of the chromosomal DNAs from the wild-type and mutant strains (Fig. 1). Chromosomal DNAs were digested with *Eco*RI and *Eco*RV simultaneously and separated by agarose-gel electrophoresis. Southern blotting was carried out using the 2574 bp *Eco*RI-*Sal*I fragment as a hybridization probe (Fig. 1). The position of the 3427 bp *Eco*RV fragment which should be detected in all the DNA samples is indicated by the arrow on the right. The arrowheads indicate the *Eco*RI-*Eco*RV fragments within which the *kan* gene cassettes were inserted. The positions of the molecular length markers are indicated on the left in bp. Chromosomal DNAs used are as follows. Lanes: 1, KK1004; 2, KK1391; 3, KK1392; 4, KK1393; 5, KK1394; 6, KK1395; 7, KK1396.

... wt *fliD*::Tn10... *fliD* *fliDS* *fliDST*... *fliS* *fliT* *fliST*

Fig. 3. Motile phenotypes of the wild-type and mutant strains. Single colonies formed on the L-broth agar plates were stabbed onto a motility agar plate and incubated for 5 h at 37 °C. Strains used are indicated on the right and as follows: wild-type, KK1004; *fliD*::Tn10 mutant, KK2601; *fliD* mutant, KK1391; *fliDS* mutant, KK1394; *fliDST* mutant, KK1396; *fliS* mutant, KK1392; *fliT* mutant, KK1393; *fliST* mutant, KK1395.

that none of the disruption mutants constructed above were motile. In order to test this possibility, these mutants were examined for motility on the motility agar plates (Fig. 3). To our surprise, the results obtained were highly complicated. After incubation at 37 °C for 5 h, mutants defective in either one of the genes in the operon showed a motile phenotype; that is, they formed swarms on motility agar plates. The *fliD* mutant (KK1391) formed minute swarms, while the *fliT* mutant (KK1393) formed swarms whose size was almost equivalent to, or somewhat smaller than, that of the swarms formed by the wild-type strain (KK1004). The *fliS* mutant (KK1392) formed swarms of an intermediate size. These results indicated that none of these genes are essential for swarm formation on the motility agar plates in *S. typhimurium*. Like the *fliD*::Tn10 mutant (KK2601), the *fliDS* and *fliDST* mutants (KK1394 and KK1396) did not form swarms under these conditions. The *fliST* mutant (KK1395) formed swarms of almost identical size to those formed by the *fliS* mutant.

Flagellation of the *fliD*, *fliS* and *fliT* mutants

In order to examine flagellation with the *fliD*, *fliS* or *fliT* mutants, cells of the individual mutants grown in L broth were negatively stained and observed by electron microscopy. As reported previously (Homma *et al.*, 1984b), the *fliD* mutant (KK1391) did not produce filaments (data not shown). The reason why the *fliD* mutant formed minute swarms on the motility agar plate is discussed later. Interestingly, the *fliS* mutant (KK1392) was found to produce flagella with filaments much shorter than those produced by the wild-type strain (KK1004) (Fig. 4). Distribution of filament length was compared between the wild-type and *fliS*-mutant strains (Fig. 5). In the wild-type cells, the length varied from 1 to 10 wave units with a mean value of 3.5 wave units. On the other hand, in the *fliS* mutant, the maximal length did not exceed 3 wave units and more than 60% of the filaments were shorter than 1 wave unit. Flagellar structures were isolated from the *fliS* mutant and inspected by electron microscopy.

Fig. 4. Electron micrographs of cells of the wild-type (KK1004) (a) and *fliS*-mutant KK1392 (b) strains. Cells grown in L broth were stained negatively with phosphotungstic acid and observed by electron microscopy. Micrographs were taken at a magnification of $\times 8000$. Bar, 1 μ m.

Fig. 5. Distribution of filament length in the wild-type (□), *fliS*-mutant (▨) and *fliT*-mutant (■) strains. The filament length of the individual flagellum was measured using the electron micrographs of cells stained negatively with phosphotungstic acid. Strains used (n = total number of flagella observed): wild-type, KK1004 (n = 180); *fliS* mutant, KK1392 (n = 215); *fliT* mutant, KK1393 (n = 133).

Fig. 6. Electron micrograph of flagellar structures produced by the *fliS* mutant. Flagellar structures were fractionated from the cells of KK1392 by the method of Suzuki *et al.* (1978). The fractionated materials were negatively stained with phosphotungstic acid and observed by electron microscopy. The micrograph was taken at a magnification of $\times 20000$. Bar, 200 nm.

Almost all of the hook-basal-body structures were found to have filament portions (Fig. 6), indicating that the *fliS* mutation affects the elongation process but not the initiation process of filament assembly. Because the *fliT*

mutant constructed above (KK1393) carries a total deletion in the *fliT* gene, it should manifest a null phenotype for the *fliT* gene. Because we could not detect any obvious difference in flagellar structure and in filament length between the wild-type and *fliT*-mutant strains, it is unlikely that FliT has a direct role in flagellar formation and function. Therefore, we did not analyse further the *fliT* mutant in this study.

Complementation analysis

The *fliS* mutant constructed above (KK1392) retains codons 1–71 of the *fliS* gene. Therefore, it might be possible that the small swarm formed by the *fliS* mutant should be attributed to the truncated FliS protein. In order to exclude this possibility, plasmid pKKD4 was introduced by transformation into the *fliDST* mutant (KK1396) in which the entire *fliS* gene has been deleted, and motility recovery of the resulting transformant was examined. Because pKKD4 carried an intact *fliD* gene and only the first eight codons of the *fliS* gene, the transformant was expected to show the null phenotype for the *fliS* gene. The transformant was found to form small swarms on motility agar plates just like the *fliS* mutant (data not shown). Therefore, we conclude that formation of small swarms is the null phenotype for the *fliS* gene.

Excretion of flagellin by the *fliS* mutant

To find out why the *fliS* mutant produces short filaments, we tested the possibility that the *fliS* mutation might affect the export process of flagellin. Export of flagellin was examined in the *fliD*-mutant background because the *fliD* mutation is known to block filament assembly, resulting in unassembled flagellin molecules being excreted into the culture media (Homma *et al.*, 1984a). Culture supernatants from the *fliD* and *fliDS* mutants (KK1391 and KK1394) were concentrated and analysed by SDS-PAGE (Fig. 7a). It was found that the amount of excreted flagellin is much smaller in the *fliDS* mutant than in the *fliD* mutant. In order to exclude the possibility that this difference might be caused by the difference in the amount of flagellin synthesized, total flagellin was analysed by Western blotting with whole cultures from the *fliD* and *fliDS* mutants (Fig. 7b). The amount of flagellin produced by the *fliDS* mutant was found to be almost identical to that produced by the *fliD* mutant. This indicates that the *fliS* mutation does not affect the process of flagellin synthesis. Therefore, we conclude that the *fliS* mutation affects the export process of flagellin.

In order to confirm our conclusion, we examined the *in vitro* reconstitution of filaments onto the hooks with exogenously supplied flagellin monomers under FliS-depletion conditions. Flagellin monomers used were purified either from the filaments produced by the wild-type strain (KK1004) or from those produced by the *fliS* mutant (KK1392). Because exogenously supplied flagellin is known to polymerize onto the hook only in the absence of the FliD protein (Kagawa *et al.*, 1983), we examined the efficiency of reconstruction of filaments onto the hooks of

Fig. 7. Electrophoretic analysis of flagellin produced by the *fliD* and *fliDS* mutants. The arrows indicate the position of flagellin. Strains used: lane 1, *fliD* mutant (KK1391); lane 2, *fliDS* mutant (KK1394). (a) SDS-PAGE of culture supernatants. Proteins in the culture supernatants were precipitated with TCA and separated by SDS-PAGE. The gel was stained with Coomassie brilliant blue. (b) Immunological detection of flagellin in the whole cultures. Aliquots of liquid cultures containing a constant number of cells were separated by SDS-PAGE. The proteins on the gel were transferred onto a nitrocellulose membrane and subjected to Western blot analysis with a polyclonal antibody against flagellin.

Fig. 8. Length distribution of filaments reconstituted onto the hooks of the *fliCD* (▨) and *fliCDS* (■) mutants by exogenously supplied flagellin monomers. The *fliCD* and *fliCDS* mutants were constructed by introducing the *fliC*::Tn10 mutation by P22-mediated transduction from KK2604 to KK1391 and KK1394, respectively. The cells of the resulting mutants were incubated with flagellin monomers prepared from the wild-type strain KK1004. After incubation for 12 h at 26 °C, the cells were negatively stained with phosphotungstic acid and observed by electron microscopy. Total number of reconstituted filaments observed: *fliCD* mutant, 239; *fliCDS* mutant, 202.

the *fliD* mutant. It was found that flagellin monomers from the *fliS* mutant polymerized with the same efficiency as those from the wild-type strain (data not shown). Next,

we examined the reconstruction of filaments onto the hooks of the *fliDS* mutant with the wild-type flagellin monomers (Fig. 7). In this experiment, the *fliC* mutants were used to prevent endogenous flagellin monomers polymerizing onto the hooks. The reconstituted filaments in the *fliCDS* mutant were found to be as long as those in the *fliCD* mutant. These results indicate that the *fliS* mutation does not affect the polymerization efficiency of the flagellin molecule and that the exogenously supplied flagellin can polymerize in the absence of FlhS as efficiently as in the presence of FlhS.

DISCUSSION

In this study, we constructed mutants in which the individual genes in the *fliD* operon on the *S. typhimurium* chromosome were specifically disrupted. Examination of flagellation with these mutants revealed that the *fliS* and *fliT* genes were both dispensable for flagellar formation and function. This result is quite inconsistent with that in *E. coli* reported by Kawagishi *et al.* (1992). They reported that introduction of plasmids carrying the *fliD* gene alone or the *fliDST* regions with the *fliS* and *fliT* genes independently inactivated did not recover the motility of an *E. coli* strain with the chromosomal *fliDST* region deleted. Based on this observation, they concluded that the *fliS* and *fliT* genes are both indispensable for flagellation in *E. coli*. Chen & Helmann (1994) reported evidence suggesting that the *fliS* gene may be also essential for flagellation in *B. subtilis*. Of course, this discrepancy could reflect a species difference among these three organisms. However, because no difference has been reported in gene requirement for flagellation between *S. typhimurium* and *E. coli* (Kutsukake *et al.*, 1980; Macnab, 1992), we anticipate that our result may be true also at least in *E. coli*. We suspect that the inability of the *E. coli* *fliDST* mutant to flagellate even in the presence of a plasmid carrying the *fliD* gene might be due to a multicopy effect of the *fliD* gene.

Although the *fliS* gene is not essential for flagellation, the *fliS* mutant produces much shorter filaments than the wild-type strain. Because almost all of the hook-basal-body structures produced by the *fliS* mutant had filament portions, the *fliS* mutation should affect the elongation step but not the initiation step of filament assembly. So far, several genes have been identified to be involved in the initiation process of filament assembly. They include *flgK*, *flgL*, *fliD* and *flgN* (Homma *et al.*, 1984b; Kutsukake *et al.*, 1994). We believe that *fliS* is the first documented gene whose mutation affects the elongation step of filament assembly. In the *fliD*-mutant background where flagellin molecules cannot assemble onto the hooks resulting in their excretion into the culture medium, the amount of flagellin excreted into the medium was shown to be much reduced by the *fliS* mutation. Because the *fliS* mutation does not affect the synthesis of flagellin, we conclude that the formation of short filaments in the *fliS* mutant is attributed to impaired flagellin transport. Ikeda *et al.* (1993) reported that a *fliD*::Tn10 mutant produces short filaments when supplied exogenously with purified FlhD. This is consistent with our conclusion because the

fliD: :Tn10 mutant is expected to be defective not only in *fliD* but also in *fliS* owing to a polar effect of Tn10 insertion.

Flagellin molecules have been postulated to be exported to the growing tips through the channel residing in the pre-existing flagellar structures (Kuwayama *et al.*, 1989). Namba *et al.* (1989) showed that the filament has a small hole through which flagellin monomers could be transported in an unfolded and stretched conformation. Using temperature-sensitive flagellation mutants of *S. typhimurium*, Vogler *et al.* (1991) identified some candidate genes involved in the export process of flagellin. They include *flhA*, *fliH*, *fliI* and *fliN*. Although their exact roles have remained unknown, it has been postulated that they may constitute the flagellum-specific export apparatus at the cytoplasmic face of the basal body. What is the role of FliS in this export pathway? It has been suggested that FliS may be neither the structural component of flagellar structure nor an integral membrane protein (Kawagishi *et al.*, 1992). Therefore, it may be localized in the cytosol. It is well known that several cytoplasmic chaperones, such as SecB, DnaK, and GroEL, are involved in the general pathway of protein secretion (Kumamoto, 1989; Phillips & Silhavy, 1990). They bind to presecretory proteins to inhibit their folding and to pass them on to the secretion apparatus (Hartl *et al.*, 1990). By analogy with this, we would like to propose a hypothesis that FliS may be a cytoplasmic chaperone specific for flagellin. FliS may bind to nascent flagellin molecules to maintain them in an unfolded state and to target them to the flagellum-specific export apparatus. In order to test this hypothesis, we are currently performing a biochemical analysis of the *fliS* gene product.

In this study, we found that the *fliD* mutant does not produce filaments in liquid medium but does form minute swarms on motility agar plates. It has been discovered that the hook structures formed by the *fliD* mutant do not support the polymerization of endogenously supplied flagellin molecules, but act as the polymerization nuclei for exogenously supplied flagellin molecules (Homma *et al.*, 1986). Because the unassembled flagellin molecules have been shown to be excreted into the medium (Homma *et al.*, 1984a), we suspect that in the motility agar plates the excreted flagellin molecules could not diffuse freely into the media and accumulated around the cells resulting in them being assembled into filaments onto the hooks deficient in the FliD protein. Inability of the *fliD*: :Tn10, *fliDS* and *fliDST* mutants to form swarms on motility agar plates might reflect the impaired excretion of flagellin into the medium owing to the *fliS* deficiency. Consistent with this, prolonged incubation or supplement of flagellin monomers into the motility agar plates could facilitate swarm formation by these mutants (our unpublished results).

Because a *fliD*: :Tn10 mutation causes over-expression of the flagellar late operons, the *fliD* operon has been postulated to contain a negative regulator gene, *rflA* (Kutsukake *et al.*, 1990). The expression of the flagellar late operons is negatively controlled by the flagellum-specific anti-sigma factor, FlgM (Ohnishi *et al.*, 1992),

whose intracellular activity is regulated by being excreted through the flagellar structure (Hughes *et al.*, 1993; Kutsukake, 1994). We showed that the secretion of FlgM is enhanced by the *fliD*: :Tn10 mutation and proposed that the *rflA* function of the *fliD* operon may be attributed to the flagellar cap protein encoded by the *fliD* gene (Kutsukake, 1994). However, at present, we cannot exclude the possibility that the *fliS* or *fliT* gene may correspond to the *rflA* gene. This problem will be solved by analysing the expression levels of the late operons and excretion levels of FlgM in the defined mutants in the *fliD* operon constructed in the present study.

The flagellar regulon of *S. typhimurium* includes more than 50 genes (Kutsukake *et al.*, 1990). Among them, three genes, *fliL*, *flhE* and *fliT*, have been reported to be totally dispensable for flagellar formation and function (Raha *et al.*, 1994; Minamino *et al.*, 1994; this study). At present, we have no idea of the roles of these genes. More careful examination of the structure and function of flagella with the corresponding mutants may be required for understanding their roles in flagellar morphogenesis and function.

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