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# Construction and Characterization of a *fimZ* Mutant of *Salmonella typhimurium*

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**The *Salmonella typhimurium fimA* gene is controlled by several ancillary *fim* genes. One of these genes, *fimZ*, appears to be involved in increasing the expression of *fimA*. A *fimZ* mutant of *S. typhimurium* was constructed by allelic exchange, and this mutant was found to be nonfimbriate. The *fimZ* mutant demonstrated decreased levels of *fimA* expression compared with the parental strain when both were grown under conditions favoring fimbrial expression. An examination of the predicted amino acid sequence, deduced from the nucleotide sequence of *fimZ*, indicated that the FimZ polypeptide possessed a DNA binding motif. Bacterial lysates, derived from strains transformed with recombinant plasmids possessing a *fimZ* gene, demonstrated DNA binding activity with a fragment containing the *fimA* promoter. Lysates without a FimZ polypeptide did not exhibit any binding activity. These data are consistent with FimZ being a transcriptional activator of *fimA*, and FimZ acts by binding to the promoter region.**

Initial investigations of the *Salmonella typhimurium fim* gene cluster have suggested that the gene encoding the major fimbrial subunit, *fimA*, is regulated in part by *fim* genes that are not immediately adjacent to *fimA* within the gene cluster (2, 23). Four genes, *fimZ*, *-Y*, *-W*, and *-U*, have been implicated in affecting *fimA* expression in *S. typhimurium* (4, 25), and one of these genes (*fimU*) encodes a tRNA molecule (25). The precise mechanism by which FimZ, -Y, and -W control *fimA* expression is unknown, but initial evidence indicates that *fimZ* encodes a positive activator of *fimA* (4, 23). The mechanism of action of the *fimZ*, *fimY*, and *fimW* determinants has yet to be elucidated.

In *Escherichia coli*, the control of *fimA* expression is a complex process involving a number of different proteins (6, 9, 13, 14). It is likely that the regulation of *fimA* expression in *S. typhimurium* is also under the control of numerous gene products. However, the two *fimA* genes do appear to be controlled, in part, by different and unrelated mechanisms. Evidence for this is as follows. Firstly, there are no *fimB* or *fimE* homologs in the *S. typhimurium fim* gene cluster. Conversely, genes related to the *Salmonella fimY* or *fimW* are not found within the *E. coli* gene cluster. Secondly, fimbriate *E. coli* strains lysogenized with an *S. typhimurium fimA-lacZ* fusion molecule demonstrate poor β-galactosidase activity (23). Therefore, under conditions in which the *E. coli fimA* gene is expressed in these strains, the *S. typhimurium fimA* gene is not. Thirdly, none of the *E. coli fim* genes affect the expression of the *S. typhimurium fimA* in its natural host, whereas ancillary *Salmonella fim* genes that alter *fimA* expression have been identified (4, 23).

Since we have demonstrated that the *S. typhimurium fimZ* gene appears to alter *fimA* expression (23), we decided to investigate further the mechanism of action of the *fimZ* gene product. The following describes the construction and characterization of an *S. typhimurium fimZ* mutant and the ability of FimZ to bind to the promoter region of the *S. typhimurium fimA* gene. Evidence that FimZ is necessary for fimbrial expression in *S. typhimurium* and that the binding by FimZ results in increased expression of *fimA* is presented.

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The fimbriate strain *S. typhimurium* LB5010 (2) was used to construct the *fimZ* mutant. *S. typhimurium* IS145 is a λ*fimA-lacZ* lysogen, and its construction and characterization have been previously described (23). *E. coli* IS145 is a derivative of *E. coli* JM109 carrying the *S. typhimurium fimA-lacZ* fusion on its chromosome (23). Different serovars of *Salmonella* were provided from the collection of J. P. Duguid (University of Dundee, Dundee, United Kingdom). Bacteria were cultured on L media and incubated at 37°C, or 30°C for lysogens, for 24 or 48 h. All plasmids used in this study are shown in Fig. 1 and are derivatives of pISF101 carrying the *S. typhimurium fim* gene cluster (24). The plasmid pISF182 possesses only the *fimZ* and *fimY* genes of the *fim* gene cluster, and it was constructed by cloning a 2.8-kb *Hpa*I DNA fragment into the *Eco*RV site of pACYC184. The plasmid pISF187 possesses a universal translation terminator inserted into a unique *Eco*RV site within *fimY*. In pISF189 the terminator was inserted at a unique *Pvu*I site within *fimZ*. The deletion derivative pISF199 was constructed by removal of nucleotides encoding the eight amino acids at the C terminus of FimZ.

**Detection of type 1 fimbriae.** Bacteria were serially subcultured in 10 ml of L broth and incubated without shaking for 48- or 72-h periods. Bacteria were harvested by centrifugation and gently resuspended in the residual fluid as previously described (20, 26). Subsequently, 30 μl of bacterial suspension was mixed with 30 μl of a 3% (vol/vol) suspension of *Candida albicans* cells or guinea pig erythrocytes in phosphate-buffered saline (PBS). The level of mannose-sensitive yeast cell agglutination or mannose-sensitive guinea pig erythrocyte hemagglutination was determined by incubation of the bacterial suspension with cells resuspended in PBS containing 3% (wt/vol) α-methyl-D-mannoside. The mannose-sensitive adhesin was considered to be present if the cells were agglutinated only in the absence of mannose within 1 min.

Fimbrial antigens were detected by using monospecific *S. typhimurium* antifimbrial serum as described elsewhere (24). Intact fimbrial appendages were detected by observation of negatively stained bacteria with an electron microscope (12).

**Construction of the *S. typhimurium fimZ* mutant.** The plasmid pISF182 possesses an intact *fimZ* gene (Fig. 1). This plasmid was linearized at the unique *Pvu*I site within *fimZ*, and a DNA molecule possessing flush ends was prepared. A *Hinc*II DNA fragment, possessing a kanamycin resistance determinant, was subsequently ligated into the *fimZ* gene. Following isolation of kanamycin-resistant transformants in *E. coli* HB101 (5), the plasmid carrying the insertionally inactivated *fimZ* gene was isolated by standard techniques (15). Subsequently, the inactivated determinant was cloned into the suicide vector pGP704 and maintained in the permissive host *E. coli* SY327 (18). After characterization of the plasmid by restriction analysis to confirm the location of the $Km^r$ cassette within the *fimZ* gene, kanamycin-resistant transformants of *S. typhimurium* that exhibited ampicillin sensitivity were isolated and characterized by Southern hybridization using DNA probes specific for the *fimZ* gene or the $Km^r$ determinant. All hybridizations were performed under high-stringency conditions as described elsewhere (11).

**β-Galactosidase assays.** Assays for β-galactosidase were performed in triplicate by the method of Miller (17) using the λ*fimA-lacZ* lysogens.

**Gel mobility shift assays.** The preparation of the 452-bp target DNA possessing the promoter region of the *S. typhimurium fimA* gene has been described previously (23). Following end labeling of the DNA fragment using the Klenow

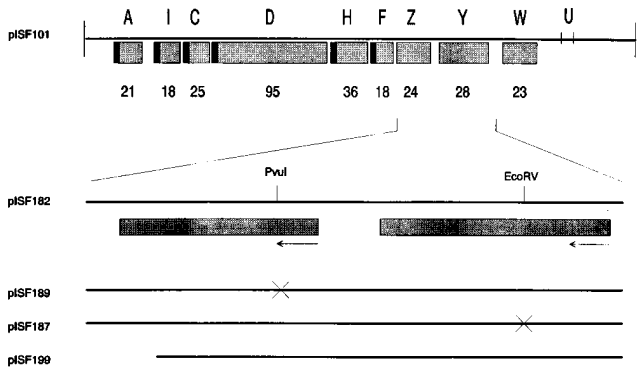


FIG. 1. Genetic organization of the *S. typhimurium fim* gene cluster. The predicted sizes (in kilodaltons) of the Fim polypeptides are as indicated, and the signal peptide region of each gene product is shown as a solid area. The arrows indicate the direction of transcription of *fimZ* and *fimY*, and the crosses indicate the site of insertion of translation stop codons into the *fimZ* and *fimY* genes. The plasmid pISF182 possesses only the *fimZ* and *fimY* genes.

fragment of DNA polymerase (15), the DNA was incubated with cell sonicates prepared from *S. typhimurium* or *E. coli* transformants (8, 10). Gel mobility shift assays were performed by standard techniques (10) except that bovine serum albumin was not added to the incubation mixtures and all volumes were adjusted with sterile distilled water. The mobility of the DNA fragment was determined by autoradiography.

**Plasmid and DNA manipulations.** All plasmids were prepared by standard techniques (15). Synthetic oligonucleotides were prepared by using an Applied Biosystems DNA synthesizer according to the manufacturer's instructions. The compositions of the synthesized oligonucleotides were derived from the appropriate nucleotide sequence of the *S. typhimurium fim* gene cluster (GenBank accession number L19338).

Restriction enzyme digestions, DNA ligations, and labeling were performed by conventional techniques.

**DNA colony blot analysis.** The genomic DNAs from bacterial isolates were tested for the presence of *fimZ* nucleotide sequences as previously described (11). The 680-bp DNA probe used in these studies was composed of sequences restricted to the *fimZ* gene of *S. typhimurium* and was prepared by PCR using the cloned gene as a template.

## RESULTS

**Characterization of the *fimZ* mutant of *S. typhimurium* LB5010.** Following transformation of *S. typhimurium* LB5010 with a plasmid possessing an insertionally inactivated *fimZ* gene carried on the pGP704 vector, a kanamycin-resistant, ampicillin-sensitive derivative was isolated. Unlike the parental strain, this isolate, designated *S. typhimurium* LBZ100, did not mediate mannose-sensitive guinea pig erythrocyte hemagglutination or mannose-sensitive yeast cell agglutination when grown under conditions for optimal expression of type 1 fimbriae (Table 1). After repeated subculture (up to 10 times) in static liquid media, no fimbriae could be observed on the bacteria. In contrast, *S. typhimurium* LB5010 was found to be fully fimbriate under identical cultural conditions.

Genomic DNA was isolated from both *S. typhimurium* LB5010 and *S. typhimurium* LBZ100. DNA preparations were restricted with *Cla*I and probed with a 1,300-bp DNA fragment comprising the Kan$^r$ gene. No sequences homologous to the probe were detected in the parental strain. By use of *S. typhimurium* LBZ100 DNA, two *Cla*I DNA fragments were shown to hybridize with the probe (Fig. 2). The larger of these DNA fragments possesses only 300 nucleotides that are homologous to the 1,300-bp DNA probe used in the assay. A faint signal corresponding to the size of this fragment was seen by autoradiography. The sizes of the two fragments (1,500 bp and >4.5 kb) are consistent with the presence of *Cla*I sites within the Kn$^r$ cassette and *fim* gene cluster.

TABLE 1. Expression of fimbriae by *S. typhimurium*

| Strain | Plasmid (phenotype) | Phenotypic expression of type 1 fimbriae by bacteria grown on or in[a]: | | Presence of fimbriae as determined by EM[b] |
|---|---|---|---|---|
| | | Agar | Broth | |
| LB5010 | | − | + | + |
| LBZ100 | | − | − | − |
| LBZ100 | pISF182 (FimZ$^+$ FimY$^+$) | − | + | + |
| LBZ100 | pISF187 (FimZ$^+$ FimY$^-$) | − | + | + |
| LBZ100 | pISF189 (FimZ$^-$ FimY$^+$) | − | − | ND[c] |

[a] Phenotypic expression was determined by mannose-sensitive guinea pig erythrocyte hemagglutination or mannose-sensitive yeast cell agglutination and reactivity with fimbrial antiserum. Agar-grown cultures were subcultured up to 3 times; broth-grown cultures were subcultured up to 10 times.

[b] Electron microscopy (EM) was performed on bacterial suspensions that demonstrated hemagglutination activity and reactivity with fimbrial antiserum.

[c] ND, not determined.

The genomic DNA preparations were digested with a number of different enzymes and probed with the 680-bp *fimZ* DNA fragment. A representative hybridization profile is shown in Fig. 2. The DNA from *S. typhimurium* LB5010 possessed the *fimZ* gene on a 2.5-kb *Eco*RV DNA fragment within the *fim* gene cluster. Insertion of the Kn$^r$ cassette into this gene resulted in hybridization of the gene probe with a 3.8-kb DNA fragment (Fig. 2).

*S. typhimurium* LBZ100 was transformed with pISF182, pISF187, or pISF189 (Table 1). When grown under conditions favoring fimbrial expression, pISF182 and pISF187 transformants were able to express type 1 fimbriae. However, *S. typhimurium* LBZ100 transformed with pISF189 did not produce fimbriae, as evidenced by its lack of agglutinating activity and reactivity with fimbria-specific serum. In addition, examination of this transformant by electron microscopy demonstrated that no fimbrial appendages were located on the bacterial surface.

**Expression of β-galactosidase by λ*fimA-lacZ* lysogens.** Previously we have described the construction of an *S. typhimurium* λ*fimA-lacZ* lysogen (23). This strain was used to prepare recombinant phages that were subsequently used to lysogenize *S. typhimurium* LBZ100. The expression of β-galac-

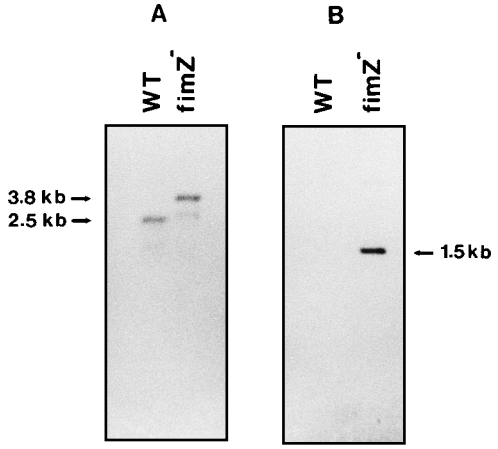


FIG. 2. Southern hybridization profiles of genomic DNA isolated from *S. typhimurium* LB5010 (WT) and LBZ100 (fimZ$^-$). (A) DNA probed with a *fimZ* gene probe. (B) DNA probed with sequences from the Kn$^r$ cassette.

TABLE 2. Expression of β-galactosidase by λ*fimA-lacZ* lysogens

| Transforming plasmid (phenotype) | β-Galactosidase production by bacteria lysogenized with λ*fimA-lacZ*[a] | | |
|---|---|---|---|
| | *S. typhimurium* LB5010 | *S. typhimurium* LBZ100 | *E. coli* JM109 |
| None | 80 | 2–3 | 0 |
| pISF182 ($FimZ^+$ $FimY^+$) | 2,000 | ND[b] | 600 |
| pISF187 ($FimZ^+$ $FimY^-$) | 500 | 900 | 0 |
| pISF189 ($FimZ^-$ $FimY^+$) | 1,800 | 1–2 | 0 |
| pISF199 ($FimZ^-$ $FimY^+$) | 1,200 | 2–3 | 0 |

[a] Strains were grown under conditions for optimal expression of type 1 fimbriae. Galactosidase activity is reported as Miller (17) units, and data represent the results from at least three independent experiments with <20% variability.
[b] ND, not determined.

tosidase by the mutant is shown in Table 2. Low levels of enzyme activity were detected in lysogens grown in broth or on agar. In contrast, the lysogen of *S. typhimurium* LB5010 expressed relatively high levels of β-galactosidase following incubation in liquid media.

The plasmids pISF187 and pISF189 possess translation termination signals in *fimY* and *fimZ*, respectively (Fig. 1). Transformants of both *S. typhimurium* LB5010 and LBZ100 lysogenized with the λ*fimA-lacZ* fusion and possessing pISF187 expressed relatively high levels of β-galactosidase (Table 2). However, with pISF189 (*fimZ* negative) only transformants of the parental strain expressed significant levels of enzyme activity. The *S. typhimurium* LBZ100 lysogen transformed with pISF189 exhibited low β-galactosidase activity in a manner similar to that of the untransformed strain. When the *E. coli* JM109 λ*fimA-lacZ* lysogen that lacks chromosomally borne *fimZ* and *fimY* alleles was used, only transformants with intact *fimZ* and *fimY* genes on the transforming plasmid could produce β-galactosidase. The relatively high levels of enzyme activity in *S. typhimurium* LB5010 λ*fimA-lacZ* transformed with pISF189 compared with the levels in the *S. typhimurium* LBZ100 and *E. coli* lysogens were most likely due to the absence of a functional *fimZ* gene in the latter strains.

**DNA binding by FimZ.** Cell extracts of pISF182 or pISF187 demonstrated DNA binding activity with a 452-bp DNA fragment possessing the promoter region of *fimA* (Fig. 3). In vitro binding could be inhibited by competition with unlabeled DNA, and no binding was observed when fractions from transformants possessing *fimA* to *fimF* were used (4). In addition, bacterial lysates prepared from strains possessing only the cloning vector of pISF182 or pISF187 did not bind to the promoter-containing region of *fimA*.

**Frequency of *fimZ* among *Salmonella* isolates.** The presence of nucleotide sequences homologous to the *fimZ* DNA probe could be detected among 14 different serovars of *Salmonella*. All these strains have been shown to be fimbriate and to express antigenically related fimbrial appendages (7, 24). In addition, genomic DNAs from *E. coli* HB101, DH5α (22), and C1a (15) possess sequences that are homologous to the *fimZ* DNA probe. Genomic DNA isolated from two nonenteric species, *Haemophilus influenzae* and *Neisseria gonorrhoeae*, did not hybridize with the *fimZ* DNA probe.

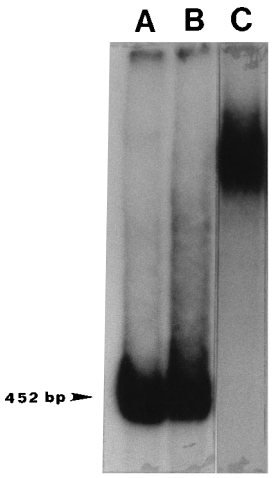


FIG. 3. Gel mobility shift assays using a DNA fragment containing the promoter region of *fimA*. Lane A, the 452-bp fragment incubated in the absence of bacterial lysates; lane B, the DNA fragment incubated with lysates prepared from *S. typhimurium* transformed with pACYC184 (the cloning vector of pISF182, pISF187, and pISF189); lane C, the DNA fragment incubated with lysates prepared from *S. typhimurium* transformed with pISF182.

## DISCUSSION

We have previously demonstrated that the *S. typhimurium* *fimZ* gene product can affect the expression of the *fimA* gene (23). Examination of the predicted amino acid sequence of the FimZ polypeptide had shown that it exhibited similarity to a number of transcriptional activators (4). These results suggested that the *S. typhimurium fimA* gene, encoding the major fimbrial subunit, is positively controlled by FimZ. If the FimZ protein is a transcriptional activator of *fimA*, then *S. typhimurium fimZ* mutants should be poorly fimbriate or nonfimbriate. Also, *fimZ* mutants should demonstrate low levels of FimA expression. In addition, binding of the *fimZ* gene product to the promoter region of *fimA* would be consistent with the hypothesis that FimZ acts as a positive regulator of *fimA*.

To examine the role of FimZ in fimbrial expression, we constructed a *fimZ* mutant of *S. typhimurium* LB5010 by allelic exchange. Southern hybridization analysis demonstrated that this mutant, *S. typhimurium* LBZ100, possesses a disrupted *fimZ* determinant on its chromosome, since a predictable change in size (from 2.5 to 3.8 kb) of an *Eco*RV DNA fragment relative to that in the wild-type strain was detected in the mutant when a *fimZ* DNA probe was used. In addition, the $Kn^r$ cassette was detected in the genomic DNA of the mutant but not in that of the wild-type strain. Confirmation that the mutated *fimZ* gene had replaced the wild-type allele was obtained by Southern hybridization analysis of DNA that had been restricted with a number of different enzymes in addition to *Eco*RV (data not shown). In all cases the hybridization profiles were consistent with replacement of the *fimZ* gene by the mutated allele.

*S. typhimurium* LBZ100 was nonfimbriate even when cultured under conditions favoring fimbrial expression. The lack of fimbriation correlated with low levels of *fimA* expression as determined by using the *fimA-lacZ* fusion. In fact, *S. typhimurium* LB5010 does express low but detectable levels of *fimA* after subculture on solid media even though no fimbriae can be detected on the bacterial surface (Table 1). However, the *fimZ* mutant does not express significant amounts of *fimA* regardless of the conditions of culture. These results are consistent with FimZ controlling FimA production rather than subsequent effects upon fimbrial transport and assembly. Also, we have demonstrated that FimZ alters FimA expression by binding to a DNA fragment possessing the promoter region of *fimA*, since only bacterial extracts of transformants possessing an intact *fimZ* gene will bind to the promoter region of the *S. typhi-*

*murium fimA* gene. By using the predicted amino acid sequence of FimZ, it is possible to demonstrate a helix-turn-helix motif in the C terminus of this polypeptide. All these results, in conjunction with our previously published studies of *fimA*-specific mRNA levels (23), are consistent with the FimZ protein being a positive regulator of *fimA*.

Complementation of *S. typhimurium* LBZ100 could be achieved by transformation of the mutant with plasmids (pISF182 and pISF187) carrying an intact *fimZ* gene. These transformants were fully fimbriate and demonstrated increased production of the *fimA* gene product (Tables 1 and 2). As shown in Fig. 1, *fimZ* lies immediately downstream of *fimY* and transcription of *fimZ* appears to start in the intergenic region between *fimZ* and *fimY*. Confirmation that only *fimZ* could complement *S. typhimurium* LBZ100 was attained by using pISF182, pISF187, pISF189, and pISF199 (Table 1). Premature termination of translation of *fimZ* on pISF189, or deletion of the C terminus (pISF199), resulted in no fimbrial assembly and low-level expression of fimbrial subunits in *S. typhimurium* LBZ100, whereas a plasmid that expresses *fimZ* but not *fimY* (pISF187) transformed the mutant to fimbriation. Interestingly, FimA expression, as measured by β-galactosidase production, depends upon both the *fimZ* gene and the *fimY* gene, since the *E. coli* JM109 lysogen requires both genes for *fimA* expression. The precise role of *fimY* in fimbrial production has yet to be determined, but initial experiments suggest that FimY may also be a DNA-binding protein. It is possible that both FimZ and FimY, in correct stoichiometric ratios, are required for optimal expression of *fimA*. However, further investigation of the role of FimY in fimbrial expression needs to be performed in order to confirm this hypothesis.

The regulation and control of *fimA* expression in *S. typhimurium* demonstrate differences from the regulation and control described for *E. coli* (13, 14). Nucleotide sequences homologous to the *fimZ* gene can be detected in many different *Salmonella* serovars. Therefore, it is likely that *fimA* expression in most *Salmonella* strains is in part controlled by a *fimZ* gene product. A search of various databases demonstrated that the primary sequence of the *S. typhimurium* FimZ protein is related (71% identical) to the predicted amino acid sequence of an *E. coli* polypeptide that may be produced by an open reading frame located in the 5′ region of the *argU* (*dnaY*) gene (19). Hybridization of nucleotide sequences representing this open reading frame of *E. coli* would explain why the *fimZ* gene probe hybridized with the *E. coli* genomic DNA. Interestingly, the *E. coli argU* gene is identical to the *S. typhimurium fimU* determinant (25). Therefore, some *E. coli* isolates possess genes related to the *Salmonella fimZ* and *fimU* determinants. However, the role, if any, of these *E. coli* genes in affecting fimbriation in these bacteria is unknown. Also, the *fimY* and *fimW* genes of *S. typhimurium* are located between *fimZ* and *fimU*, whereas the *E. coli argU* and the open reading frame are adjacent to each other. It is currently unknown if the *E. coli* open reading frame encodes a polypeptide and if such a gene product is involved in fimbrial expression in *E. coli*.

In both *E. coli* and *Salmonella* spp. the regulation of *fimA* expression appears to involve a complex regulon (6, 9, 13, 16). Initial studies have demonstrated that in addition to *fimZ*, both *fimY* and *fimW*, as well as the tRNA gene *fimU* (25), play a role in fimbrial expression (4, 23). It is unknown whether any of the additional gene products, such as FimY or FimW, directly interact with FimZ to mediate *fimA* expression or if these polypeptides regulate *fimZ*. It is probable that additional genes are also involved in the regulation of fimbrial expression because in other fimbrial systems gene products encoded by determinants that are not linked to the fimbrial gene cluster have been detected (1, 3, 6, 9, 16, 21). It appears that each fimbrial system has evolved to possess its own unique features of regulation that enable the bacteria to modulate fimbrial expression in their appropriate environment.

Currently, we are identifying the precise binding site of FimZ relative to the promoter region of *fimA*. In addition, the role of *fimY* and *fimW* in *fim* gene expression is being investigated. Since *fimZ*, *fimY*, and *fimW* can affect the production of the FimA subunit in *S. typhimurium* (4, 23), the interaction of these genes and their products will need to be elucidated in order to more fully understand *fim* gene expression in *Salmonella*.

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