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Role of FimW, FimY, and FimZ in Regulating the Expression of Type I Fimbriae in *Salmonella enterica* Serovar Typhimurium[▽]

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Type I fimbriae in *Salmonella enterica* serovar Typhimurium are surface appendages that facilitate binding to eukaryotic cells. Expression of the *fim* gene cluster is known to be regulated by three proteins—FimW, FimY, and FimZ—and a tRNA encoded by *fimU*. In this work, we investigated how these proteins and tRNA coordinately regulate *fim* gene expression. Our results indicate that FimY and FimZ independently activate the P_{fimA} promoter which controls the expression of the *fim* structural genes. FimY and FimZ were also found to strongly activate each other's expression and weakly activate their own expression. FimW was found to negatively regulate *fim* gene expression by repressing transcription from the P_{fimY} promoter, independent of FimY or FimZ. Moreover, FimW and FimY interact within a negative feedback loop, as FimY was found to activate the P_{fimW} promoter. In the case of *fimU*, the expression of this gene was not found to be regulated by FimW, FimY, or FimZ. We also explored the effect of *fim* gene expression on *Salmonella* pathogenicity island 1 (SPI1). Our results indicate that FimZ alone is able to enhance the expression of *hilE*, a known repressor of SPI1 gene expression. Based on our results, we were able to propose an integrated model for the *fim* gene circuit. As this model involves a combination of positive and negative feedback, we hypothesized that the response of this circuit may be bistable and thus a possible mechanism for phase variation. However, we found that the response was continuous and not bistable.

Type I fimbriae in *Salmonella enterica* serovar Typhimurium are proteinaceous surface appendages that carry adhesions specific for mannosylated glycoproteins (9). Type I fimbriae are involved in serovar Typhimurium pathogenicity by facilitating the binding to and invasion of intestinal epithelial cells (43). In orally inoculated mice, a wild-type strain has been shown to cause more infections and deaths than a *fim* mutant strain (18). A *fim* mutant has also been shown to exhibit severalfold weaker binding to HEp-2 and HeLa cells, and the defect in binding could be restored by complementing the *fim* system on a plasmid (4). Apart from type I fimbriae, mutations in different *Salmonella* fimbrial systems—*lpf*, *pef*, and *agf*—have all also been shown to greatly reduce virulence in mice (47). These systems appear to work synergistically in order to facilitate colonization of the ileum (5). In serovar Typhimurium, the *fim* gene cluster possesses all of the genes necessary for type I fimbrial production. This gene cluster is composed of six structural genes, three regulators, and a tRNA specific for rare arginine codons (AGA and AGG). The structural genes *fimA*, *fimI*, *fimC*, *fimD*, *fimH*, and *fimF* are all expressed in one transcript from the P_{fimA} promoter (26, 36–38). The regulators *fimZ*, *fimY*, and *fimW* are all expressed from independent promoters (44, 46, 48). The tRNA encoded by *fimU* is located at one end of the cluster and is required for the effective translation of the regulatory genes that all carry rare arginine codons (42).

Type I fimbriation is environmentally regulated with *fim* gene expression favored in static liquid medium, whereas growth on solid medium inhibits expression (17). Moreover, serovar Typhimurium cultures in fimbriae-inducing conditions contain cells in both fimbriated and nonfimbriated states (35). While the regulation of *fim* gene expression has been studied extensively in *Escherichia coli*, far less is known about the regulation in serovar Typhimurium (1, 27). In particular, despite homology between the structural genes for type I fimbriae in *E. coli* and serovar Typhimurium, their expression is regulated in completely different manners. No homologs of *E. coli* regulators, FimB and FimE, are present in serovar Typhimurium (24, 28). Also, the serovar Typhimurium P_{fimA} promoter is inactive in *E. coli*, indicating that the P_{fimA} promoter is regulated by different factors in these two organisms (48). In serovar Typhimurium, the expression of the structural genes is regulated by three transcription factors, FimY, FimZ, and FimW (44, 46, 48). Both FimZ and FimY are essential for the expression of the structural genes from the P_{fimA} promoter (48). In particular, the deletion of either the *fimY* or *fimZ* gene reduces expression from the P_{fimA} promoter and prevents serovar Typhimurium from making type I fimbriae. FimZ has been shown to bind the P_{fimA} promoter and promote transcription (13, 48). FimY, on the other hand, is thought to facilitate the activation of the P_{fimA} promoter, as direct binding has not been observed (44). FimW is a negative regulator of *fim* gene expression (45). FimW has also been suggested to autoregulate its expression, as enhanced P_{fimW} activity has been observed in the Δ*fimW* mutant. In DNA-binding assays, FimW was not observed to bind any of the *fim* promoters. However, FimW was found to interact with FimZ in a LexA-based two-hybrid system in *E. coli* (45). Thus, a possible mechanism for FimW-mediated repression may be that it binds FimZ and prevents it from activating transcription. However, an analysis of the

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FimW amino acid sequence predicts that it has a DNA-binding domain. Moreover, it is related to a broad range of prokaryotic transcription factors, with its closest relatives being BpdT from *Rhodococcus* spp. and an uncharacterized response regulator, TodD, from *Pseudomonas putida* (29, 30). Thus, FimW may also act by an alternate mechanism involving DNA binding.

In addition to these transcription factors, the *fimU* tRNA also plays a role in *fim* gene expression (42). All three regulators—FimZ, FimY, and FimW—contain a number of the rare arginine codons, AGA and AGG, recognized by the *fimU* tRNA. In the case of FimY, Δ*fimU* mutants have been shown to be nonfimbriated due to the inefficient translation of *fimY* mRNA. This translational regulation results from FimY having three rare arginine codons within its first 14 amino acids. The phenotypic effect of the Δ*fimU* mutation could, however, be overcome by expressing *fimU* from a plasmid or by changing these three rare arginine codons in *fimY* to ones more efficiently translated.

As a pathogen, serovar Typhimurium invades host cells by a process in which effector proteins are injected into the target cells with the help of the *Salmonella* pathogenicity island 1 (SPI1) type III secretion system (12, 14). SPI1 gene expression is regulated by a number of proteins, with the critical activator being HilA (2). The expression of *hilA*, in turn, is regulated by three AraC-like transcriptional activators, *hilC*, *hilD*, and *rtsA* (19, 21, 22, 32, 40, 41). HilD activity is controlled by HilE; this protein binds HilD and is thought to prevent it from activating the P_{hilA} promoter (6, 8). FimY and FimZ have been previously shown to regulate SPI1 gene expression by repressing *hilA* expression through their activation of the P_{hilE} promoter (7).

In this work, we investigated the gene circuit regulating *fim* expression. Using genetic approaches, we found that FimZ and FimY activate each other's expression and that each protein can independently activate the P_{fimA} promoter. Moreover, FimZ and FimY were found to be weak autoactivators. Our data also suggest that FimW-mediated repression occurs at the level of *fimY* transcription. With regard to *fimU*, we found that none of the *fim* regulatory genes had any effect on its transcription. As the *fim* gene circuit involves a combination of positive and negative feedback, we tested whether induction was bistable. However, we found the cell population responded homogeneously when induced. Finally, we looked at the link between the *fim* and SPI1 gene circuits and found that the P_{hilE} promoter is activated solely by FimZ. Collectively, these results allow us to propose an integrated model for the regulation of the *fim* gene circuit in serovar Typhimurium.

MATERIALS AND METHODS

General techniques and growth conditions. All culture experiments were performed in Luria-Bertani (LB) broth (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl) at 37°C unless otherwise noted. Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml, chloramphenicol at 20 μg/ml, and kanamycin at 40 μg/ml. All experiments involving the growth of cells carrying pKD46 were performed at 30°C as previously described (15). Loss of the helper plasmid pKD46 was achieved by growth in nonselective conditions on LB agar at 42°C. The removal of the antibiotic cassette from the FLP recombinant target (FRT)-chloramphenicol/kanamycin-FRT insert was obtained by the transformation of pCP20 into the respective strain and selection on ampicillin at 30°C. The loss of the helper plasmid pCP20 was obtained by growth at 42°C under nonselective conditions on LB agar (10). Integrations into the λ*attB* sites of the serovar Typhimurium and *E. coli* genomes were done using the helper plasmid pInt-ts as described previously (25). The loss of the helper plasmid pInt-ts was obtained by growth at 42°C under nonselective conditions. Primers were purchased from IDT, Inc. Enzymes were purchased from New England Biolabs and Fermentas and used according to the manufacturer's recommendations.

TABLE 1. Strains used during this study

Strain	Genotype or characteristic[a]
14028	Wild-type serovar Typhimurium
CR311	Δ*fimY*::FRT *cm* FRT
CR312	Δ*fimZ*::FRT *cm* FRT
CR313	Δ*fimYZ*::FRT *cm* FRT
CR314	Δ*fimW*::FRT *cm* FRT
CR315	Δ*fimU*::FRT *cm* FRT
CR316	Δ*fimU*::FRT
CR317	14028 *att*λ::pVenus::P_{fimA} *venus*
CR318	14028 *att*λ::pVenus::P_{fimY} *venus*
CR319	14028 *att*λ::pVenus::P_{fimZ} *venus*
CR320	14028 *att*λ::pVenus::P_{fimw} *venus*
CR321	14028 *att*λ::pVenus::P_{fimU} *venus*
CR322	Δ*fimY*::FRT
CR323	Δ*fimY*::FRT *att*λ::pVenus::P_{fimA} *venus*
CR324	Δ*fimY*::FRT *att*λ::pVenus::P_{fimY} *venus*
CR325	Δ*fimY*::FRT *att*λ::pVenus::P_{fimZ} *venus*
CR326	Δ*fimY*::FRT *att*λ::pVenus::P_{fimw} *venus*
CR327	Δ*fimY*::FRT *att*λ::pVenus::P_{fimU} *venus*
CR328	Δ*fimZ*::FRT
CR329	Δ*fimZ*::FRT *att*λ::pVenus::P_{fimA} *venus*
CR330	Δ*fimZ*::FRT *att*λ::pVenus::P_{fimY} *venus*
CR331	Δ*fimZ*::FRT *att*λ::pVenus::P_{fimZ} *venus*
CR332	Δ*fimZ*::FRT *att*λ::pVenus::P_{fimw} *venus*
CR333	Δ*fimZ*::FRT *att*λ::pVenus::P_{fimU} *venus*
CR334	Δ*fimYZ*::FRT
CR335	Δ*fimYZ*::FRT *att*λ::pVenus::P_{fimA} *venus*
CR336	Δ*fimYZ*::FRT *att*λ::pVenus::P_{fimY} *venus*
CR337	Δ*fimYZ*::FRT *att*λ::pVenus::P_{fimZ} *venus*
CR338	Δ*fimYZ*::FRT *att*λ::pVenus::P_{fimw} *venus*
CR339	Δ*fimYZ*::FRT *att*λ::pVenus::P_{fimU} *venus*
CR340	Δ*fimW*::FRT
CR341	Δ*fimW*::FRT *att*λ::pVenus::P_{fimA} *venus*
CR342	Δ*fimW*::FRT *att*λ::pVenus::P_{fimY} *venus*
CR343	Δ*fimW*::FRT *att*λ::pVenus::P_{fimZ} *venus*
CR344	Δ*fimW*::FRT *att*λ::pVenus::P_{fimw} *venus*
CR345	Δ*fimW*::FRT *att*λ::pVenus::P_{fimU} *venus*
CR346	14028 *att*λ::pVenus::P_{hilE} *venus*
CR347	Δ*fimYZ*::FRT *att*λ::pVenus::P_{hilE} *venus*
CR348	Δ*fimYZ*::FRT Δ*fimW*::FRT *att*λ::pVenus::P_{fimY} *venus*

[a] All *Salmonella* strains are isogenic derivatives of the serovar Typhimurium strain 14028. Strains are from this study except for the wild-type 14028, which is from the American Type Culture Collection.

Strain and plasmid construction. All bacterial strains and plasmids used in this study are described in Tables 1 and 2, respectively. All serovar Typhimurium strains are isogenic derivatives of strain 14028 (American Type Culture Collection [ATCC]). The generalized transducing phage of serovar Typhimurium P22 HT105/1int-201 was used in all transductional crosses (16).

The plasmids pKD3 and pKD4 were used as templates to generate scarred FRT mutants as described previously (14). The Δ*fimYZ* mutant was made using primers SS105F and SS105R. The Δ*fimZ* mutant was made using primers SS105FII and SS105F. The Δ*fimY* mutant was made using the primers SS105RII and SS105R. The Δ*fimW* mutant was made using primers SS152F and SS152R. The Δ*fimU* mutant was made using the primers SS165F and SS165R. All mutations were checked by PCR using primers that bound outside the deleted region. Prior to the removal of the antibiotic resistance marker, the constructs resulting from this procedure were moved into a clean wild-type background (14028) by P22 transduction.

In order to construct the fluorescent Venus reporter plasmid (34), PCR was used to amplify Venus from pBS7 using primers LC294F and LC296R. The resulting PCR product was used as a template with primers LC295F and LC296R to add three out-of-frame stop codons and a synthetic Shine-Dalgarno sequence before the Venus start codon. The resultant PCR product was then digested with EcoRI and HindIII and subcloned into the EcoRI and HindIII cut sites of pQE80L (Qiagen),

TABLE 2. Plasmids used during this study

Plasmid	Relevant characteristics	Source or reference[a]
pKD46	*bla* P_{BAD} *gam beto exo* pSC101 *oriTS*	15
pCP20	*bla cat* λ*c*I857 λPRflp pSC101 *oriTS*	10
pKD3	*bla* FRT *cm* FRT *oriR6K*	15
pKD4	*bla* FRT *kan* FRT *oriR6K*	15
plnt-ts	*bla lnt oriR6K*	29
pQE80L	*bla lacI*^{q} ColE1	Qiagen
pAH125	*kan lacZ att*λ *oriR6K*	29
pVenus	*kan venus att*λ *oriR6K*	
P_{fimA}-Venus	*kan* P_{fimA} *venus att*λ *oriR6K*	
P_{fimY}-Venus	*kan* P_{fimY} *venus att*λ *oriR6K*	
P_{fimZ}-Venus	*kan* P_{fimZ} *venus att*λ *oriR6K*	
P_{fimw}-Venus	*kan* P_{fimw} *venus att*λ *oriR6K*	
P_{fimU}-Venus	*kan* P_{fimU} *venus att*λ *oriR6K*	
P_{hilE}-Venus	*kan* P_{hilE} *venus att*λ *oriR6K*	
pPROTet.E	*cm* $P_{LtetO-1}$ *ori* ColE1	Stratagene
pSS012 (pPROTet.E tetR)	*cm* $P_{LtetO-1}$ *tetR* ori ColE1	45
pSS039 (pFimY)	*cm* $P_{LtetO-1}$ *fimY tetR ori* ColE1	
pSS040 (pFimZ)	*cm* $P_{LtetO-1}$ *fimZ tetR ori* ColE1	
pSS041 (pFimW)	*cm* $P_{LtetO-1}$ *fimW tetR ori* ColE1	
pSS042 (pFimY*)	*cm* $P_{LtetO-1}$ *fimY* tetR ori* ColE1	

[a] Plasmids are from this study unless specified otherwise.

yielding pQE80L-*Venus*. The plasmid pQE80L was digested with EcoRI and NheI, and the fragment was cloned into the conditional-replication, integration, and modular (CRIM) plasmid pAH125 digested with EcoRI and NheI (25). The resulting CRIM plasmid was called pVenus. Venus transcriptional fusions were made by amplifying the promoter of interest and then cloning these PCR fragments into the multiple cloning site of pVenus. The *fimA* transcriptional fusion was made using primers SS104F and SS104R. The *fimY* transcriptional fusion was made using primers SS037F and SS037R. The *fimZ* transcriptional fusion was made using primers SS103F and SS103R. The *fimW* transcriptional fusion was made using primers SS154F and SS154R. The *fimU* transcriptional fusion was made using primers SS162F and SS162R. The *hilE* transcriptional fusion was made using primers SS024F and SS024R. The PCR fragments were then digested with KpnI and EcoRI (sequences underlined) and cloned into the multiple cloning site of the pVenus vector. The resulting transcriptional fusions were integrated into the serovar Typhimurium and *E. coli* chromosomes at the λ*attB* site using λInt produced from the CRIM helper plasmid pInt-ts, thus creating single-copy transcriptional fusions. In the case of serovar Typhimurium, the integrated plasmid was moved into different mutant strains by P22 transduction.

Expression plasmids for *fimY*, *fimZ*, and *fimW* were made by cloning the respective gene into the multiple cloning site of pPROTet.E (Clontech) under the control of a strong promoter, $P_{LTetO-1}$, resulting in plasmids pFimY, pFimZ, and pFimW (33). The plasmid pFimZ was made first by amplifying the *fimZ* gene using the primers SS106F and SS106R. The PCR product was then digested with EcoRI and KpnI and cloned into pPROTet.E. The plasmid pFimY was made by amplifying the *fimY* gene using the primers SS107F and SS107R. The PCR product was then digested with SalI and BamHI and cloned into pPROTet.E. The plasmid pFimW was made by amplifying the *fimW* gene using the primers SS160F and SS160R. The PCR product was then digested with EcoRI and HindIII and cloned into pPROTet.E. In order to mutate the first three arginine rare codons at positions 7, 9, and 14 in *fimY*, primers SS162F and SS107R were used to amplify *fimY* with the rare arginine codons mutated to consensus arginine codons. The resulting PCR product was used as a template with primers SS167F and SS107R. The amplified product was digested with EcoRI and BamHI and cloned into the multiple cloning site of pPROTet.E. The plasmid is called pFimY*.

In our expression plasmids, in the absence of TetR, the $P_{LTetO-1}$ promoter is constitutively active. To regulate the expression levels from the $P_{LTetO-1}$ promoter, the *tetR* gene was also cloned downstream of the gene target into the plasmids as previously described (39). In this arrangement, in the absence of the inducer anhydrotetracycline (aTc), expression from the promoter is inhibited due to TetR. The inhibition, however, is relieved upon the addition of 100 ng/ml of aTc, and expression from the $P_{LTetO-1}$ promoter then takes place. All constructs were sequenced prior to transforming into the wild-type and mutant strains. The sequences for all the primers used in this study are given in Table 3.

Fluorescence assays. As an indirect measure of gene expression, end-point and dynamic measurements of the fluorescent reporter system were made using a Tecan Safire2 microplate reader. For fluorescence end-point measurements, 1 ml culture was grown at 37°C overnight and then subcultured 1:1,000 in fresh medium and grown in static conditions for 24 h at 37°C. A total of 100 μl of the culture was then transferred to a 96-well microplate, and the relative fluorescence and optical density at 600 nm (OD_{600}) measured. The fluorescence readings were normalized with the OD_{600} to account for cell density. For time course measurements, overnight cultures at 37°C were subcultured to an OD of 0.05 in fresh medium and allowed to grow to an OD of 0.15. A total of 100 μl of the culture was then transferred to a 96-well microplate and overlaid with 25 μl of oil to prevent evaporation. The temperature was maintained at 37°C, and fluorescence and OD readings were taken every 5 min. All experiments were done in triplicate and average values with the standard deviations reported.

Single-cell measurements were done similarly by growing the cells in noninducing conditions with vigorous shaking at 37°C. Overnight cultures were subcultured to an OD of 0.05 in fresh medium (LB) and grown in inducing conditions of high oxygen and no shaking at 37°C. Samples were collected at different time points by spinning the cells down, resuspending them in phosphate-buffered saline supplemented with chloramphenicol (34 μg/ml) to stop all translation and arrest the cells in their respective state, and finally storing on ice. All flow cytometry experiments were performed on a BD LRS II system (BD Biosciences). The data extraction and analysis for the flow cytometry experiments were done using FCS Express version 3 (De Novo Software).

RESULTS

FimZ and FimY are activators and FimW is a repressor of *fim* gene expression. FimZ and FimY have previously been reported as activators of *fim* gene expression in serovar Typhimurium (44, 48). Both have also been reported as essential for fimbriation, as the deletion of either one results in the loss of expression from the P_{fimA} promoter (49). To understand the roles of FimZ and FimY in the *fim* gene circuit, we measured expression from the P_{fimA}, P_{fimZ}, P_{fimY}, and P_{fimW} promoters in the wild type and the Δ*fimZ*, Δ*fimY*, Δ*fimYZ*, and Δ*fimW* mutants (Fig. 1). Chromosomally integrated Venus transcriptional reporters were employed as indirect measures of promoter activities (34). In the cases of all four promoters, activity levels were found to be about two times less active in the Δ*fimZ*, Δ*fimY*, and Δ*fimYZ* mutants than in the wild type. For all four promoters, note that no further reduction in promoter activity was observed in the double mutant. In a Δ*fimW* mutant, the activities of all four promoters were approximately two times higher than the wild-type levels. While these results agree with previously published data regarding the *fim* system in serovar Typhimurium, they still do not tell us how FimW, FimY, and FimZ individually contribute to P_{fimA} activation.

FimY and FimZ are strong activators of each other's expression and weak activators of their own expression. To determine the relative effect of FimY and FimZ on *fim* gene expression, the P_{fimY} and P_{fimZ} promoter activities were measured in a Δ*fimYZ* mutant in which either FimZ or FimY was expressed from a strong, aTc-inducible promoter on a plasmid (see Materials and Methods). Using this system, we found that expressing FimZ in the Δ*fimYZ* mutant led to a more than 10-fold increase in P_{fimY} activity (Fig. 2A). Likewise, expressing FimY in the Δ*fimYZ* mutant led to about a 10-fold increase in P_{fimZ} levels. In addition to their ability to activate each other's promoters, FimY and FimZ were found to increase expression from their own promoters roughly threefold.

Even though *E. coli* makes type I fimbriae, the serovar Ty-

TABLE 3. List of primers used in the study

Primer	Sequence	Characteristic
SS105F	TGT CCG TTA TTG TGG CTC CCG AAC GAT AAT TCG CCG GGA GGA TGG TAG TGT GGG GTC TCC	*fimYZ* knockout primer
SS105R	ATC AAT CAG TTT CTT TAA TAT TTC ACC ATG ATT CAC CTG CCA TGG GAA TTA GCC ATG GTC C	*fimYZ* knockout primer
SS105FII	TTA TAA AAC GAA GGA CGC ATA ACA GTC TGA GGC ATA CAA ATG GGA ATT AGC CAT GGT CC	*fimZ* knockout primer
SS105RII	GTA ATT TCT TAA AAA ATC TTA TTC ACC AAA ACG TTA CTT CGA TGG TAG TGT GGG GTC TCC	*fimY* knockout primer
SS152F	TAT TTC ACC ATG ATT CAC CTG CCG TGT AGG ATA TTT TTT TGT GTA GGC TGG AGC TGC TTC	*fimW* knockout primer
SS152R	GGT GAG ATA TTT CGT AAG CCT TGT AAA AAG TTA AGT GAG TCA TAT GAA TAT CCT CCT TAG	*fimW* knockout primer
SS165F	CTC GCG TTT CGT CTA CAC GAA GTC TTC ACT TCA CAA GGC GGT GTA GGC TGG AGC TGC TTC	*fimU* knockout primer
SS165R	GGA AAA TAA GGA GGA AAT AAA GAA GCG TAA CAC GTT GAT TCA TAT GAA TAT CCT CCT TAG	*fimU* knockout primer
LC294F	GAT TAA CTT TAT AAG GAG GAA AAA CAT ATG AGT AAA GGA GAA GAA CTT TTC	*venus* gene
LC296R	ATA AAG CTT TTA TTT GTA TAG TTC ATC CAT GCC ATG	*venus* gene
LC295F	CTC GAA TTC CCT AAC TAA CTA AAG ATT AAC TTT ATA AGG AGG A	*venus* gene
SS104F	TTT GGT ACC AAA TCT GTG AGG CCG GAT TG	*fimA* promoter
SS104R	GGG GAA TTC GTA GAG GTC ATT AAT TTA TG	*fimA* promoter
SS037F	TCT GGT ACC AAA ATA TAT TAG AGT TAA CC	*fimY* promoter
SS037R	AAA GAA TTC CCC TGC GTG GTA CGC TGC GC	*fimY* promoter
SS103F	TTT GGT ACC ATA AAA CCT CCG CTA TAA CA	*fimZ* promoter
SS103R	GGG GAA TTC CCA TAA TGA TAA CAG ATG CA	*fimZ* promoter
SS154F	GGG GGT ACC GGA TTC GAA CCT GCG ACC CA	*fimW* promoter
SS154R	GGG GAA TTC TTT TCC GGG TAA TTT CTT CA	*fimW* promoter
SS162F	GGG GGT ACC CGT TTC GCT TAA ATG ATA AC	*fimU* promoter
SS162R	GGG GAA TTC CTA TCC AAC TGA GCT AAG GG	*fimU* promoter
SS024F	AAG GGT ACC ATG ACG TTG CGT AGC GTT GG	*hilE* promoter
SS024R	GGT GAA TTC GAA AGA ACG TTC CAT TTT CC	*hilE* promoter
SS106F	GGG GAA TTC TAA CAG TCT GAG GCA TAC AA	*fimZ* gene
SS106R	TTT GGT ACC TTA CAA TAA TTC GTG TGA TT	*fimZ* gene
SS107F	CCT GTC GAC ATA TTA GAG CAA TGG AAA A	*fimY* gene
SS107R	CCC GGA TCC TTA AAA AAT GTC GTG GAA AG	*fimY* gene
SS160F	ATA GAA TTC GCC GTG TAG GAT ATT TTT TT	*fimW* gene
SS160R	ATA AAG CTT TTA TTA CTT ACT GAG TAA GAA ATG AAG G	*fimW* gene
SS162F	GGG GGT ACC ATG CGC AGC GTA CCA CGC CGG GAA CGA CAC CGC CGT TTA CGA AAT GCT AA	*fimY** gene
SS167F	GGG GAA TTC TTT ATA AGG AGG AAA AAC ATA TGC GCA GCG TAC CAC GCC GG	*fimY** gene

phimurium *fim* promoters by themselves are inactive in this organism. Therefore, we performed an identical set of experiments with *E. coli* using the serovar Typhimurium proteins and promoters. Overall, the results were identical to those for serovar Typhimurium (Fig. 2B). In particular, FimZ expression led to a more than 10-fold increase in P_{fimY} promoter activity, and FimY expression led to a 10-fold increase in P_{fimZ} activity. Both FimZ and FimY were again found to weakly activate expression from their own promoters. The goal of these experiments was to remove the effect of any serovar Typhimurium-specific regulatory mechanisms, thus allowing us to more confidently conclude that the observed results are due to direct interactions. Collectively, these results show that FimY and FimZ strongly activate each other's expression and weakly activate their own expression. This cross-regulation also explains why both FimY and FimZ are required for strong P_{fimA} promoter activity, as the expression of each is dependent on the other.

FimZ and FimY can independently activate expression from the P_{fimA} promoter. Next, we looked at how FimZ and FimY independently affected P_{fimA} expression. To investigate this problem, we measured P_{fimA} promoter activity in a Δ*fimYZ* mutant in which either FimY or FimZ was expressed using the aTc-inducible system. FimZ expression was found to strongly (>15-fold) activate the P_{fimA} promoter, whereas FimY could only weakly (more than twofold) activate it (Fig. 3). We also performed these experiments with *E. coli* with similar results (data not shown). Based on these results, we conclude that FimZ and FimY can both independently activate the P_{fimA} promoter. In the case of FimY, the weak activation of the P_{fimA} promoter is likely due to its strong dependence on *fimU* tRNA (see below) (42).

FimY activates the P_{fimW} promoter, and FimW represses the P_{fimY} promoter. FimW has previously been observed to repress *fim* gene expression (45). Consistent with these results, we observed that P_{fimA}, P_{fimW}, P_{fimY}, and P_{fimZ} promoter activities were all elevated in a Δ*fimW* mutant (Fig. 1). To understand the mechanism of FimW-mediated repression, we first sought to identify the proteins that regulate expression from the P_{fimW} promoter. To answer this question, we measured the level of expression from the P_{fimW} promoter in a Δ*fimYZ* mutant in which FimW, FimY, and FimZ were independently expressed using the aTc-inducible system. In the case of FimW and FimZ, expression had no effect on P_{fimW} promoter activity

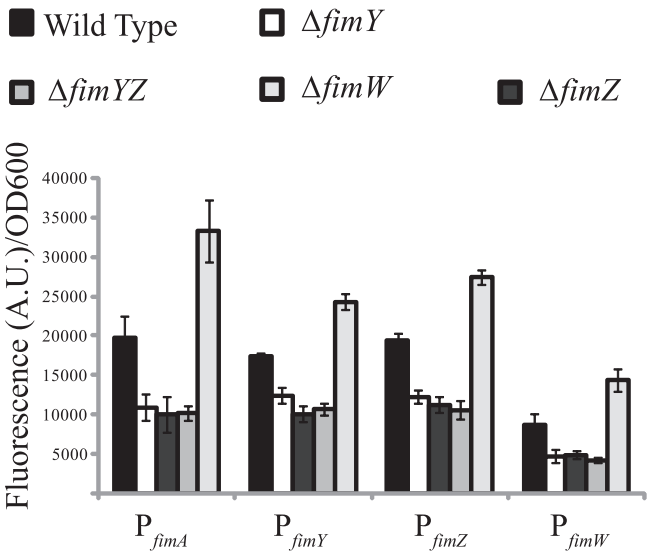

FIG. 1. FimY and FimZ are activators and FimW is a repressor of *fim* gene expression. Shown is a comparison of the P_{fimA}, P_{fimY}, P_{fimZ}, and P_{fimW} promoter activities in the wild type and the Δ*fimY*, Δ*fimZ*, Δ*fimYZ*, *and* Δ*fimW* mutants. Data are averages of the results from three experiments. Each experiment was done in triplicate.

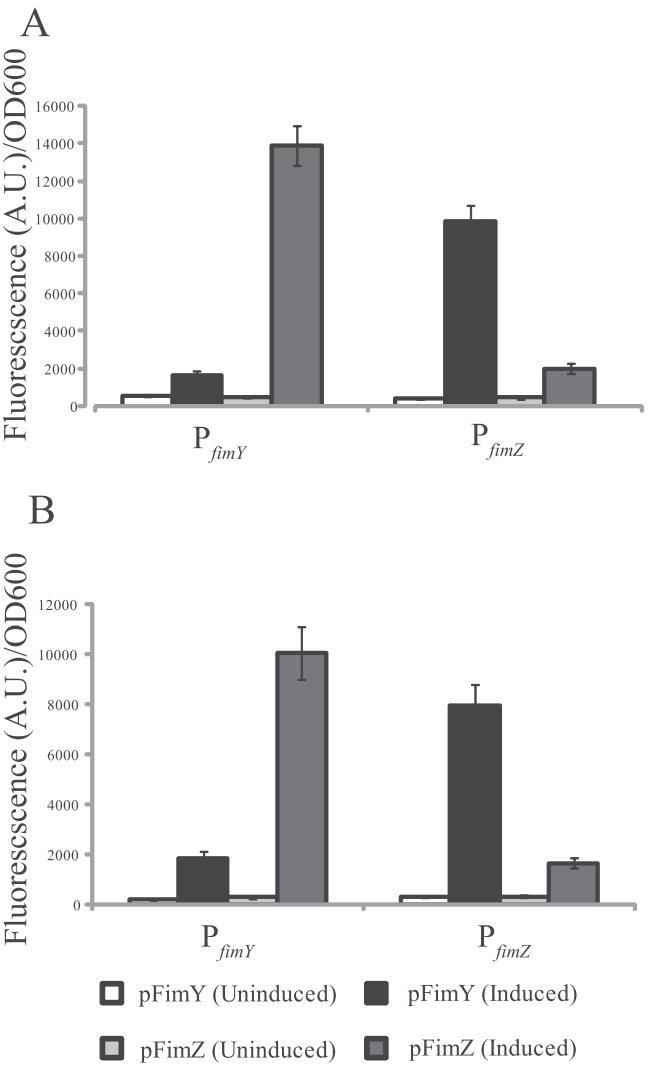

FIG. 2. FimY and FimZ are strong activators of each other's expression and also weak autoactivators. Shown is a comparison of the P_{fimY} and P_{fimZ} promoter activities in a serovar Typhimurium Δ*fimYZ* mutant (A) and *E. coli* (B) in which FimY and FimZ are independently expressed from an aTc-inducible promoter on a plasmid. Note that *tetR* is also expressed from this plasmid in order to achieve aTc-inducible expression. Data are averages of the results from three experiments. Each experiment was done in triplicate.

(data not shown). However, in the case of FimY, we observed a significant increase in P_{fimW} promoter activity (1,052 ± 381 relative fluorescence units [RFU]/OD [uninduced] versus 14,718 ± 1,032 RFU/OD [induced]). Similar results were also obtained when these experiments were performed with *E. coli* (data not shown). To identify the regulatory targets of FimW, we measured the expression of the P_{fimA}, P_{fimZ}, and P_{fimY} promoters in a Δ*fimW* Δ*fimYZ* mutant in which FimW was expressed using the aTc-inducible system. In the cases of the P_{fimA} and P_{fimZ} promoters, we found that FimW expression had no effect. However, in the case of the P_{fimY} promoter, FimW expression led to about a threefold decrease in P_{fimY} activity (7,462 ± 319 RFU/OD [uninduced] versus 2,781 ± 188 RFU/OD [induced]). Based on these results, we conclude that FimY activates expression from the P_{fimW} promoter and that FimW represses expression from the P_{fimY} promoter.

The P_{fimU} promoter is not regulated by FimW, FimY, or FimZ. Both *fimY* and *fimZ* contain rare arginine codons (AGA and AGG) and need *fimU*, a tRNA specific for rare arginine codons, for effective translation. In a Δ*fimU* mutant, P_{fimA} activity was less than 10-fold compared to the wild-type levels (wild type, 16,723 ± 1,173 RFU/OD; the Δ*fimU* mutant, 1,389 ± 261 RFU/OD). The expression of FimY in the Δ*fimU* mutant using the aTc-inducible system, however, did not increase P_{fimA} activity (988 ± 319 [uninduced] versus 1,343 ± 166 [induced]). Replacing the rare arginine codons in the *fimY* gene with consensus ones did restore P_{fimA} activity to the wild-type levels (817 ± 73 RFU/OD [uninduced] versus 11,294 ± 462 RFU/OD [induced]). These experiments are consistent with previously published results (45) and indicate that *fimU* is essential for effective *fimY* translation.

As *fimU* has a strong effect on P_{fimA} promoter activity, we hypothesized that it may be subject to regulation by the other proteins within the circuit. To test this hypothesis, we measured P_{fimU} promoter activity in different regulatory mutants. Contrary to our hypothesis, we did not observe any change in P_{fimU} promoter activity in any mutant (wild type, 26,717 ± 1,381 RFU/OD; the Δ*fimZ* mutant, 28,991 ± 2,164 RFU/OD; the Δ*fimY* mutant, 25,884 ± 1,983 RFU/OD; the Δ*fimYZ* mutant, 26,516 ± 1,772 RFU/OD; and the Δ*fimW* mutant, 24,829 ± 2,073 RFU/OD). Likewise, we did not observe any change in P_{fimU} promoter activity when FimW, FimY, and FimZ were expressed using the aTc-inducible system in wild-type serovar Typhimurium or *E. coli* (data not shown). Based on these results, we conclude that the P_{fimU} promoter is not regulated by any *fim* protein.

FimZ alone is able to regulate SPI1 gene expression. Previous studies have shown that both FimY and FimZ regulate

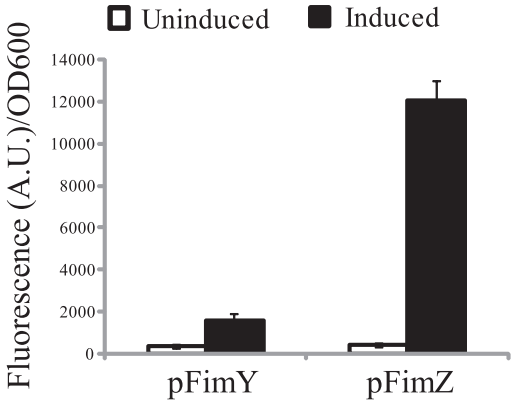

FIG. 3. FimY and FimZ can independently activate expression from the P_{fimA} promoter. Shown is a comparison of P_{fimA} promoter activities in a Δ*fimYZ* mutant in which FimY and FimZ are independently expressed from an aTc-inducible promoter on a plasmid. Data are averages of the results from three experiments. Each experiment was done in triplicate.

SPI1 expression through their activation of the P_{hilE} promoter (7). HilE, in turn, is known to bind HilD and repress the HilD-mediated activation of the P_{hilA}, P_{hilC}, P_{rtsA}, and P_{hilD} promoters (6, 20). To test which protein activates the P_{hilE} promoter, we independently expressed FimY and FimZ in a Δ*fimYZ* mutant using the aTc-inducible system and then measured the level of expression from the P_{hilE} promoter. Of the two, only FimZ was found to affect P_{hilE} expression (1,089 ± 421 RFU/OD [uninduced] versus 17,654 ± 2,234 RFU/OD [induced]). Similar results were also observed for *E. coli* (data not shown).

We note that these results are contrary to those previously reported, for which it was shown that both FimY and FimZ were necessary for activation of the P_{hilE} promoter (7). One possible explanation for the discrepancy involves how the two gene products were selectively expressed. In the original study, a DNA fragment containing the *fimYZ* gene cluster was cloned onto a plasmid and expressed using the tetracycline promoter. To study their relative effects, each gene was selectively inactivated using a universal translational terminator. As part of the P_{fimY} promoter and the whole P_{fimZ} promoter were left intact in their construct, transcriptional inference may have occurred between the various promoters. In our design, we selectively cloned each gene and then expressed it from an inducible promoter, eliminating any potential interfering effects from having the native promoters still present.

Dynamics of *fim* gene expression. Finally, we wished to investigate the dynamics of *fim* gene expression. We first measured P_{fimA} promoter activity in the wild type and the Δ*fimY*, Δ*fimZ*, Δ*fimYZ*, and Δ*fimW* mutants using a microplate reader (Fig. 4A). Consistent with our end-point measurements, we found that the P_{fimA} promoter was weakly expressed in the Δ*fimY*, Δ*fimZ*, and Δ*fimYZ* mutants. Likewise, expression was enhanced in a Δ*fimW* mutant. Note that the microplate experiments tell us only about the average response of the population and nothing about how individual cells are behaving. To test whether the cells were responding homogeneously, we also performed single-cell measurements of P_{fimA} promoter activity at selected times in the wild type and a Δ*fimW* mutant using

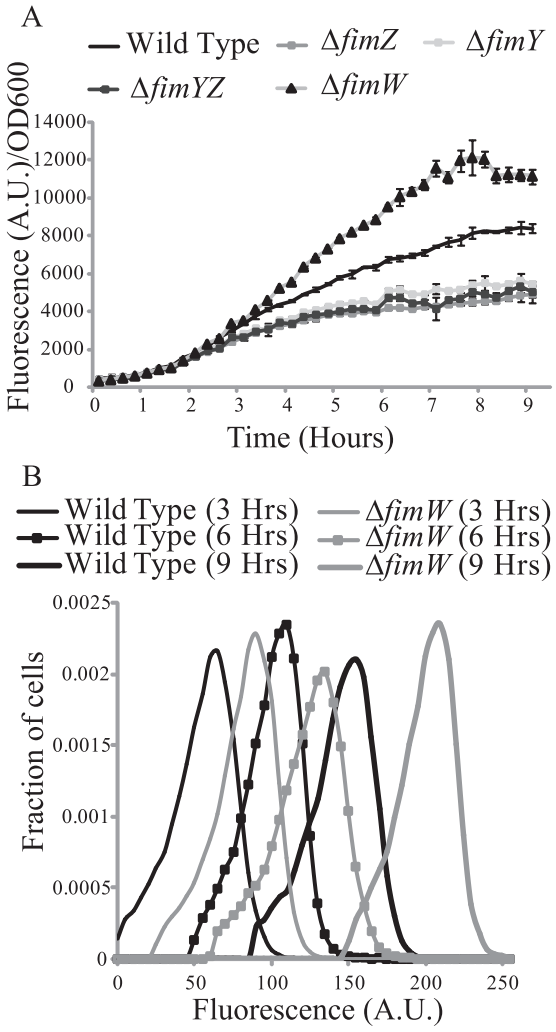

FIG. 4. Dynamics of P_{fimA} promoter activity. (A) Population average P_{fimA} activity as a function of time in the wild type and the Δ*fimY*, Δ*fimZ*, Δ*fimYZ*, and Δ*fimW* mutants. Data are averages of the results from a single experiment with an average of six independent cultures. The experiment was repeated thrice, and identical results were observed. (B) Histogram of single-cell P_{fimA} promoter activity at selected times in the wild type and a Δ*fimW* mutant. Single-cell measurements of promoter activity were obtained using flow cytometry. Population distribution data are from a single experiment. The experiment was repeated thrice, and identical results were observed (data not shown).

flow cytometry (Fig. 4B). Our results indicate that individual wild-type and Δ*fimW* mutant cells are responding homogeneously with respect to P_{fimA} promoter activity at all times tested. In other words, we did not observe any phase variation or heterogeneity with regard to P_{fimA} promoter activity in our kinetic experiments.

DISCUSSION

In this work, we investigated the regulatory gene circuit controlling the expression of type I fimbriae in serovar Typhimurium. Using genetic approaches, we demonstrated that FimY and FimZ independently activate the P_{fimA} promoter. Of the two, FimZ was found to be the dominant activator. We also

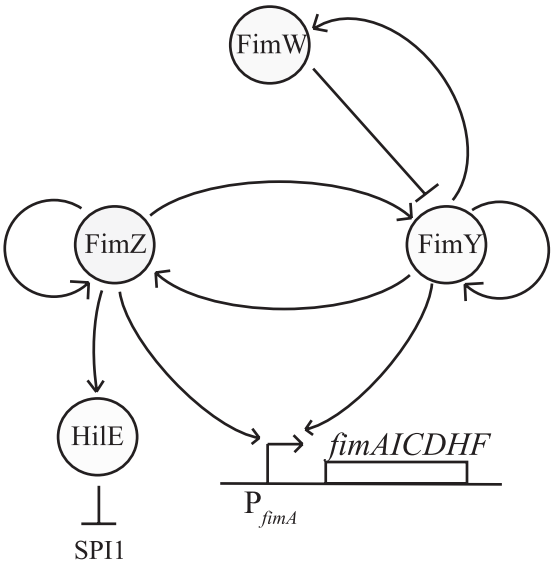

FIG. 5. Model for the type I fimbria gene circuit in serovar Typhimurium.

found that FimY and FimZ strongly activate each other's expression and weakly activate their own expression. In addition to these two positive regulators, a third regulator, FimW, is known to repress *fim* gene expression. We found that FimW negatively regulates *fim* gene expression by repressing expression from the P_{fimY} promoter. Furthermore, FimW participates in a negative feedback loop as FimY was found to enhance P_{fimW} expression. Interestingly, these results suggest that FimY is both an activator and a repressor of *fim* gene expression, as it can directly activate the P_{fimZ}, P_{fimY}, and P_{fimA} promoters and indirectly repress them by enhancing FimW expression. In addition to these regulators, type I fimbriation is also dependent on the expression of a rare arginine codon tRNA, *fimU*. However, our results showed that the P_{fimU} promoter is not regulated by FimY, FimZ, or FimW. The results suggest that *fimU* does not play a role in the internal regulation of the circuit. Finally, we demonstrated that the previously observed coordinate regulation of SPI1 gene expression by the *fim* gene circuit (7) occurs through the activation of *hilE* expression by FimZ. Based on these results, we are able to propose the following model for the *fim* gene circuit in serovar Typhimurium (Fig. 5).

According to our model, the induction of the *fim* circuit begins with the activation of the P_{fimY} and P_{fimZ} promoters, resulting in small amounts of *fimY* and *fimZ* being expressed. FimY and FimZ then rapidly accumulate in the cell due to the positive feedback loop formed by the cross-activation of the P_{fimY} and P_{fimZ} promoters by these two proteins. The expression of the type I fimbrial structural genes from the P_{fimA} promoter commences when the concentration of FimY and FimZ within the cell rises beyond a critical level. These two regulators can independently activate the P_{fimA} promoter; however, their expression is correlated, as each activates the other's expression. Moreover, FimY and FimZ protein expression levels are controlled by a negative feedback loop involving FimW. In this loop, FimY activates the expression of the P_{fimW} promoter, and FimW represses the expression of the P_{fimY} promoter. We hypothesize that this negative feedback loop involving FimW prevents the runaway expression of FimY and FimZ arising from their participation in interacting positive feedback. Specifically, we hypothesize that when FimY and FimZ reach their optimum expression levels, the FimW negative feedback loop is activated and halts expression from the P_{fimY} and P_{fimZ} promoters.

While our model for the *fim* circuit explains internal regulation, it still does not explain how the circuit is activated. In particular, we do not know which factors induce the P_{fimY} and P_{fimZ} promoters. We suspect that these factors activate both promoters, as each alone exhibits some activity in a Δ*fimYZ* mutant (Fig. 1). In addition to these factors, another open question concerns whether *fimU* plays a role in regulating circuit dynamics. While it is tempting to speculate that *fimU* expression is tuned in response to environmental signals and thus affects circuit dynamics, more likely this gene is constitutively expressed like other tRNAs.

Our results also indicate that the FimW-mediated inhibition of *fim* gene expression is through repression of the P_{fimY} promoter. Earlier reports suggested that FimW binds FimZ and somehow inhibits the FimZ-dependent activation of *fim* promoters (45). Moreover, FimW was not found to bind to the P_{fimW} promoter. Based on these results, FimW would appear to repress the P_{fimY} promoter by preventing FimZ from activating it. However, we found that FimW is able to repress the P_{fimY} promoter in the absence of FimZ. Our results would suggest that FimW directly binds the P_{fimY} promoter and represses transcription independently of FimZ. Consistent with our model, FimW has a C-terminal LuxR-type helix-turn-helix DNA domain (SM00421) (31). However, at this time we have no direct experimental support for such a mechanism. Moreover, an equally likely hypothesis is that repression by FimW is indirect. Further experiments are clearly required to determine the mechanism of FimW-mediated repression and distinguish between these different putative models.

A final unanswered question concerns the role of the positive and negative feedback loops in the *fim* gene circuit. Our initial hypothesis was that these feedback loops would result in bistability. In particular, interacting positive and negative feedback loops are known to be sufficient ingredients for bistability (23). This bistability could potentially explain the phase variation observed in type I fimbriation during growth in inducing conditions (35). To test whether the *fim* circuit exhibited bistability, we measured P_{fimA} activity at single-cell resolution as a function of time. Contrary to our initial hypothesis, we did not observe a heterogeneous or switch-like response in induction, the telltale indicator of bistability. Rather, we observed a continuous or rheostat-like response in both the wild type and a Δ*fimW* mutant (3). One possibility is that there is a lack of correlation between *fim* gene expression and the production of type I fimbria in serovar Typhimurium (11). Another is that the environment may dictate the response characteristics. For example, under conditions different from those used in our study, Duguid and coworkers observed subpopulations of cells expressing type I fimbriae, indicative of phase variation (17). With these in mind, we hypothesize that the bacteria exhibit type I fimbria phase variation under specific environmental

conditions and that the regulation of this process involves posttranscriptional mechanisms as well.

ACKNOWLEDGMENTS

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