Microbiol. Immunol., **46**(1), 1−10, 2002

FimZ Binds the *Salmonella typhimurium fimA* Promoter Region and May Regulate Its Own Expression with FimY

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Received June 18, 2001; in revised form, October 10, 2001. Accepted October 15, 2001

***Abstract*: The FimZ protein, an activator of FimA production in *Salmonella typhimurium*, acts in conjunction with FimY to facilitate the expression of type 1 fimbriae. The predicted amino acid sequence of FimZ suggests that this protein may be a DNA-binding protein related to BvgA, a sensory regulator of virulence gene expression in *Bordetella pertussis*. Purification of FimZ following overexpression of the protein by a strong inducible promoter and gel mobility shift assays confirm that FimZ is a 25-kDa polypeptide that binds to the promoter region of *fimA*. The region of DNA protected from DNase I digestion by FimZ binding is located between 47 and 98 nucleotides upstream from the *fimA* transcription initiation site. This region possesses a pair of 7-base pair tandem repeats, of which at least one is necessary for FimZ binding. One copy of the 7-base pair sequence is also located in the *fimZ* promoter region. In addition, expression from a *fimZ-lacZ* reporter construct confirms that FimZ plays a role in its own expression. Both FimZ and FimY are required for high-level expression of FimZ, which suggests that these two fimbrial proteins are involved in regulating both FimA and FimZ.**

***Key words*: *Salmonella typhimurium*, Type 1 fimbriae, Activator**

The *Salmonella typhimurium fim* gene cluster encodes the expression of the mannose-sensitive type 1 fimbriae that mediate adherence to a range of eucaryotic cells and tissues (3, 9, 11, 19). The complete nucleotide sequence of the gene cluster has been determined and comprises a number of genes that are responsible for the ordered assembly and binding specificity of the fimbrial appendages on the surface of the bacteria (9). Most of these genes code for proteins that are believed to exhibit functions related to those reported in *Escherichia coli* fimbrial and pilus systems (12, 17, 18, 21). However, the regulation of the *S. typhimurium* major fimbrial subunit gene (*fimA*) is controlled by a mechanism different to that reported for the *E. coli fimA* gene. Genes homologous to the *E. coli fimB* and *fimE* determinants that control the inversion of the *fimA* promoter region are not found within the *S. typhimurium fim* gene cluster (6, 7, 13, 24). Also, the expression of an *S. typhimurium fimA-lacZ* reporter using *E. coli* as a host is not observed even under conditions that result in increased expression of the *E. coli fimA* gene. However, in the *E. coli* host, the *S. typhimurium fimA* gene is expressed in the presence of two *Salmonella* proteins, FimZ and FimY (33, 40).

The predicted amino acid sequence of FimZ relates to a family of transcriptional activators that are sensory regulators of two-component systems (10, 32). However, examining the *fim* gene cluster does not indicate the presence of a gene product that demonstrates homology to sensory kinases of these systems. Consistent with the role of FimZ as an activator of *S. typhimurium fimA* expression, *fimZ* mutants are non-fimbriate and exhibit only low levels of *fimA* expression (40). Restoration of FimA production and the fimbriate phenotype can be achieved by introducing plasmids that carry only the *fimZ* gene of the gene cluster into a *fimZ* mutant (40). These transformants are fully fimbriate, indicating that they demonstrate a high level of *fimA* gene expression. However, they lack the ability to demonstrate fimbrial phase variation, the switching phenomenon of individual cells between a state of expression (fimbriate) and non-

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Abbreviations: BSA, bovine serum albumin; LB, Luria-Bertani; IPTG, isopropyl-β-D-thiogalactoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

expression (non-fimbriate), or vice versa, to produce populations containing a mixture of both cell types (13). This constitutive activity is most likely due to the presence of relatively large amounts of FimZ in the transformants since *fimZ* is carried on multi-copy recombinant plasmids in these strains.

Since FimZ plays a central role in *fimA* expression by *S. typhimurium*, we have identified the specific FimZ DNA-binding region within *fimA* promoter. Also, since FimZ regulates *S. typhimurium fimA* expression and is related to a family of sensory regulators, we constructed a *fimZ-lacZ* fusion to investigate the expression of this important regulator of the fimbrial system.

Materials and Methods

Bacterial strains, plasmids and media. Table 1 lists the bacterial strains and plasmids used in this study. Basically, all the DNA manipulations were performed by conventional procedures (29). The FimZ expression plasmid, pKYZ100, was constructed as follows. The entire *fimZ* gene was cloned on an *Eco*RI-*Bam*HI DNA fragment by conventional polymerase chain reaction (PCR) techniques using pISF101 as a template. The primers 5′-GCTAACGGAATTCGGACGCATAACATAACAGTCTGAGG-3′ and 5′-GCTGAAGGATCCCTCCCGAACGATAATTCGCC-3′ containing appropriate restriction enzyme sites (underlined, *Eco*RI and *Bam*HI respectively), were used to generate a 692-bp PCR product that was cloned into the expression vector pT7-7 containing a φ10 T7 RNA polymerase promoter (36). High-level production of FimZ by transformants was achieved following induction by isopropyl-β-D-thiogalactoside (IPTG) as previously described (36). The plasmid vector pMC1403 (8), containing a promoterless *lacZ* gene, was used to construct the *fimZ-lacZ* fusion (pISF239) molecule. The *fimZ* transcription initiation site has previously been characterized by primer extension and was located 227 nucleotides upstream of the *fimZ* initiation codon (unpublished data). A 700-bp DNA fragment carrying the *fimZ* promoter region was cloned into the *Eco*RI site of pMC1403. The promoter-containing region of *fimZ* was prepared by PCR using pISF101 as a template and 5′-GATCTGTGGAATTCCGAAGTAACGTTTTGGTG-3′ and 5′-CATGTGGTGAATTCCAGGATAAGTGCGCAGAT-3′ as primers (*Eco*RI sites are underlined). Transformants possessing a recombinant molecule comprising an in-frame fusion between the *fimZ* and *lacZ* genes were used to prepare plasmid DNA, and the fidelity of the

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant features	Reference or source
Salmonella typhimurium		
LB5010	Wild type; fimbriate with complete *fim* gene cluster	(34)
LBZ100	LB5010 *fimZ*: : *kan* Kan^r	(40)
Escherichia coli		
BL21 (DE3)	F^- *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ (DE3)	(36)
DH_5 α	*deo*R *end*A1 *gyr*A96 *hsd*R17 (r_k^- m_k^+) *rec*A1 *rel*A1 *sup*E44 *thi*-1 Δ (*lac*ZYA-*arg*FV169) φ80*lac*ZΔM15 F^-	(15)
Plasmids		
PT7-7	Cloning vector that contains T7 promoter; Am^r	(36)
pMC 1403	Promoterless *lacZ* fusion system	(8)
pKYZ	629-bp DNA fragment containing *fimZ* cloned into pT7-7; Am^r	This study
pISF101	12.8-kbp DNA fragment containing *fim* gene cluster of *S. typhimurium* cloned into pACYC184; Cm^r Tc^r	(9)
pISF182	*fimZ* and *fimY* cloned into pACYC 184; $FimZ^+$ $FimY^+$; Cm^r	(40)
pISF187	pISF182 with translational terminator inserted into *fimY*; $FimZ^+$ $FimY^-$; Cm^r	(40)
pISF189	pISF182 with translational terminator inserted into *fimZ*; $FimZ^-$ $FimY^+$; Cm^r	(40)
pISF239	*fimZ-lacZ* reporter fusion	This study
pBluescript II KS (−)	Cloning vector; Am^r	Stratagene
pKYA452	452-bp DNA fragment containing *fimA* promoter region cloned into pBluescript II KS (−); Am^r	This study
pKYA390	pKYA452 with the AATAAGA sequence adjacent to 5′ end of *fimA* promoter deleted; Am^r	This study
pKYA241	241-bp *Bst*EII-*Eco*RV fragment from *fimA* promoter DNA cloned into pBluescript II KS (−); Am^r	This study
pKYA211	211-bp *Eco*RV-*Eco*RI fragment from *fimA* promoter DNA cloned into pBluescript II KS (−); Am^r	This study

fusion was determined by DNA sequencing through the fusion junction. The pKYA452 DNA fragment possessing the *fimA* promoter region was prepared from pISF141 restricted with *Bst*EII and *Rsa*I, and purified with an agarose gel (14). The ends of the DNA fragment were made flush by Klenow polymerase (New England Biolabs, Beverly, Mass., U.S.A.) and this fragment was subcloned into the *Eco*RV site of pBluescript II KS (−) (Stratagene, La Jolla, Calif., U.S.A.). Deletions of the *fimA* promoter region were derived from pKYA452 by PCR or by enzyme restriction. All bacterial strains were cultured on Luria-Bertani (LB) medium and incubated at 37 C, or 30 C for lysogens, for 24 or 48 hr.

Preparation of FimZ. FimZ was purified from transformants of *E. coli* BL21 (pKYZ100) following growth in 4 liters of LB broth at 30 C to an OD_{600} of 1.0. IPTG was added to a final concentration of 1 mM, and cultures were incubated for an additional 16 hr. The bacteria were collected by centrifugation and resuspended in 50 ml of ice-cold Buffer A (20 mM Tris pH 8.0, 1 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol). The protease inhibitor phenylmethylsulfonyl fluoride (100 μg/ml) was added prior to disruption in a French pressure cell at 18,000 psi (1 psi = 8.89 kPa). Following lysis, the soluble cell extract and cell debris were separated by centrifugation at 11,000 × *g* for 30 min at 4 C. Ammonium sulfate was added to the supernatant to achieve 30% saturation, and the protein was then collected by centrifugation and redissolved in 30 ml of Buffer A. After dialysis against Buffer A, the protein extract was applied to a previously equilibrated Q-Sepharose FF column (2.6 × 40 cm). Using an automated FPLC system (Amersham Pharmacia Biotech, Piscataway, N.J., U.S.A.), FimZ was eluted against a linear gradient of 0−1 M NaCl in Buffer A. Column fractions were subsequently examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (22). The Protein Structure Facility, University of Iowa determined the N-terminal amino acid sequence of the purified FimZ.

Gel mobility shift assays. The gel mobility shift assay was based on the methods previously published (40). For 5′-labeling, DNA fragments were treated with calf intestinal phosphatase (New England Biolabs), and labeled with [γ-^{32}P]-ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase (Promega, Madison, Wisc., U.S.A.). All labeled DNA fragments were purified with a 5% polyacrylamide gel. The labeled DNA was preincubated in 1 × DNA binding buffer (5 mM Tris-HCl pH 7.5, 25 mM KCl, 0.5 mM EDTA, 2.5% glycerol, 0.5 mM dithiothreitol) (18 μl total volumes) at 37 C for 5 min, after which 2 μl volumes of protein samples diluted in 2 × DNA binding buffer were added. The binding reactions were carried out at 37 C for 15 min, mixed with 1 μl of loading dye (50% glycerol, 0.1% Xylene Cyanol), and subsequently applied to a non-denaturing 5% polyacrylamide-3% glycine gel. Electrophoresis was performed at 200 volts for 2 hr, after which the gel was dried and subjected to autoradiography.

DNase I footprint assays. DNase I (Roche Diagnostics GmbH, Mannheim, Germany) was dissolved in 1 × low salt dilution buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 μg/ml BSA) to a final concentration of 5 mg/ml. After the binding reaction, a DNase I (2.5 μg per assay) digest was performed at 37 C for 30 sec. The digestion was stopped by adding 100 μl of termination buffer (1% Sarkosyl, 0.1 M NaCl, 0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml proteinase K, 25 μg/ml calf thymus DNA) and the mixture was incubated at 37 C for 15 min. The DNA samples were extracted with phenol: chloroform (1:1), ethanol-precipitated, and finally resuspended in 7 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue). The samples were analyzed by electrophoresis through a 6% polyacrylamide/8 M urea sequencing gel, and compared to a Maxam-Gilbert sequence reaction performed on the same DNA fragments (23, 30, 38).

Results

Purification of FimZ

Cell extracts from IPTG-induced cultures of *E. coli* (pYZ100) were observed by SDS-PAGE to possess large amounts of a 25-kDa polypeptide that was absent from un-induced cultures (Fig. 1, Panel A). The size of this polypeptide was consistent with that (24.8 kDa) predicted from the nucleotide sequence of *fimZ*. This polypeptide was further purified using ion-exchange chromatography. SDS-PAGE analysis of the column eluates indicated that the 25-kDa protein was eluted at approximately 400−500 mM NaCl in a linear gradient of 0−1 M (Fig. 1, Panel B). The purified protein was subjected to amino acid sequencing from the N-terminus, and analysis of this sequence confirmed that the first 10 amino acids of the 25-kDa polypeptide were identical to those predicted from the nucleotide sequence of the *fimZ* gene.

Gel Mobility Shift Assays

Gel mobility shift assays were performed to determine whether FimZ was able to bind to the target DNA fragments and cause a mobility shift. A representative gel is shown in Fig. 2. The purified FimZ and FimZ-containing cell extracts exhibited identical ability to cause a shift of the DNA fragment possessing the *fimA* promoter (Fig. 2, Lanes 3, 4). Bacterial extracts derived from *E.*

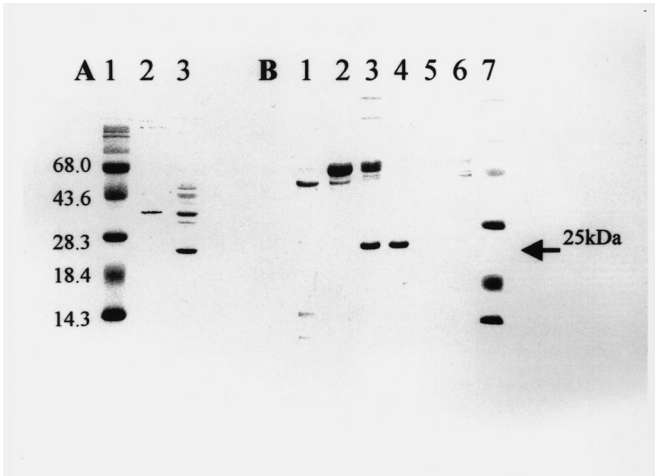

Fig. 1. SDS-polyacrylamide gel analysis of FimZ. Panel A, Lane 1, molecular weight standards; Lanes 2 and 3, bacterial lysates from un-induced (Lane 2) and IPTG-induced (Lane 3) cultures of *E. coli* BL21 (pKYZ100). Panel B, Lanes 1 and 2, French pressure lysates (Lane 1) and ammonium sulfate precipitates (Lane 2) from *E. coli* BL21 transformed with the cloning vector pT7-7. Lane 3, ammonium sulfate (30% saturation) precipitate from lysates of *E. coli* BL21 (pKYZ100). Lanes 4–6, 20 µl fractions eluted from a Q-sepharose column at 450 mM NaCl (Lane 4), 200 mM (Lane 5) and 800 mM NaCl (Lane 6). Lane 7, molecular weight standards. The arrow in Panel B represents the size of the FimZ polypeptide and the source of protein used to determine the N-terminal amino acid sequence.

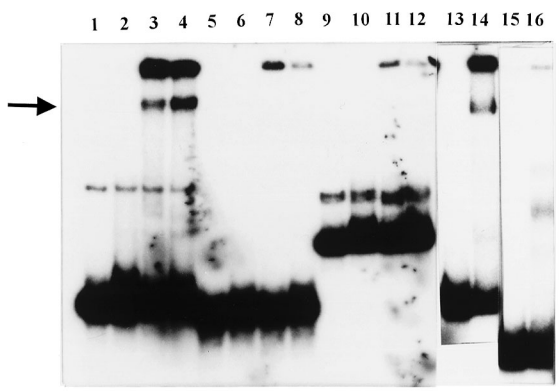

Fig. 2. Gel mobility shift assay. The 452-bp *fimA* promoter DNA was used in Lanes 1–4; the 390-bp derivative DNA is in Lanes 5–8; a 746-bp *metF* promoter DNA is in Lanes 9–12; the 241-bp derivative DNA is in Lanes 13, 14; the 211-bp derivative DNA is in Lanes 15, 16. Lanes 1, 5, and 9, DNA incubated with no protein; Lanes 2, 6, 10, 13, and 15, DNA incubated with 5 µg of cell extract possessing no FimZ; Lanes 3, 7, and 11, DNA incubated with 5 µg of FimZ-containing cell extract; Lanes 4, 8, 12, 14, and 16, DNA incubated with 1 µg of purified FimZ. The arrow indicates the DNA-protein complex.

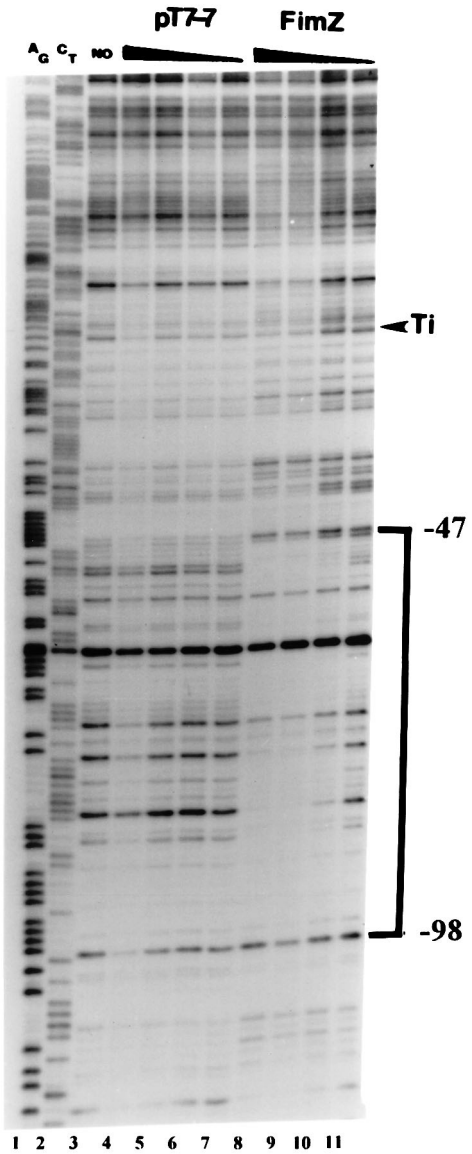

Fig. 3. DNase I footprint analysis of the *fimA* promoter region by FimZ. Lanes 1 and 2, the A+G and C+T chemical sequence analysis of the promoter region. Lane 3, DNA fragments incubated with no protein. Lanes 4–7, DNA incubated with 500, 250, 125, and 62.5 ng, respectively, of protein prepared from bacterial extracts of *E. coli* transformed with the expression vector pT7-7. Lanes 8–11, DNA incubated with 500, 250, 125, and 62.5 ng, respectively, of protein from cell extract prepared from *E. coli* (pKYZ100) containing FimZ. The region of DNA protected from DNase I digestion is indicated by the brackets. The numbers indicate the location of the protected region relative to the transcription initiation site of *fimA* (Ti).

coli BL21 (pT7-7) carrying the cloning vector alone, and used at protein concentrations equivalent to those employed for FimZ binding, did not demonstrate any DNA binding activity for the *fimA* promoter region (Fig. 2, Lanes 2, 6, 10). FimZ did not affect the mobility of a *S. typhimurium*-derived DNA fragment possessing the non-*fim* promoter *metF* that is controlled by the heterologous *Salmonella* DNA binding protein (Fig. 2,

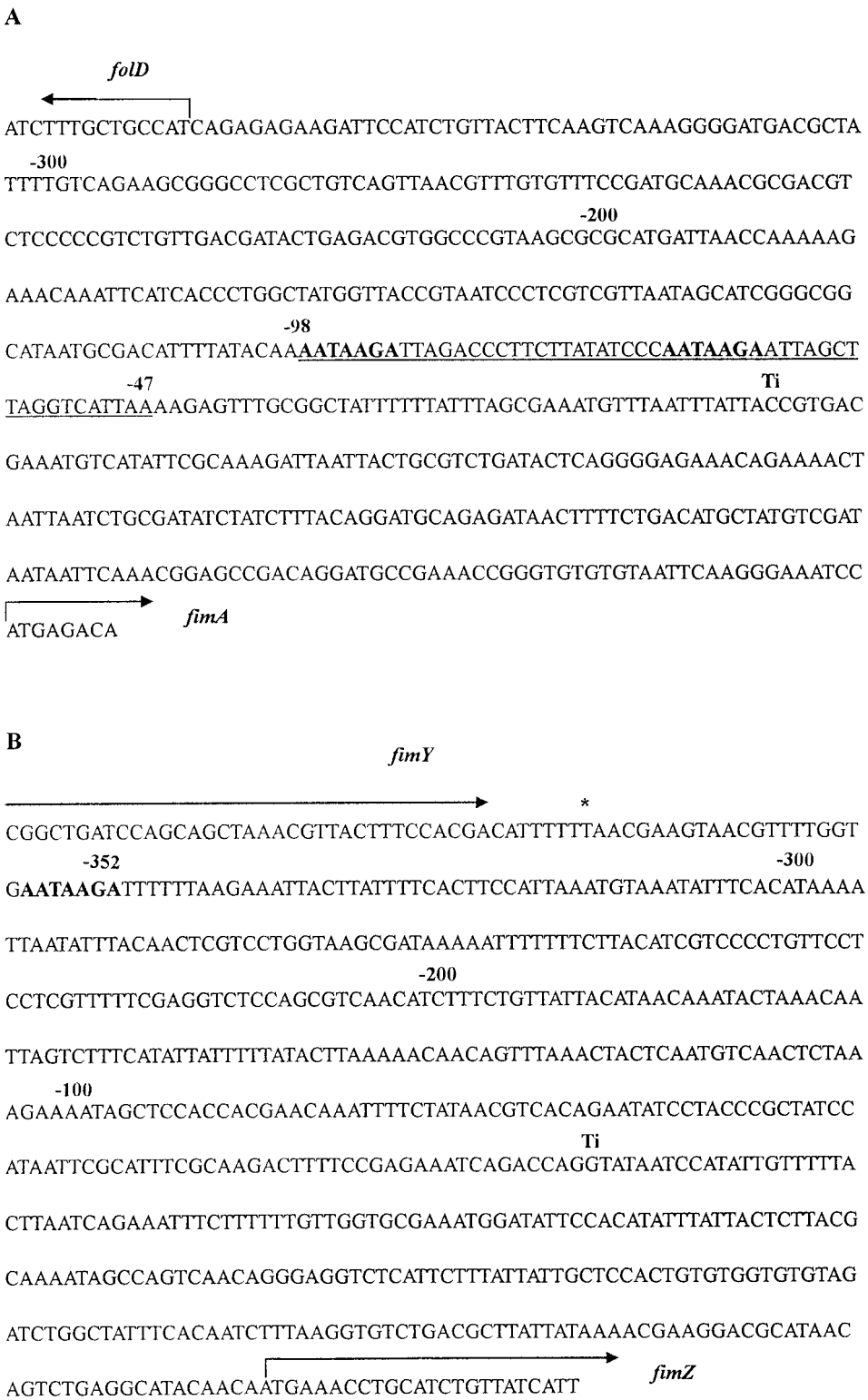

Fig. 4. Sequence analysis of *fimA* and *fimZ* promoter regions. Panel A, the underlined region shows the FimZ-protected area within *fimA* promoter. A pair of direct repeat AATAAGA are in boldface. Transcription initiation site of *fimA* is previously determined by nuclease S1 protection assay (9) to be located 195-bp upstream from the *fimA* initiation codon and denoted by Ti. The nucleotide sequences of N-terminal parts of tetrahydrofolate dehydrogenase (*folD*) and *fimA* gene products are indicated by bent arrows. Panel B, transcription initiation site of *fimZ* is determined by primer extension to be located 227-bp upstream from the *fimZ* initiation codon (unpublished data) and is denoted by Ti. AATAAGA sequence is shown in boldface. The nucleotide sequences of N-terminal part of *fimZ* product and C-terminal part of *fimY* product are indicated by bent arrows. Nucleotide marked with an asterisk is the stop codon of *fimY*.

Lanes 11, 12). However, not only was a shift in the electrophoretic mobility of the DNA fragment possessing the *fimA* promoter observed, but also the use of FimZ resulted in aggregation of this DNA region such that some labeled DNA did not migrate into the gel and remained within the wells of the gel (Fig. 2, top portions of Lanes 3, 4, 11, and 12).

Location of FimZ-Binding Region on the fimA *Promoter*

A DNase I footprint assay was performed to determine the specific region of the *fimA* promoter to which FimZ binds. As shown in Fig. 3, when a DNA fragment was incubated with FimZ-containing extracts from *E. coli* BL21 (pKYZ100), a 52-bp region of the fragment was protected from DNase I digestion. This region was not protected when cell extracts from *E. coli* BL21 (pT7-7) were used. Figure 4 shows the nucleotide sequences of the protected region, and this DNA region extends from 47-bp to 98-bp upstream from the transcription initiation site previously reported for *fimA* (33). A pair of 7-bp direct repeats (5′-AATAAGA-3′) were found within the protected region (Fig. 4, Panel A).

Since a 452-bp DNA fragment carrying the *fimA* promoter region was employed in both the gel mobility shift and DNase I footprinting assays, deletions within this fragment, carried on plasmid pKY452, were constructed. Plasmid pKY241 possessed *Salmonella*-derived DNA in which the 52-bp-protected region was present, but deletions downstream from this region were made. Plasmid pKY211 comprised DNA lacking the 52-bp-protected region. In plasmid pKY390, 12 nucleotides of the 52-bp FimZ-protected region, including one of the 7-bp direct repeat sequences, were deleted. As indicated in Fig. 2, Lane 14, pKY241 was also retarded in gel mobility shift assays following incubation with FimZ. The DNA fragments from pKY390 and pKY211 that lacked the intact 52-bp FimZ-protected region indicated by footprint analysis were not retarded in gel shift assays (Fig. 2, Lanes 7, 8, and 16). Aggregation of labeled DNA fragments that remained within the wells of the gel were also observed as described above when using FimZ protein (Fig. 2, top portions of Lanes 7, 8, 14, and 16). Figure 5 summarizes the results of gel mobility shift assays.

Expression of β-Galactosidase from the fimZ-lacZ *Reporter in* E. coli *and* S. typhimurium

Examination of the nucleotide sequence of the *fimZ* promoter region indicated one copy of the sequence, 5′-AATAAGA-3′ was identical to the directly repeated sequence found in the FimZ binding region of the *fimA* promoter (Fig. 4, Panel B). Accordingly, the ability of FimZ to regulate its own expression was analyzed. The expression of FimZ was investigated *in vivo* using the *fimZ-lacZ* fusion carried on plasmid pISF239. In an *E. coli* host previously shown not to express the *S. typhimurium fimA* subunit gene in the absence of FimZ and FimY (33, 40), a relatively low level of FimZ expression was detected (Table 2) even when the fusion was carried on a medium copy-number vector. As indicated

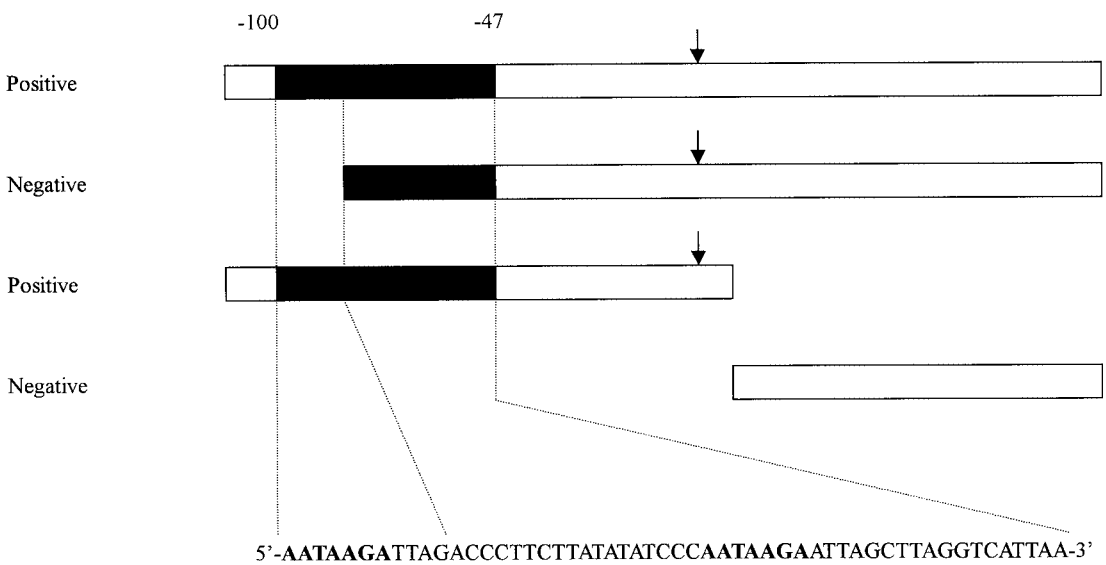

Fig. 5. Summary of the gel mobility shift assays. The ability of FimZ to bind to the *fimA* promoter DNA and its derivatives is indicated as positive, while negative indicates that FimZ does not cause a mobility shift of these DNA fragments. The solid black boxes denote the FimZ protection region determined by the DNase I footprint assay. The sequences represented by the solid areas are as indicated. A pair of direct repeat AATAAGA are in boldface. Transcription initiation site of *fimA* is designated by arrow.

Table 2. Expression of β-galactosidase by *fimZ-lacZ* reporter constructs in *E. coli* and *S. typhimurium*

Strain	Plasmid (relevant genotypes)	β-Galactosidase expression[a)]
E. coli		
$DH_5\alpha$	pISF239 (*fimZ-lacZ*)	24
$DH_5\alpha$	+pISF182 (*fimZ*$^+$ *fimY*$^+$)[b)]	3468
$DH_5\alpha$	+pISF187 (*fimZ*$^+$ *fimY*$^-$)	36
$DH_5\alpha$	+pISF189 (*fimZ*$^-$ *fimY*$^+$)	65
S. typhimurium		
LB5010	pISF239 (*fimZ-lacZ*)	603
LBZ100	pISF239 (*fimZ-lacZ*)	196
LBZ100	+pISF182 (*fimZ*$^+$ *fimY*$^+$)	1088
LBZ100	+pISF187 (*fimZ*$^+$ *fimY*$^-$)	755
LBZ100	+pISF189 (*fimZ*$^-$ *fimY*$^+$)	771

[a)] β-Galactosidase activity is reported in Miller (25) units.
[b)] pISF239 + pISF182.

in Table 2, *E. coli* (pISF239) transformed with pISF187 or pISF189 merely exhibited small increases in FimZ expression even though these plasmids encode functional FimZ (pISF187) or FimY (pISF189) polypeptides (33, 40). However, more than a 100-fold increase in FimZ expression was detected in the presence of pISF182 that produced both FimZ and FimY.

The *fimZ* mutant *S. typhimurium* LBZ100 did not produce type 1 fimbriae on its surface and demonstrated low levels of *fimA* expression (40). Transformants of this strain possessing pISF239 exhibited detectable levels of β-galactosidase activity (Table 2). However, *fimZ-lacZ* transformants of the parental strain *S. typhimurium* LB5010, bearing an intact *fimZ* on the chromosome, resulted in higher levels of FimZ expression than did the *fimZ* mutant. Introduction of cloned copies of *fimZ* or *fimY* into *S. typhimurium* LBZ100 resulted with FimZ expression slightly higher than that produced by *S. typhimurium* LB5010. The presence of many copies of both *fimZ* and *fimY* was responsible for maximal expression of FimZ (Table 2).

Discussion

The predicted amino acid sequence of the *S. typhimurium* fimbrial regulator, FimZ, exhibits a high degree of homology to a variety of bacterial transcriptional activators (10, 32). The N-terminal domain of FimZ is similar in sequence to a family of response regulators including BvgA. BvgA is a DNA-binding protein involved in virulence gene expression in *Bordetella pertussis* (10, 31). Examination of the amino acid sequence of the C-terminal domain of FimZ reveals a helix-turn-helix motif and this, along with our previous reports of FimZ affecting *fimA* expression (33, 40), indicates that FimZ can bind to the *fimA* promoter region. Binding of FimZ to the *fimA* promoter could be demonstrated *in vitro* and the location of binding was precisely detected by DNase I footprint assays.

The FimZ-protected region extends from approximately 47 to 98 base pairs upstream from the transcription initiation site of *fimA*. This region shares characteristics with the activator binding site for class I transcription factors (20). Most activator-binding sites of this class are a short distance upstream from the relevant transcription initiation site, and protein-protein contact between RNA polymerase and the activator has been suggested to increase the rate of isomerization of the polymerase-promoter complex from the closed to the open state (20). Transcriptional factors belonging to this class include Ada, OxyR, CRP, OmpR, TrpI, and IHF (20). Removal of the 52-bp FimZ-binding region from *fimA* promoter resulted in abrogation of FimZ binding but deletion of nucleotides outside of the protected region did not affect this binding. A pair of 7-bp direct repeats, predicted to be separated by two helical turns, are present within the binding region. Removing one of these repeats causes the FimZ binding to be lost, suggesting that these sequences participate in FimZ binding.

We have previously reported that both FimZ and FimY are necessary for *fimA* expression *in vivo* (9, 33). However, our *in vitro* results indicate that FimZ alone will bind to the *fimA* promoter. Such binding leads to an electrophoretic profile consistent with a DNA fragment which exhibits very slow mobility with a large aggregation of FimZ-DNA complexes. This profile is dissimilar to that which would be predicted by an increase in molecular size when one, two, or three protein molecules bind to the DNA. The wells of the gel loaded with FimZ/DNA mixtures frequently possessed labeled DNA fragments that did not migrate into the gel. FimZ/DNA

with FimZ-binding region mixtures had more protein-DNA complex within the wells than those of FimZ/DNA with no FimZ-binding region. It is possible that the FimZ prepared in our study has some non-specific DNA-binding activity. However, when FimZ binds *in vitro* to the *fimA* promoter, many molecules of FimZ are associated with the DNA fragments, creating a large DNA-protein complex that has difficulty entering the gel. The observed aggregation of FimZ on the *fimA* promoter *in vitro* is not likely to occur where additional regulatory proteins may play a role in *fimA* expression. In addition, this aggregation is not seen when crude bacterial lysates possessing FimZ are used in the gel-shift assays (40), and is therefore a result of using over-expressed or purified protein in these assays. The predicted amino acid sequence of FimZ and its ability to bind to the *fimA*-promoter region suggest that this protein may be a response regulator; it is unknown whether FimZ needs to be phosphorylated in order to bind. It has been demonstrated that response regulators can be phosphorylated *in vitro* by small molecule phosphate donors such as phosphoramide and acetyl phosphate (16). No differences in the binding activity of FimZ to the *fimA* promoter were observed when acetyl phosphate was used as a donor (data not shown).

Analysis of the nucleotide sequences upstream from *fimZ* indicated the presence of a single 7-bp sequence that is between 358 and 352 base pairs upstream from the *fimZ* transcription initiation site and is identical to the direct repeats found within the *fimA* promoter. Since we could neither demonstrate the binding of FimZ to its promoter DNA nor identify the precise FimZ site of binding to its promoter region *in vitro* by gel mobility shift or DNase I footprint assays (data not shown), we decided to determine whether FimZ could regulate its own expression by using a *fimZ-lacZ* reporter construct. The observation that putative DNA-binding proteins can be shown to control their target gene expression without demonstrable binding *in vitro* has been reported for several systems (1, 2, 5). In fact, by using a *fimZ-lacZ* reporter construct that possesses an intact *fimZ* promoter region, we were able to demonstrate that FimZ expression is significantly reduced in a *fimZ* mutant, *S. typhimurium* LBZ100. The Miller units seen with the *fimZ* mutants introduced with cloned copies of *fimZ* or *fimY* are higher than those seen with the *fimZ* mutant, but are only slightly higher than those seen with the wild type *S. typhimurium* LB5010. Many copies of *fimY* alone restore the FimZ expression in the *fimZ* mutant back to the wild type strain since there is no functional FimZ in either chromosomal DNA or the introduced plasmid. However, in *S. typhimurium* LBZ100+pISF187 (*fimZ*$^+$ *fimY*$^-$), many copies of *fimZ* and one copy of *fimY* in chromosomal DNA exhibit almost the same level of FimZ expression. There is no result demonstrating that many copies of *fimZ* alone can activate FimZ expression; that many copies of *fimY* alone can activate FimZ expression could be explained by gene dosage effect. Notably, the *in vivo* expression of FimZ is highest in the presence of both FimZ and FimY. It is possible that FimZ and FimY, in correct stoichiometric ratios, are required for optimal expression of FimZ. This result corresponds to the observations previously reported for the expression of *fimA in vivo* (33). It was also demonstrated that transcription from the cloned *fimY* promoter was not detected in the *E. coli* host unless both FimY and FimZ were present (37). Consequently, we propose that both FimZ and FimY act cooperatively to regulate their target genes. However, in contrast to the case of the previously reported *fimA* expression (33, 40), a relatively low level of FimZ expression occurs in the absence of the FimZ protein. The sequence of the FimZ polypeptide is 71% identical to the predicted amino acid sequence of an *E. coli* polypeptide, and can be produced by an open reading frame located 155-bp upstream from the transcriptional start of the *argU* gene (26). The presence of this *S. typhimurium* FimZ counterpart in *E. coli* may explain the FimZ expression level in the *E. coli* host. Our data indicate that FimZ affects FimZ expression *in vivo*, and that FimZ may, therefore, be autoregulatory. However, this activation is maximally achieved in the presence of FimY. The molecular interaction of both FimZ and FimY proteins must be determined to understand the role of these two gene products in *Salmonella* fimbrial expression.

The precise role of the 7-bp repeat AATAAGA sequence, which is 352-bp upstream from the *fimZ* transcription initiation site, was not elucidated in this study. This sequence is at a large distance from the *fimZ*-promoter region. If this sequence is critical for *fimZ* activation, it could be an enhancer-like element as documented in other bacterial systems (39). Nonetheless, further study is required to explore the function of this AATAAGA sequence.

Although FimZ is closely related to the response regulator BvgA and possesses the highly conserved motifs associated with this class of regulator, the *fimZ* gene is not immediately adjacent to a determinant that is likely to encode a sensory kinase associated with two-component regulatory systems. Upstream from *fimZ* lies *fimY* (9), and an examination of the amino acid sequence of the FimY does not reveal the presence of highly conserved amino acids found within sensory kinases. Also, FimY is a relatively small polypeptide (27 kDa) with no apparent membrane-spanning regions. Hence, if FimZ is a component of a sensory regulatory system, it is not

closely linked to its cognate sensory kinase. Examples of other proteins that are related to response regulators and also are not contiguous with identifiable kinases have been reported in *Salmonella* (4, 35). In addition, the dependence of *fimA* activation on both FimZ and FimY *in vivo* indicates that if FimZ is a sensory regulator, FimY may act as a third component in such a system along with an as-yet-unidentified sensory kinase. The influence of cultural conditions on the expression of type 1 fimbriae by *S. typhimurium* has been comprehensively described by Duguid and coworkers (11, 27, 28). Therefore, environmental factors probably play a role in affecting *Salmonella* fimbrial expression, and FimZ is a putative candidate in this process.

This work was supported by a grant from the National Research Initiative of the USDA, U.S.A. (97-35204-4616) and in part by a grant from the National Science Council, Taiwan (88-2313-B-059-017). JKT was supported by a predoctoral fellowship from a National Institutes of Health Parasitism Training Grant, U.S.A. (TEA107511). The authors wish to thank Peggy Wang and Ted Knoy for editorial suggestions for this article.

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