Structure and expression of the *fliA* operon of *Salmonella typhimurium*

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The *fliA* gene encodes the flagellum-specific sigma factor σ^{28} in *Salmonella typhimurium*. The transcription *in vivo* and *in vitro* of this gene was analysed and it was found that there are two promoters for the expression of this gene. One is a class 2 promoter which is recognized by σ^{70} -RNA polymerase in the presence of the FlhD and FlhC activator proteins. The other is a class 3 promoter which is recognized by σ^{28} -RNA polymerase. Therefore, the *fliA* operon is under dual positive control from FlhD/FlhC and from FliA itself. The nucleotide sequence downstream of the *fliA* gene was determined. The sequence contains two ORFs following the *fliA* gene. On the basis of their sequence homology, it is concluded that these two correspond to the *fliZ* and *fliY* genes of *Escherichia coli*. Northern blot analysis revealed that the *fliZ* gene is transcribed from the *fliA* promoters, whereas the *fliY* gene is transcribed from both the *fliA* promoters and its own FlhD/FlhC-independent promoter. A *fliZ*-disruption mutant was constructed by inserting a kanamycin-resistance gene cassette into the *fliZ* gene on the chromosome. The mutant showed poor motility, and introduction of a *fliZ*⁺ plasmid into this mutant restored the wild-type level of motility. These results suggest that the *fliZ* gene may be required for expression of maximal motility.

Keywords: *Salmonella*, flagellum-specific sigma factor, primer extension, *in vitro* transcription, gene disruption

INTRODUCTION

The flagellum of *Salmonella typhimurium* is composed of three structural parts, a basal body, a hook and a filament, and is constructed in this order (Macnab, 1996). More than 50 genes are required for flagellar formation and function. These flagellar genes constitute at least 14 different operons, and most of them are clustered in four regions on the chromosome (Kutsukake *et al.*, 1988; Macnab, 1996). Transcription of these operons forms a highly organized cascade called the flagellar regulon, and is coordinated with the flagellar assembly hierarchy (Kutsukake *et al.*, 1990; Kutsukake & Iino, 1994). According to the relative positions in the transcriptional hierarchy, the flagellar operons are grouped into three classes, 1, 2 and 3. Class 1 contains only one operon, *flhD*, consisting of two genes, *flhD* and

flhC, whose products are both required for class 2 expression. Class 2 contains the operons responsible for formation of the flagellar basal structure, the hook-basal body complex. Class 2 also contains the *fliA* gene, which encodes an alternative sigma factor, σ^{28} , needed for class 3 expression (Ohnishi *et al.*, 1990). Class 3 contains operons responsible for filament formation, flagellar rotation and chemotaxis. FliA-dependent expression of class 3 is under negative control from an anti-sigma factor, FlgM (Gillen & Hughes, 1991; Ohnishi *et al.*, 1992; Kutsukake & Iino, 1994; Kutsukake *et al.*, 1994b; Iyoda & Kutsukake, 1995; Chadsey *et al.*, 1998). The *flgM* gene is expressed from both class 2 and class 3 promoters (Gillen & Hughes, 1993; Kutsukake, 1994; Kutsukake *et al.*, 1994a). In addition to class 3 expression, the FliA-FlgM regulatory system is known to be involved in regulation of class 1 and class 2 expression (Kutsukake & Iino, 1994; Kutsukake, 1997b). However, a molecular mechanism underlying this control has remained unknown.

Escherichia coli contains a similar number of flagellar genes, and their expression is organized in a similar

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Table 1. Bacterial strains and plasmids used

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
<i>Escherichia coli</i>		
BL21(DE3)	T7 expression host	Studier & Moffatt (1986)
EKK22	<i>hsd flhD</i>	Ohnishi <i>et al.</i> (1990)
<i>Salmonella typhimurium</i>		
KK1004	Wild-type	Kutsukake <i>et al.</i> (1988)
KK1397	<i>fliZ::kan</i>	This study*
KK2040	<i>flhD::Tn10</i>	Kutsukake <i>et al.</i> (1988)
KK2091	<i>fliA::Tn10</i>	Kutsukake <i>et al.</i> (1988)
Plasmids		
pACYC184	Cloning vector, Cm ^r Tc ^r	Chang & Cohen (1978)
pBR322	Cloning vector, Ap ^r Tc ^r	Bolivar <i>et al.</i> (1977)
pET17b	T7 expression vector, Ap ^r	Novagen
pSTV29	Cloning vector, Cm ^r	Takara
pTrc97A	<i>tac</i> expression vector, Ap ^r	Amann <i>et al.</i> (1988)
pTrc99C	<i>tac</i> expression vector, Ap ^r <i>lacI^q</i>	Amann <i>et al.</i> (1988)
pUC4K	<i>kan</i> gene cassette, Ap ^r	Pharmacia
pUC19	Cloning vector, Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pKK1059	pBR322 <i>fliAZY</i> ⁺	Ohnishi <i>et al.</i> (1990)†
pKK1064	pBR322 <i>fliA</i> ⁺	Ohnishi <i>et al.</i> (1990)†
pSIIA1	pTrc99C <i>fliA</i> ⁺	Kutsukake <i>et al.</i> (1994b)†
pSIIA100	pTrc97A $\Delta(tac)$ p _{<i>fliA</i>} -t _{<i>rrnB</i>}	S. Iyoda, unpublished†
pSIHD1	pET17b <i>flhD</i> ⁺	S. Iyoda, unpublished
pSIHC2	pACYC184 <i>flhC</i> ⁺	S. Iyoda, unpublished
pBRIA2	pBR322 p _{<i>fliA</i>}	This study†
pUCIZ1	pUC19 <i>fliZ</i> '	This study*
pKKIZ2	pUCIZ1 <i>fliZ</i> ' (PvuII) 'f <i>liZ</i>	This study*
pKKIZ3	pUCIZ2 <i>fliZ::kan</i>	This study*†
pTHI22	pSTV29 <i>fliZ</i> ⁺	This study†

* Construction procedures are described in detail in Methods.

† *S. typhimurium* chromosomal regions carried by these plasmids are shown in Fig. 1(b).

cascade fashion (Komeda *et al.*, 1980; Komeda, 1982, 1986). The FlhD and FlhC proteins of *E. coli* were purified and shown to act as the activators for σ^{70} -dependent expression of the class 2 operons (Liu & Matsumura, 1994). Like FliA of *S. typhimurium*, FliA of *E. coli* was shown to be an alternative sigma factor specific for flagellar operons (Liu & Matsumura, 1995). Mytelka & Chamberlin (1996) reported that the *fliA* gene constitutes an operon together with two genes, *fliZ* and *fliY*, in *E. coli*. They postulated that these two genes may not be essential for motility but have a regulatory role. Expression of the *fliA* operon of *E. coli* was characterized *in vivo* and *in vitro*, and was shown to be under dual control from class 2 and class 3 promoters (Mytelka & Chamberlin, 1996; Liu & Matsumura, 1996). Therefore, the *fliA* operon of *E. coli* is transcribed both by σ^{70} -RNA polymerase in the presence of FlhD and FlhC and by σ^{28} -RNA polymerase.

Though the *fliA* gene product has been characterized extensively in *S. typhimurium* (Ohnishi *et al.*, 1990; Kutsukake *et al.*, 1994b), the entire structure and

expression of the *fliA* operon have been less well-characterized in this organism. In the present work, we analysed the DNA sequence and transcriptional control of the *fliA* operon of *S. typhimurium*. As in *E. coli*, the *fliA* operon was found to contain two additional genes, *fliZ* and *fliY*, and to be transcribed from both the class 2 and class 3 promoters. However, we obtained evidence suggesting that the *fliY* gene is also transcribed from its own non-flagellar promoter. A disruption mutant was constructed by inserting a kanamycin-resistance gene cassette into the *fliZ* gene on the chromosome. The mutant showed poor motility, and maximal motility was recovered by introducing a *fliZ*⁺ plasmid into this mutant. This suggests that the *fliZ* gene, though not essential, is required for expression of maximal motility.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in the present study are listed in Table 1. Ordinary culture media including L broth, L-agar plates, motility agar plates and minimal medium were prepared as

described previously (Kutsukake, 1997a). Ampicillin, chloramphenicol and kanamycin were used at final concentrations of 50, 25 and 25 $\mu\text{g ml}^{-1}$, respectively. In order to induce genes fused to the *tac* promoter on the expression plasmids, IPTG was added to a final concentration of 1 mM.

Oligonucleotide primers, chemicals and enzymes. Customized primers were purchased from Life Technologies, Kurabo or Pharmacia. Unless otherwise specified, all the chemicals were purchased from Nacalai Tesque. *E. coli* RNA polymerase core enzyme and holoenzyme were purchased from Epicentre Technologies and Boehringer Mannheim, respectively. *Taq* DNA polymerase and Moloney murine leukaemia virus reverse transcriptase were purchased from Promega and Amersham, respectively. Other DNA-modifying enzymes were purchased from Toyobo or Nippon Gene.

Protein purification. The *S. typhimurium* FlhA protein formed inclusion bodies in the IPTG-treated cells of EKK22 harbouring pSIIA1. From these inclusion bodies, FlhA was purified according to the purification procedure for *E. coli* σ^{70} developed by Igarashi & Ishihama (1991). The *S. typhimurium* FlhD/FlhC complex was purified from the soluble fraction of the IPTG-treated cells of BL21(DE3) carrying both pSIHD1 and pSIHC2 according to the purification procedure for the *E. coli* FlhD/FlhC complex described by Liu & Matsumura (1994).

Primer extension analysis. Total RNA was isolated from *S. typhimurium* cells with a TRIzol reagent (Gibco-BRL) according to the manufacturer's instructions. The primer used for detection of the *flhA* transcripts was IAP1 (GAGTGT-TATCCATTACAC). Radiolabelling of the primer and the primer extension reaction were carried out according to the procedures described previously (Kutsukake & Ide, 1995). The extension products were separated on a DNA sequencing gel alongside sequencing ladders of the *flhA* promoter region made using the same primer.

In vitro transcription. *In vitro* RNA synthesis was performed using *E. coli* RNA polymerase according to the method described by Kajitani & Ishihama (1983) with the following modifications. The reaction mixture contained 50 mM Tris/HCl, pH 7.8, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 25 $\mu\text{g BSA ml}^{-1}$, 3 mM magnesium acetate, 150 μM each ATP, GTP and CTP, 50 μM UTP with 10 μCi (3.7×10^5 Bq) [α - ^{32}P]UTP (ICN), 1 μg template DNA and 1.5 μg RNA polymerase. Mixtures without substrates were prepared in a 35 μl volume and incubated at 37 °C for 30 min. The reaction was started by adding 15 μl prewarmed substrate mixture. After incubation at 37 °C for 10 min, the reaction was stopped by adding 50 μl stop solution (0.6 M sodium acetate, pH 5.5, 20 mM EDTA, 200 $\mu\text{g tRNA ml}^{-1}$). The transcripts were extracted with phenol and precipitated with ethanol. The precipitated materials were dissolved in 20 μl loading solution (8 M urea, 0.025 % bromophenol blue, 0.025 % xylene cyanol) and electrophoretically separated in a 4 % polyacrylamide gel containing 8 M urea. The transcript bands were visualized by autoradiography on an X-ray film.

DNA sequencing and PCR. DNA sequences were determined by the dideoxy chain-termination method with an automated DNA sequencer model ABI 373S (Perkin Elmer) using various synthetic primers. Sequence data were analysed by use of the software GENETYX (Software Development). PCR was performed using *Taq* DNA polymerase according to the manufacturer's instructions.

Northern blot hybridization. Total RNA was isolated from *S. typhimurium* cells as described above, and 20 μg of each

Fig. 1. Structure and transcriptional organization of the *flhA* operon of *S. typhimurium* (a) and the chromosomal regions cloned on the recombinant plasmids (b). Only restriction sites used in the present study are shown: Bm, *Bam*HI; Ei, *Eco*RI; Ev, *Eco*RV; Hd, *Hind*III; Kp, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Sl, *Sal*I. The probes used in the Northern blotting analysis are indicated by hatched bars. P1, P2 and P3 indicate the transcription start sites, and wavy arrows represent RNA transcripts. t, Possible transcription-termination site; *kan*, *kan* gene cassette; *p_{tac}* and *t_{rrnB}*, *tac* promoter and the *rrnB* terminator, respectively.

sample was separated electrophoretically on an agarose gel. DNA probes used were probes A, Z and Y, which are internal to the *flhA*, *flhZ* and *flhY* genes, respectively (Fig. 1). Probes A and Y were made by excising the 0.6 kb *Eco*RI–*Sac*I and 0.4 kb *Kpn*I–*Eco*RV fragments from pKK1059, respectively. Probe Z was prepared by excising the 0.4 kb *Bam*HI–*Pst*I fragment from the amplified product by PCR using primers IZP1 (GGGGATCCACGGTGCAGCAACCTAAAAG) and IYP3 (GGGAATTCATAGTTAGTCCCCAGCCCCAA) and pKK1059 as a template. These probes were radiolabelled with a *Bca*BEST labelling kit (Takara) and [α - ^{32}P]dCTP (ICN) according to the manufacturer's instructions. RNA blotting and hybridization were performed according to the method of Baga *et al.* (1985). Radioactive bands were visualized using a BAS2000 image analyser (Fuji).

Construction of a *flhZ*-disruption mutant. The 0.84 kb *Pst*I–*Pvu*II fragment excised from pKK1064 was inserted into pUC19 at the *Pst*I/*Hinc*II site to yield pUCIZ1. The 0.79 kb *Pvu*II–*Kpn*I fragment excised from pKK1059 was inserted into pUCIZ1 at the *Sma*I/*Kpn*I site to yield pKKIZ2. The kanamycin-resistance gene cassette (*kan*) excised from pUC4K with *Bam*HI was inserted into pKKIZ2 at the *Bam*HI site to yield pKKIZ3. This plasmid carries the *kan* gene inserted into the *flhZ* gene. The chromosomal *flhZ* gene of a wild-type strain, KK1004, was replaced with this *flhZ::kan* allele according to the method described previously (Kutsukake, 1994; Kutsukake *et al.*, 1994a). The structure of the chromosomal *flhZ* gene was examined by Southern blot analysis with a DIG DNA labelling and detection kit (Boehringer Mannheim). One

clone with the desired structure was named KK1397 and used in this study.

RESULTS AND DISCUSSION

Primer extension analysis of the *fliA* gene

In order to identify the promoters of the *fliA* gene, primer-extension-mediated mapping of the transcriptional start sites was carried out. RNAs were isolated from KK1004 (wild-type), KK2040 (*flhD*::Tn10) and KK2091 (*fliA*::Tn10) harbouring pBRIA2, which carries the promoter region of the *fliA* gene. In the RNA sample from KK1004, two positive signals were observed (Fig. 2). They map to adenine nucleotides positioned 29 and 18 bases upstream of the initiation codon of the *fliA* gene. These start sites were designated P1 and P2, respectively.

The P1 signal was absent in the RNA sample from KK2040 but present in that from KK2091. This indicates that the P1 signal is dependent on FlhD/FlhC but not on FliA. Therefore, we conclude that P1 is a start site from a class 2 promoter. The sequence around the -10 region of P1 resembles the -10 consensus sequence of the σ^{70} -dependent promoter, but a sequence corresponding to the -35 region is not evident. Instead, the sequence TTATTCC, which is conserved in the upstream regions of the class 2 promoters of *S. typhimurium* (Kutsukake & Ide, 1995), is present at the region centred 42 bp upstream of P1. The DNA sequence to which the FlhD/FlhC complex binds was reported in the *E. coli fliA* promoter (Liu & Matsumura, 1994). It corresponds to the DNA region 30–80 bp upstream of P1. Within this region, the sequence AAATAACCC, centred 63 bp upstream of P1, is exactly conserved between *E. coli* and *S. typhimurium*. This suggests that these nine nucleotides may be responsible for FlhD/FlhC binding.

The P2 signal was absent in both RNA samples from KK2040 and KK2091. This indicates that P2 is dependent on both FlhD/FlhC and FliA. Because the *fliA* gene is under positive control of FlhD/FlhC (Kutsukake *et al.*, 1990), we conclude that P2 is a start site from a class 3 promoter. Consistent with this, both the -10 and -35 regions of P2 are homologous to the -10 and -35 consensus sequences of the class 3 promoter (Arnosi & Chamberlin, 1989; Kutsukake *et al.*, 1990). These results agree with our previous observation that the *fliA* gene is under positive control of its own product (Kutsukake & Iino, 1994).

In vitro transcription of the *fliA* gene

In order to confirm that the promoters identified above are actually class 2 and class 3 promoters, *in vitro* transcription analysis of the *fliA* gene was performed using purified transcription factors (Fig. 3). The DNA template used was a supercoiled DNA of pSIIA100, in which the *fliA* promoter region is located about 350 bp upstream of the terminator sequence of the *rrnB* operon. When core enzyme or σ^{70} -holoenzyme was used without other transcription factors, no transcription occurred.

Fig. 2. Transcriptional mapping of the *fliA* operon by primer extension analysis (a) and the structure of the *fliA* promoter region (b). (a) RNAs were isolated from KK1004 (lane 1), KK2040 (lane 2) and KK2091 (lane 3). The transcription start sites were identified by primer extension with primer IAP1. The sequencing ladder (lanes G, A, T and C) was made with the same primer. P1 and P2 indicate the class 2 and class 3 transcription start sites, respectively. (b) The sequence complementary to the primer used is indicated by a broken line. The P1 start site is labelled as +1. The -10 region of P1 is underlined, while the -10 and -35 regions of P2 are doubly underlined. The sequences conserved in other class 2 promoters of *S. typhimurium* and in the *E. coli fliA* promoter are indicated by wavy lines. Sequence data were taken from Ohnishi *et al.* (1990).

When σ^{70} -holoenzyme was used with FlhD/FlhC, one transcript of about 350 nucleotides was produced. This is a class 2 transcript and must correspond to the P1 transcript. When core enzyme or σ^{70} -holoenzyme was used with FliA, one slightly shorter transcript was produced. This is a class 3 transcript and must correspond to the P2 transcript. These were further confirmed by determining the transcription start sites of the *in vitro* transcripts by primer extension analysis (data not shown).

Fig. 3. *In vitro* transcription of the *fliA* operon. The DNA template used was a supercoiled DNA of pSIIA100, which carries the class 2 and class 3 *fliA* promoters located about 350 bp upstream of the *rrnB* terminator on pTrc97A (Fig. 1b). Transcription factors added in the reaction mixture are indicated above each lane: core, RNA polymerase core enzyme; σ^{70} -holo, σ^{70} -containing RNA polymerase holoenzyme; FliA, FliA protein; FlhD/FlhC, FlhD/FlhC complex. All the reactions were carried out using 1.5 μ g *E. coli* RNA polymerase. When required, 40 pmol of the FliA protein or the FlhD/FlhC complex was added to the reaction mixture, except for lane 7. In the reaction mixture of lane 7, 10 pmol FliA and 40 pmol FlhD/FlhC were added simultaneously. Open and filled triangles indicate the class 2 and class 3 transcripts, respectively. Positions of RNA size markers are indicated in nucleotides on the left.

Sequence analysis of the region downstream of the *fliA* gene

Plasmid pKK1059 contains the *fliA* gene and its adjacent region of the *S. typhimurium* chromosome (Fig. 1). Ohnishi *et al.* (1990) reported the DNA sequence of the 1.4 kb *EcoRV*–*PvuII* fragment of this plasmid. The reported sequence contains the *fliA* gene and its downstream region of 0.2 kb. Examination of this downstream sequence revealed that it contains the 5' portion of an ORF (*orf1*) which starts from either of the ATG codons 8 bp and 59 bp downstream of the termination

codon of the *fliA* gene and extends to the *PvuII* site. Because there is no potential transcription-termination signal in the intergenic region, *orf1* is likely to be transcribed together with *fliA*.

In order to know the entire structure of the *fliA* operon, we determined the sequence of the *PvuII*–*BamHI* fragment of pKK1059 (accession no. AB010947). It was found that *orf1* is followed by two additional ORFs, *orf2* and *orf3*. *orf2* starts from either of the ATG codons 99 bp and 147 bp downstream of the termination codon of *orf1*. The intergenic region between *orf1* and *orf2* contains sequences similar to the –35 and –10 consensus sequences of the σ^{70} -dependent promoter, suggesting that *orf2* may be transcribed by σ^{70} -RNA polymerase. Two pairs of inverted repeats which may function as the transcriptional terminators are present in the downstream region of *orf2*. *orf3* starts from the ATG codon 154 bp downstream of the termination codon of *orf2* and extends to the *BamHI* site.

As expected, the determined sequence is homologous to the previously sequenced *E. coli* *fliAZY* locus (Mytelka & Chamberlin, 1996; Itoh *et al.*, 1996). The deduced amino acid sequences of Orf1, Orf2 and Orf3 show homology to FliZ, FliY and YedO of *E. coli*, respectively. In all cases, they are more than 80% identical in amino acid sequence in these two bacteria. Therefore, we conclude that *orf1*, *orf2* and *orf3* are the *S. typhimurium* counterparts of the *fliZ*, *fliY* and *yedO* genes, respectively (Fig. 1). In the *fliZ* and *fliY* genes, deduced amino acids between the first and second ATG codons are very diverse both in number and in sequence between *E. coli* and *S. typhimurium*. This suggests that the second ATG codons are more plausible translation start sites in these two genes. Consistent with this, the second ATG codon of the *fliZ* gene has a much better match for the ribosome-binding site. If this inference is correct, *fliZ* and *fliY* should encode proteins of 183 and 266 amino acids, respectively, in both bacteria.

Northern blot analysis of the *fliA* operon

Transcriptional organization of the *fliAZY* locus of *S. typhimurium* was examined by Northern blot analysis using probes A, Z and Y, which are internal to the *fliA*, *fliZ* and *fliY* genes, respectively (Fig. 4). RNAs used were isolated from KK1004 (wild-type) and KK2040 (*flhD::Tn10*). When probe A or probe Z was used, two major transcripts of about 2300 and 1400 nucleotides

Fig. 4. Northern blot analysis of the *fliA*, *fliZ* and *fliY* genes. RNAs were isolated from KK1004 (lane 1) and KK2040 (lane 2). Probes used were probe A (a), probe Z (b) and probe Y (c). Molecular sizes (in nucleotides) are indicated on the left.

Fig. 5. Motility phenotype of the *fliZ*::*kan* mutant. (a) Single colonies of KK1004 (left) and KK1397 (right) were stabbed onto a motility agar plate and incubated for 5 h at 30 °C. (b) Single colonies of KK1004 harbouring pSTV29 (top) and KK1397 harbouring pSTV29 (bottom, left) or pTII22 (bottom, right) were stabbed onto a motility agar plate containing chloramphenicol and incubated as above.

were detected in RNAs from KK1004. Because neither of these transcripts was detected in RNAs from KK2040, these must be class 2 and/or class 3 transcripts. When probe Y was used, two major transcripts of about 2300 and 900 nucleotides were detected in RNAs from KK1004. In RNAs from KK2040, the longer transcript was not observed, while the shorter one was still detected. Therefore, the 900-nucleotide transcript is not under control of the flagellar regulon. Together with the data from the transcription start site analysis described above, we conclude that the 2300- and 1400-nucleotide transcripts correspond to the cotranscription products of the *fliA*, *fliZ* and *fliY* genes and the *fliA* and *fliZ* genes, respectively. These are likely to start from the *fliA* promoters identified above. The signal of the 2300-nucleotide transcript was much weaker than that of the 1400-nucleotide transcript, indicating that most of the

transcripts of the *fliA* operon terminate between the *fliZ* and *fliY* genes. On the other hand, the 900-nucleotide transcript may correspond to a single cistron transcript of the *fliY* gene, which is likely to start from the σ^{70} -type promoter residing in the intergenic region between *fliZ* and *fliY*. Therefore, the *fliY* gene may be transcribed both from its own non-flagellar promoter and from the flagellar promoters of the *fliA* operon. Transcription presumably terminates at the potential termination signals in the intergenic region between *fliY* and *yedO*. Therefore, the *yedO* gene does not belong to the *fliA* operon.

Disruption of the *fliZ* gene

The results described above indicate that at least the *fliZ* gene is under control of the flagellar regulon. This suggests that FliZ may play a role in flagellar formation or function. Motility of the cells of the polar, Tn10-insertion *fliA* mutant KK2091 was restored by introduction of pKK1064, which carries the *fliA* gene but not the *fliZ* gene (data not shown). This indicates that the *fliZ* gene is not essential for motility development. Next, we constructed a *fliZ*-disruption mutant of *S. typhimurium* according to the procedures described in Methods. The obtained mutant, KK1397, formed smaller swarms than the wild-type strain KK1004 on a motility agar plate at 30 °C (Fig. 5). This effect was reversed by introducing pTII22, which carries the *fliZ* gene but not the *fliY* gene (Fig. 5). These results indicate that FliZ is required for expression of maximal motility in *S. typhimurium*.

In our earlier studies on the flagellar regulon of *S. typhimurium* (Kutsukake *et al.*, 1990; Kutsukake & Iino, 1994; Kutsukake, 1997b), we used exclusively strain KK2091 (*fliA*::Tn10) as a *fliA* mutant. In this study, we show that the *fliA* operon includes the *fliZ* gene. This indicates that the *fliZ* gene is not expressed efficiently in KK2091 owing to a polar effect of the Tn10 insertion. Because FliZ is likely to play a role in flagellar formation or function, it is possible that at least one or some of the observed effects of the *fliA*::Tn10 mutation could be attributed to a lowered expression of the *fliZ* gene. Experiments are now in progress to test this possibility.

Function of the *fliY* and *yedO* genes

Mytelka & Chamberlin (1996) reported that a *fliZY*-deletion mutant of *E. coli* showed weak motility. We show here that the *fliZ* mutant which carries an intact *fliY* gene also exhibited weak motility. This suggests that the impaired motility of the *E. coli fliZY*-deletion mutant may be caused by a defect of the *fliZ* gene. Based on the sequence homology of FliY with extracellular solute-binding proteins, Mytelka & Chamberlin (1996) proposed a hypothesis that *E. coli* FliY may modulate motility of the cell through binding a certain amino acid or amino acid analogue in the periplasmic space. We show here that the *S. typhimurium fliY* gene is transcribed from a non-flagellar promoter immediately up-

stream of the gene. This raises the possibility that the *fliY* gene may not be a member of the flagellar genes. Of course, we cannot exclude the possibility that this gene belongs to the flagellar genes, because the Northern blot analysis with probe Y (Fig. 4) suggests that the *fliA* flagellar and *fliY* non-flagellar promoters may contribute almost equally to *fliY* transcription in the wild-type cells. In order to test these possibilities, we are currently attempting to construct a *fliY*-disruption mutant.

According to the *E. coli* genome project (Itoh *et al.*, 1996), *yedO* is registered as a gene encoding a protein homologous to 1-aminocyclopropane-1-carboxylate (ACC) deaminase of *Pseudomonas*. This enzyme is involved in degradation of ACC, a precursor of the phytohormone ethylene (Campbell & Thomson, 1996), which is unlikely to be related to flagellar formation and function. The upstream region of the *yedO* gene does not contain sequences homologous to any flagellum-related promoters. These facts suggest that this gene may not be a member of the flagellar genes. At present, the function and expression of the *yedO* gene remain unknown.

Concluding remarks

Through *in vivo* and *in vitro* studies on the transcription of the *fliA* operon of *S. typhimurium*, we showed that this operon is under dual positive control from FlhD/FlhC and from FliA. Nucleotide sequence and Northern blot analyses revealed that the *fliA* operon includes two additional genes, *fliZ* and *fliY*, though the *fliY* gene is also transcribed from its own non-flagellar promoter. A *fliZ*-disruption mutant showed poor motility, suggesting that this gene, though not essential, is required for expression of maximal motility.

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