Structure and expression of the fliA operon of Salmonella typhimurium

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The fliA gene encodes the flagellum-specific sigma factor $\sigma^{28}$ 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 $\sigma^{70}$-RNA polymerase in the presence of the FlhD and FlhC activator proteins. The other is a class 3 promoter which is recognized by $\sigma^{28}$-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 flbc, 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, $\sigma^{28}$, 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; Chadey et al., 1998). The fliG 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...
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 σ70-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 σ70-RNA polymerase in the presence of FlhD and FlhC and by σ28-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 analyzed 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 µg 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. *Tag* 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 pSIA1. From these inclusion bodies, FlhA was purified according to the purification procedure for *E. coli* α7 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 pSISH1 and pSICH2 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 flA transcripts was IAP1 (GAGTGGTT-TATCCATTACAC). Radiolabelling of the primer and the primer extension reaction were carried out according to the procedures described previously (Kutsukake & Ide, 1993). The extension products were separated on a DNA sequencing gel alongside sequencing ladders of the flA 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 30 mM Tris/ HCl, pH 7.8, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 25 µg BSA ml⁻¹, 3 mM magnesium acetate, 150 µM each ATP, GTP and CTP, 50 µM UTP with 10 µCi (3.7 x 10⁸ Bq) [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 µg 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 dyeodeoxy 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 *Tag* 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 sample was separated electrophoretically on an agarose gel. DNA probes used were probes A, Z and Y, which are internal to the flA, flIZ and flY genes, respectively (Fig. 1). Probes A and Y were made by excising the 0.6 kb EcoRI-Sacl and 0.4 kb KpnI-EcoRV fragments from pKK1059, respectively. Probe Z was prepared by excising the 0.4 kb BamHI-PstI fragment from the amplified product by PCR using primers IZP1 (GGGATCCCGTGTCGCCAACCCTAAAG) and IYP3 (GGGATCTAGTATGCTCCACCGCA) and pKK1059 as a template. These probes were radiolabelled with a BcaBEST labelling kit (Takara) and [α-³²P]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 flZ-disruption mutant.** The 0.84 kb PstI-PvuII fragment excised from pKK1064 was inserted into pUC19 at the PstI/HincII site to yield pUCIZ1. The 0.79 kb PvuII-KpnI fragment excised from pKK1059 was inserted into pUCIZ1 at the Smal/KpnI site to yield pKKIZ2. The kanamycin-resistance gene cassette (kan) excised from pUC4K with BamHI was inserted into pKKIZ2 at the BamHI site to yield pKKIZ3. This plasmid carries the kan gene inserted into the flIZ gene. The chromosomal flIZ gene of a wild-type strain, KK1004, was replaced with this flIZ::kan allele according to the method described previously (Kutsukake et al., 1994). The structure of the chromosomal flIZ 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 σ70-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 & Iide, 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 AAATACCC, 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 (Arnosti & 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 pSHA100, in which the fliA promoter region is located about 350 bp upstream of the terminator sequence of the rrvB operon. When core enzyme or σ70-holoenzyme was used without other transcription factors, no transcription occurred. When σ70-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 σ70-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).
Salmonella fliA operon

The template used was a supercoiled DNA of pS11A100, which carries the class 2 and class 3 fliA promoters located about 350 bp upstream of the rnaB terminator on pTrc97A (Fig. 1b). Transcription factors added in the reaction mixture are indicated above each lane: core, RNA polymerase core enzyme; $\sigma^{70}$-holo, $\sigma^{70}$-containing RNA polymerase holoenzyme; FliA, FliA protein; FlhD/FlhC, FlhD/FlhC complex. All the reactions were carried out using 1.5 $\mu$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 markers are indicated in nucleotides on the left.

**Fig. 3.** In vitro transcription of the fliA operon. The DNA template used was a supercoiled DNA of pS11A100, which carries the class 2 and class 3 fliA promoters located about 350 bp upstream of the rnaB terminator on pTrc97A (Fig. 1b). Transcription factors added in the reaction mixture are indicated above each lane: core, RNA polymerase core enzyme; $\sigma^{70}$-holo, $\sigma^{70}$-containing RNA polymerase holoenzyme; FliA, FliA protein; FlhD/FlhC, FlhD/FlhC complex. All the reactions were carried out using 1.5 $\mu$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 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 $\sigma^{70}$-dependent promoter, suggesting that orf2 may be transcribed by $\sigma^{70}$-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 ($fliD::Tn10$). When probe A or probe Z was used, two major transcripts of about 2300 and 1400 nucleotides...
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 σ70-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 pTllZ2, 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, fiiZ 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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