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T-POP Array Identifies EcnR and PefI-SrgD as Novel Regulators of Flagellar Gene Expression▽†

Christopher E. Wozniak,[1]‡ Changhan Lee,[1] and Kelly T. Hughes[2]*

Department of Microbiology, University of Washington, Seattle, Washington 98195,[1] and Department of Biology, University of Utah, Salt Lake City, Utah 84112[2]

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The T-POP transposon was employed in a general screen for tetracycline (Tet)-induced chromosomal loci that exhibited Tet-activated or Tet-repressed expression of a *fliC-lac* transcriptional fusion. Insertions that activated flagellar transcription were located in flagellar genes. T-POP insertions that exhibited Tet-dependent *fliC-lac* inhibition were isolated upstream of the *ecnR*, *fimZ*, *pefI-srgD*, *rcsB*, and *ydiV* genes and in the flagellar gene *flgA*, which is located upstream of the anti-σ^{28} factor gene *flgM*. When expressed from the chromosomal P_{araBAD} promoter, EcnR, FimZ, PefI-SrgD, and RcsB inhibited the transcription of the flagellar class 1 *flhDC* operon. YdiV, which is weakly homologous to EAL domain proteins involved in cyclic-di-GMP regulation, appears to act at a step after class 1 transcription. By using a series of deletions of the regulatory genes to try to disrupt each pathway, these regulators were found to act largely independently of one another. These results identify EcnR and PefI-SrgD as additional components of the complex regulatory network controlling flagellar expression.

Flagella are rotary propellers that some bacteria use to swim through liquid environments. A single flagellum can grow to 10 times the length of the cell and be composed of tens of thousands of protein subunits (35). *Salmonella* strains have an average of eight flagella per cell (43). By enabling bacteria to swim toward nutrients and away from harmful substances, these structures provide bacteria with a competitive advantage. The price for this advantage is about 2% of the cell's biosynthetic resources in order to build the flagella and 0.1% of the cell's energy for their rotation (16, 35). Therefore, flagella are likely to be expressed only when they are needed. Bacteria upregulate flagellar synthesis under low-nutrient conditions (62, 63). Flagellar genes are shut down after attachment to surfaces during biofilm formation (2, 42). Flagellar synthesis is inhibited after entry into host cells, which would prevent the recognition of flagellin by the immune system (8, 9, 14). Flagella are also regulated in response to temperature, DNA supercoiling, water availability, and other conditions (6, 56).

The approximately five dozen genes involved in flagellar synthesis and chemotaxis in *Salmonella enterica* serovar Typhimurium are organized into a transcriptional hierarchy of three classes. At the top of the hierarchy is the class 1 promoter. The class 1 promoter is thought to integrate many different signals in the decision on when to express flagellar genes. In *Salmonella*, the class 1 promoter contains at least six transcriptional start sites (61) and expresses the *flhDC* genes, which encode the $FlhD_4C_2$ activator complex (34, 58). The $FlhD_4C_2$ activator complex directs σ^{70} and RNA polymerase to initiate transcription at class 2 promoters. Gene products expressed from the class 2 promoters are used to build the basal body, which is the motor structure that spans the membranes and peptidoglycan and consists of the rotor, the rod, bushings, and the flagellar secretion system. Class 2 gene products are also used to assemble the flexible, extracellular rod-filament linker called the hook. A class 2 promoter transcribes the gene for the flagellar sigma factor σ^{28}, which activates transcription from class 3 promoters. Class 3 promoters transcribe the genes for the filament subunits, motor force generators, and chemosensory system (6).

In *Salmonella*, a number of nonflagellar proteins are known to affect flagellar synthesis. These regulators include eight proteins that contain DNA-binding domains. One is the cyclic AMP-catabolite gene activator protein (CAP) complex that activates *flhDC* transcription (27, 28, 30, 62). CAP binds DNA when cyclic AMP levels are high during growth under nutrient-limiting conditions. In *Escherichia coli*, DNA footprinting demonstrated previously that CAP binds to a specific site within the *flhDC* promoter region (49). A second regulator of flagellar transcription is RcsB, which is the response regulator component of the phosphotransfer relay system that controls capsule synthesis (5, 11). The RcsCDB system responds to multiple external stimuli, including high osmolarity, desiccation, and low temperature with high zinc concentrations (36). RcsB binds to a specific site termed the RcsAB box within the class 1 promoter region to inhibit motility (57). A third DNA-binding protein that affects motility is FimZ. FimZ is a response regulator that activates type 1 fimbrial genes. Type 1 fimbriae are utilized for surface adhesion, but it is not known to what signals FimZ responds. FimZ inhibits motility at the level of class 1 transcription (7). The iron-regulatory protein Fur and the nucleoid proteins Fis and H-NS activate flagellar transcription (4, 25, 28). It has been demonstrated previously for Fis in *Salmonella* and for Fur and H-NS in *E. coli* that these regula-

* Corresponding author. Mailing address: Department of Biology, University of Utah, 257 S. 1400 E., Salt Lake City, UT 84112-0840. Phone: (801) 585-6950. Fax: (801) 585-9735. E-mail: hughes@biology.utah.edu.

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‡ Present address: Department of Biology, University of Utah, Salt Lake City, UT 84112.

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tors can bind directly to the *flhDC* promoter (25, 49, 51). Finally, SlyA and RtsB are pathogenesis-related DNA-binding proteins that affect motility or flagellar expression (13, 50). SlyA promotes the expression of the filament subunits (50), and RtsB binds to and inhibits the class 1 flagellar promoter (13).

Proteins that do not contain DNA-binding domains also regulate motility in *Salmonella* and tend to affect steps after class 1 transcription. CsrA is an RNA-binding protein involved in carbon storage regulation that stabilizes the *flhDC* transcript in *E. coli* (59) and is important for motility in *Salmonella* (32). DnaK is a heat shock response chaperone that directly interacts with $FlhD_4C_2$ to promote the formation of active $FlhD_4C_2$ complexes (52). ClpXP is a protease that is also induced by the heat shock response but inhibits motility by degrading $FlhD_4C_2$ (9, 54, 55). Proteins that adjust the levels of or respond to the global signaling molecule cyclic-di-GMP (c-di-GMP) also affect motility. The GGDEF domain protein AdrA increases c-di-GMP levels, reduces motility, and promotes biofilm formation (47). The EAL domain proteins YhjH and STM1827 decrease c-di-GMP levels, increase motility, and inhibit biofilm formation (45–47). The YcgR protein inhibits motility and contains a PilZ domain that binds c-di-GMP (46). In *E. coli*, these c-di-GMP-related proteins have been shown to promote the assembly of the motor force generator proteins and alter the directional bias of flagellar rotation (19, 26).

Because flagella can be an advantage or a handicap depending on the situation, it is not surprising that flagellar synthesis is regulated in response to many different signals. However, the more than a dozen proteins described above may not constitute the entire regulatory network in *Salmonella*. A genetic approach was utilized in this study to search for additional regulators of flagellar synthesis. The T-POP transposon, which contains an inducible promoter, was allowed to transpose to random locations throughout the *Salmonella* genome. Depending on the orientation of the transposon, the inducible promoter could initiate the transcription of adjacent genes or produce antisense RNA to inhibit translation. This transposon was used to screen for genes that either inducibly activated or inhibited the transcription of the filament subunit gene *fliC*. In addition to insertions that directly controlled known regulators of flagellar transcription, two new loci were found to inhibit flagellar synthesis when overexpressed. Deletions in and *lac* fusions to these negative regulator genes were used to organize the corresponding proteins into pathways. These additional regulators provide further insight into when and how flagellar synthesis is controlled.

MATERIALS AND METHODS

Bacterial strains and general techniques. The strains used in this study were derived from *Salmonella enterica* serovar Typhimurium strain LT2. Many of these strains are listed in Table 1. Additional strains were constructed from the alleles indicated in Table 1 and are listed in Table S1 in the supplemental material. Cultures of bacterial strains and phage P22 lysates were prepared as described previously (18). Chlortetracycline (50 μg/ml; autoclaved) or tetracycline (Tet; 15 μg/ml) was used to induce transcription from the *tetA* promoter within the T-POP transposon, and 0.2% arabinose (Ara) was used to induce transcription from the *araBAD* promoter. Tet^s selections and transductions were performed as described previously (37, 38), except that Luria broth (LB) was used instead of nutrient broth. Primers were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table S2 in the supplemental material. PCR products were sequenced at the DNA sequencing facility (Department of Biochemistry) at the University of Washington or at the DNA sequencing core facility at the University of Utah. Strain constructions utilizing λ *red* recombination were performed using plasmid pKD46 as described previously (60). MES (morpholineethanesulfonic acid) buffer (100 mM) was used in acidic motility plates to maintain the pH near 5.1 (39). A position-specific score matrix algorithm was used to search intergenic regions with the consensus sequences for σ^{28} and $FlhD_4C_2$, as described in an earlier study (60).

Screen for random T-POP insertions affecting flagellar transcription. The screen was performed as described in Results. The T-POP transposon was mobilized by P22-mediated transduction into recipient strains expressing Tn*10* transposase from a plasmid. The frequency of hot-spot transposition was reduced by using the mutant Tn*10* transposase with altered target specificity from plasmid pNK2880. To compensate for the decreased transposition frequency of the mutant transposase with altered target specificity, the donor strain (TH3923) had the Tn*10d*Tc[Δ*25*] transposon inserted downstream from a Mud-P22 insertion in an F plasmid. Because an induced Mud-P22 packages primarily adjacent chromosomal DNA, 10 to 50% of the phage particles should carry the T-POP DNA sequence (33).

The screen was undertaken by undergraduate students, graduate students, and postdoctoral fellows in a number of genetics classes. We roughly estimate that a half million transposon insertions were screened for inducible expression of the *fliC-lac* transcriptional fusion. Arbitrary PCR and sequencing were used to identify individual T-POP insertions (33). Many of the insertions that showed linkage to markers near *fimZ*, *rcsB*, and *ydiV* by P22 transduction were not considered further.

Isolation of MudJ insertions. Transposon insertions of the MudJ transcriptional reporter were isolated near the P*ecnR*::T-POP insertion in strain TH9291, the *feoB*::T-POP insertion in strain TH9293, the P*pefI*::T-POP insertion in strain TH8774, and the *rcsD*::T-POP insertion in strain TH8758. Random MudJ insertions were generated (22), and between 24,000 and 90,000 colonies were pooled for each strain. Phage stocks were prepared on the pooled colonies. A wild-type strain (TH437) was transduced with these pools of random insertions, and the cells were spread onto LB-Tet-kanamycin (Kan)-EGTA plates to select for transductions that had brought in the T-POP insertion (by selecting for Tet^r) and nearby MudJ insertions (by selecting for MudJ-encoded Kan^r). To screen for MudJ *lac* fusions under the control of the *tetA* promoter in the T-POP, transductants were patched onto lactose indicator medium (LB–X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside], MacConkey agar-lactose [Mac-Lac], or triphenyl tetrazolium chloride-lactose) containing either chlortetracycline or no inducer. To screen for MudJ insertions that blocked the inhibition of motility, transductants were poked into Tet-Kan-EGTA motility plates. The locations of insertions linked to *ecnR*, *pefI-srgD*, and *rcsB* were determined by PCR and sequencing.

For *feoB*, transposons were checked for linkage to the *feoB*::T-POP insertion regardless of the strains' motility or their Lac phenotypes. The left ends of linked MudJ insertions were identified using arbitrary PCR and sequencing as described previously (33), except that primer Mud-R3 (AGCAATTTTTTACTATCTTTCGCG) was used for the primary reaction and primer Mud-R4 (TTTGCACTACAGGCTTGCAAGCCC) was used for the secondary reaction and for sequencing. The isolation of MudJ transposons in *E. coli* F plasmid genes *ybiB* and *ycbB* indicated that F plasmid DNA had transferred into the *feoB* gene. Through PCR and sequencing, the F plasmid junctions were located. The left end (*tetA* side) of the T-POP was inserted 82 bp downstream from the start of *ybjA*, with P*tetA* transcribing toward the start codon. The right end (*tetR* side) of the T-POP was inserted 73 bp downstream from the start of *ybjA*, with P*tetR* transcribing toward the stop codon. A junction that fused F plasmid DNA 3,149 bp after the start of *ycbB* to F plasmid DNA 11 bp after the end of *ybhB* was also sequenced. Altogether, 6,016 bp of F plasmid DNA flanked by T-POPs had transposed into *feoB*.

A transposon insertion of the MudJ transcriptional reporter into a fimbrial gene was also isolated. Random MudJ insertions were generated in strain TH8757. Strain TH13235 (P*fimZ*::T-POP Δ*araBAD*::*fimZ*) was transduced with a phage stock prepared on 180,000 pooled colonies. Tet-sensitive plates containing Kan were used to select for MudJ insertions that had replaced P*fimZ*::T-POP. Insertions in fimbrial genes could be distinguished on lactose indicator media by looking for an increase in *lac* expression when *fimZ* was induced from the arabinose promoter. Arbitrary PCR and sequencing were used to locate the left end of a MudJ insertion in *fimH*.

Expression of genes at the *ara* locus. Genes were placed under the control of the arabinose promoter at the *ara* locus by amplifying the coding sequence and then using λ *red* recombination to replace Δ*araBAD*::*tetRA* in TH6706 with the amplified DNA. Primers were designed with about 40 bp of homology to the arabinose locus (for recombination) and about 20 bp of homology to the cloned

TABLE 1. *Salmonella* strains used in this study

Strain	Genotype and/or description	Source or reference[a]
TH437	LT2	J. Roth
TH2089	*fliL5100*::MudJ	Lab collection
TH2142	*hisD9953*::MudJ *his-9944*::MudI	22
TH2775	*flhC5213*::MudJ	G. Chilcott
TH3730	P*flhDC5451*::Tn*10d*Tc[Δ*25*]	24
TH3923	pJS28 (Ap^r P22-9$^+$) F′114(Ts) *lac*$^+$ *zzf-20*::Tn*10*[*tetA*::MudP] *zzf-3833*::Tn*10d*Tc[Δ*25*] *leuA414 hsdSB* Fels2$^-$	Lab collection
TH3933	*motA5461*::MudJ	Lab collection
TH4702	pKD46 (λ *red* recombinase plasmid; Amp^r; temp-sensitive replication)	10
TH4881	pNK2880 (pTn*10*-tpnase Amp^r *ats-l ats-2*) *fliC5050*::MudJ *fljB5001*::MudCm	Lab collection
TH6608	*fimD6025*::MudJ	Lab collection
TH6609	Δ*fimA6008*::FCF	Lab collection
TH6706	pKD46 Δ*araBAD925*::*tetRA*	P. Aldridge
TH8757	*ydiV240*::Tn*10d*Tc[Δ*25*] (260 bp upstream of *ydiV* start)	
TH8758	*rcsD241*::Tn*10d*Tc[Δ*25*] (2,145 bp downstream of *rcsD* start)	
TH8768	*fliG6340*::Tn*10d*Tc[Δ*25*] (785 bp downstream of *fliG* start)	
TH8769	*fliA6341*::Tn*10d*Tc[Δ*25*] (8 bp upstream of *fliA* start)	
TH8770	*fliE6342*::Tn*10d*Tc[Δ*25*] (70 bp upstream of *fliE* start)	
TH8773	*fimZ53*::Tn*10d*Tc[Δ*25*] (26 bp upstream of *fimZ* start)	
TH8774	*pefI*::Tn*10d*Tc[Δ*25*] (238 bp upstream of *pefI* start)	
TH9260	*flgA6418*::Tn*10d*Tc[Δ*25*] (535 bp downstream from *flgA* start; *tetA* facing *flgA* start)	
TH9291	*ecnR*::Tn*10d*Tc[Δ*25*] (21 bp upstream of *ecnR* start; *tetR* facing *ecnR* start)	
TH9293	*feoB*::Tn*10d*Tc[Δ*25*] (1,082 bp downstream of *feoB* start; includes 6 kb of F plasmid sequence)	
TH9386	Δ*araBAD921*::*ecnR*$^+$	J. Karlinsey
TH9878	*pefI*::Tn*10d*Tc[Δ25] *srgA*::MudJ (~200 bp downstream of *srgD* stop codon)	
TH9883	*pefI*::Tn*10d*Tc[Δ25] *pefI*::MudJ (77 bp upstream of *pefI* start)	
TH10068	*ecnR*::MudJ (inserted 561 bp into *ecnR*)	
TH10099	Δ*fimZ*::Kan	B. Jones
TH10165	*srgD*::MudJ (inserted 198 bp into *srgD*)	
TH11115	Δ*araBAD964*::*srgD*$^+$ (start codon through 14 bp after stop codon of *srgD*)	Y. Su
TH11116	Δ*araBAD965*::*pefI*$^+$ (start codon through stop codon of *pefI*)	Y. Su
TH11117	Δ*araBAD966*::*pefI*$^+$-*srgD*$^+$ (start codon of *pefI* through 14 bp after stop codon of *srgD*)	Y. Su
TH11418	*feoB*::Tn*10d*Tc[Δ*25*] STM3508::MudJ	J. Hoggan
TH12194	Δ*araBAD981*::*pefI*$^+$-*srgD*$^+$ (250 bp upstream of *pefI* start codon through 193 bp after stop codon of *srgD*)	
TH12195	Δ*araBAD982*::*pefI*$^+$-*srgD*$^+$ (start codon of *pefI* through 193 bp after stop codon of *srgD*)	
TH12196	Δ*araBAD983*::*pefI*$^+$-*srgD*$^+$ (250 bp upstream of *pefI* start codon through 14 bp after stop codon of *srgD*)	
TH12882	Δ*araBAD995*::*ydiV*$^+$	
TH12883	Δ*araBAD996*::*rcsB*$^+$	
TH12888	Δ*rcsDBC*::FKF	
TH12890	Δ*ecnR*::FKF	
TH12892	Δ*pefI-srgD*::FKF	
TH12902	Δ*ydiV*::FKF	B. Cookson
TH12904	*rcsB*::MudJ (inserted ~200 bp into *rcsB*)	
TH12905	Δ*araBAD997*::*fimZ*$^+$	
TH13235	*fimZ53*::Tn*10d*Tc[Δ*25*] Δ*araBAD997*::*fimZ*$^+$	
TH13373	*motA5461*::MudJ *feoB*::Tn*10d*Tc[Δ*25*] (no F plasmid DNA)	
TH13498	*fimH56*::MudJ	
TH13554	Δ*araBAD1005*::FCF (replaces region from the start codon of *araB* through the stop codon of *araD*)	
TH13742	*ydiV*::*lacZ* (constructed from pKG136 and TH12902 by procedure of Ellermeier et al. [12])	K. Winterberg
TH13833	P$_{wza}$*wza*::Kan	57
TH14258	*feoB*::Tn*10d*Tc[Δ*25*] *feoB*::MudJ (475 bp downstream from *feoB* start)	
TH14595	*feoB*::Tn*10d*Tc[Δ*25 ycbB*::MudJ]	
TH14596	*feoB*::Tn*10d*Tc[Δ*25 ybiB*::MudJ]	
TH14787	*pefI*::Tn*10d*Tc[Δ25] P*flhD7781* (A−198G)	
TH14788	*pefI*::Tn*10d*Tc[Δ25] P*flhD7782* (insertion of an A between bases −190 and −191)	
TH14789	*pefI*::Tn*10d*Tc[Δ25] P*flhD7783* (G−211A)	
TH14790	*pefI*::Tn*10d*Tc[Δ25] P*flhD7784* (G−368A)	
TH14791	Δ*araBAD921*::*ecnR*$^+$ P*flhD7785* (insertion of an A between bases −190 and −191)	
TH14792	Δ*araBAD921*::*ecnR*$^+$ P*flhD7786* (G−211A, G−213A)	
TH14793	Δ*araBAD921*::*ecnR*$^+$ P*flhD7787* (G−211A)	
TH14794	Δ*araBAD921*::*ecnR*$^+$ P*flhD7788* (C−189T)	
TH14795	Δ*araBAD921*::*ecnR*$^+$ P*flhD7789* (A−198G)	
TH14897	Δ*araBAD983*::*utr-tetRA-srgD* (*tetRA* deletes all of the *pefI* coding sequence upstream of the *srgD* start codon)	
TH14945	Δ*araBAD1019*::*utr-srgD* (*pefI* is cleanly deleted from the TH12196 strain)	

[a] Strains for which no source or reference is given were constructed for this study.

gene (for amplification). These constructs replaced the sequence spanning the *araB* start codon through the *araD* stop codon with that spanning the start codon through the stop codon of the cloned gene. Tet-sensitive plates were used to select for recombinants following λ *red* recombination. In the case of *pefI-srgD*, a 250-bp sequence of DNA starting upstream of *pefI* and continuing through 14 bp after the stop codon of *srgD* was used to replace the ribosome-binding site from 13 bp upstream of the *araB* start codon through the stop codon of *araD* (resulting in strain TH12196). To delete *pefI* from this construct, a *tetRA* cassette was inserted (generating strain TH14897) and then replaced with a clean deletion of *pefI* (producing strain TH14945). Other P_{ara}::*pefI-srgD* variants that began with the *pefI* start codon or ended 193 bp after *srgD* were constructed.

Construction of deletions. Genes were deleted using the FLP recombination target (FRT)-Kan-FRT cassette (FKF) or the FRT-chloramphenicol-FRT cassette (FCF) as described by Datsenko and Wanner (10). FKF and FCF insertion-deletion alleles were constructed in strain TH4702 and designed to leave 10 codons at the beginning and end of each gene intact. Kan^r or chloramphenicol-resistant colonies were selected, and the location of each insertion was confirmed by PCR.

Motility plates. Motility plates were prepared as described previously (18). Single colonies were poked into the plates using toothpicks, and the plates were incubated for 6 or 24 h. For motility assays requiring the induction of a T-POP or P*ara* construct, colonies were picked from plates that contained Tet or arabinose, respectively. At least six independent colonies were checked for each strain assayed. Adobe Photoshop was used to adjust the levels for pictures of motility plates in order to increase the contrast between the swarms and the background.

β-Galactosidase assays. Ten microliters of an overnight culture (in LB, LB-Ara, LB-Ara-Tet, or LB-chlortetracycline) was subcultured into 3 ml of fresh medium (LB, LB-Ara, LB-Ara-Tet, or LB-chlortetracycline). Tubes were incubated with shaking at 37°C until the contents reached a mid-log-phase density of 60 Klett units, which corresponds to an optical density at 600 nm of about 0.6. Cultures were put on ice, spun down, and resuspended in 3 ml of cold buffered saline. Culture samples of between 50 μl and 0.5 ml were added to 0.55 ml of complete Z-buffer (Z-buffer plus 5 μl of 10% sodium dodecyl sulfate and 100 μl of chloroform) (37) and adjusted to give a total aqueous volume of 1.05 ml with buffered saline. The assay was continued as described previously (37). For each strain, assays were performed for three independent biological replicates.

RESULTS

Isolation of T-POP insertion mutations that induce or inhibit flagellar gene transcription. Strain TH4881 (pNK2880 [pTn*10*-tpnase Amp^r *ats-l ats-2*] *fliC5050*::MudJ *fljB5001*::MudCm) was mutagenized with the T-POP transposon Tn*10d*Tc[Δ*25*], which is a derivative of Tn*10*. The T-POP contains the *tetA* and *tetR* genes needed for resistance to Tet and forms stable insertions because it lacks the transposase gene. In the T-POP transposon Tn*10d*Tc[Δ*25*], the transcription terminator region of the *tetA* transcript is deleted. Insertions of this T-POP element into the chromosome result in Tet-inducible transcription from the *tetA* promoter into adjacent chromosomal DNA (44). A transcriptional fusion of the *lac* operon to the σ^{28}-dependent *fliC* gene (*fliC5050*::MudJ) was present in the recipient strain as a reporter to screen for Tet-dependent changes in flagellar transcription. Strains expressing the *fliC-lac* fusions grew as Lac^+ (red) colonies on Mac-Lac indicator medium. Following transposition, four classes of T-POP insertion mutants were obtained based on their phenotypes on Mac-Lac and Mac-Lac-Tet media. (i) The majority of T-POP insertion mutants displayed a Lac^+ phenotype on both Mac-Lac and Mac-Lac-Tet plates, indicating that the transposon insertion had no significant effect on *fliC-lac* transcription. (ii) A small percentage of the T-POP insertion mutants had a Lac^- (white) phenotype on both Mac-Lac and Mac-Lac-Tet plates. These mutants carried insertions that themselves resulted in the inhibition of *fliC-lac* transcription. For example, insertions in flagellar genes required for the formation of the hook-basal body (HBB) accumulate the anti-σ^{28} factor FlgM, resulting in the inhibition of *fliC-lac* transcription (21, 29, 30). (iii) About 0.5% of the T-POP insertion mutants showed normal *fliC-lac* transcription on Mac-Lac plates but had a Lac^- phenotype on Mac-Lac-Tet plates. This phenotype indicated that *fliC-lac* expression was inhibited by the transcription of chromosomal DNA near the T-POP. (iv) About 0.1% of the insertion mutations resulted in a Lac^- phenotype on Mac-Lac plates and a Lac^+ phenotype on Mac-Lac-Tet plates. In this case, the act of T-POP insertion resulted in the inhibition of the *fliC-lac* reporter but expression was restored by the transcription of genes adjacent to the site of insertion. Mutations in the third and fourth classes, which yielded inducible Lac phenotypes, were analyzed further.

Characterization of T-POP insertions resulting in Tet-induced Lac phenotypes. The fourth class of T-POP insertion mutants that had a Tet-induced Lac^+ phenotype was characterized by DNA sequence analysis. In three cases, the T-POP had inserted upstream of the *fliAZY* operon, upstream of the *fliE* operon, or 785 bp into the 996-bp *fliG* gene. These insertion mutations were polar: the flagellar promoters did not transcribe through the T-POP insertion elements. The inhibition of transcription of the *fliAZY* operon, which includes the σ^{28} structural gene (*fliA*), would prevent all class 3 flagellar gene transcription. The inhibition of transcription of *fliE* or the genes downstream of *fliG* that are needed for the completion of the HBB would result in the accumulation of FlgM in the cell and the inhibition of σ^{28}-dependent class 3 flagellar gene transcription. The *fliG* gene containing the T-POP insertion is likely still functional in its role in HBB assembly. It is known that the first 245 amino acids of FliG are enough to interact with FliM and FliN in order to build the C-ring rotor complex and full-length flagella, but these flagella do not rotate due to a defective interaction with the stator complex (53). For the *fliA*, *fliE*, and *fliG* mutants, the addition of Tet would induce the transcription of flagellar structural or regulatory genes from the *tetA* promoter and restore *fliC-lac* expression.

The third class of T-POP insertion mutants exhibited a Tet-inducible Lac^- phenotype. The insertions in these mutants potentially inhibited flagellar transcription by inducing the expression of an adjacent gene on the chromosome. These T-POP insertions were moved into a wild-type background by P22-mediated transduction and characterized for the effect of Tet on motility. The transductants exhibited either a Tet-induced Mot^- phenotype or a Mot^- phenotype with or without Tet. As confirmed by DNA sequence analysis or linkage tests and complementation analysis, all of the T-POP mutations that produced a Mot^- phenotype with or without Tet were inserted in the HBB assembly gene *flgA*. While insertions in genes required for HBB assembly normally accumulate high levels of the anti-σ^{28} factor FlgM inside the cell, insertions in *flgA* have a polar effect on the downstream *flgM* gene and do not accumulate as much FlgM (30). The remaining T-POP insertions generating Tet-inducible Mot^- phenotypes were located in six other regions of the chromosome that are not associated with the flagellar regulon (Fig. 1). The motility of one representative T-POP insertion mutant for each of these six regions is shown in Fig. 2A. For three of these regions, T-POP insertions were isolated upstream of the *fimZ*, *rcsB*, and *ydiV* genes (Fig. 1A, B, and C), which have been identified previously as regu-

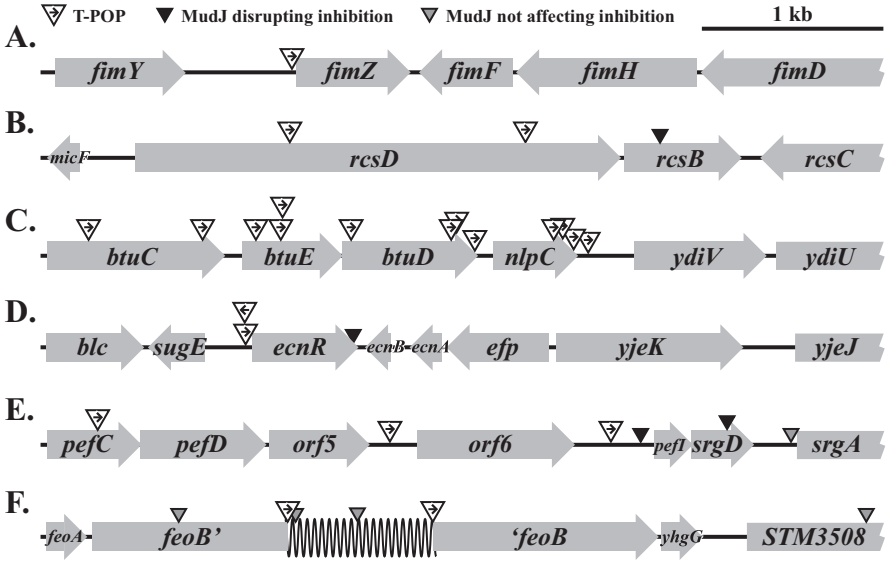

FIG. 1. T-POP insertions at six different loci inhibited flagellar transcription when induced with Tet. Each T-POP insertion is represented by a triangle containing an arrow. The arrow indicates the direction in which transcripts initiated from the *tetA* promoter proceed into the adjacent chromosomal DNA. The smaller, solid triangles represent polar MudJ insertions which either disrupt the inhibition of flagellar transcription or have no effect on inhibition by the T-POP insertions. As depicted in panel F, the only isolated insertion in *feoB* consisted of 6 kb of *E. coli* F plasmid DNA (wavy line) flanked by T-POP insertions.

lators of flagellar transcription (5, 7, 11, 48, 57). RcsB and FimZ are response regulators with C-terminal DNA-binding domains. YdiV shows weak homology to EAL domain proteins, which break down the signaling molecule c-di-GMP (20). However, YdiV inhibits motility while other EAL domain proteins like YhjH and STM1827 stimulate motility (45–48). To confirm that FimZ, RcsB, and YdiV were responsible for inhibiting flagellar transcription, each corresponding gene was cloned after the arabinose promoter at the *araBAD* locus on the chromosome. All three constructs inhibited motility when arabinose was added (Fig. 2B).

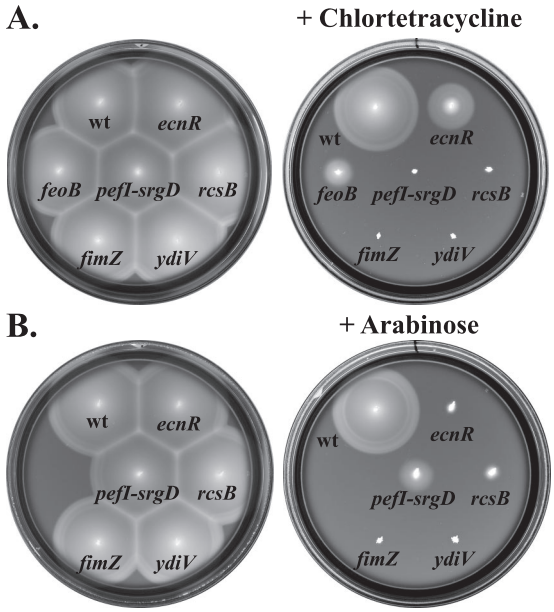

FIG. 2. Motility of T-POP insertion strains with or without induction by autoclaved chlortetracycline (A) or P_{ara} constructs with or without arabinose induction (B). For the colonies poked into motility plates containing chlortetracycline or arabinose, the expression of negative regulators had already been induced by growth on LB-Tet or LB-Ara plates. Pictures were taken after 6 h. Strains are indicated by the affected gene. wt, wild type.

Two T-POPs were isolated upstream of the *ecnR* gene (Fig. 1D). While transcription from the *tetA* promoter of one of the T-POP insertions proceeded away from *ecnR* into the upstream gene, an insertion in *ecnR* disrupted the inhibition of motility in the strain. Some transcripts originating from the *tetR* promoter, which initiates transcription in the opposite direction from the *tetA* promoter, apparently did not terminate before the end of the transposon. A P_{ara}::$ecnR^+$ construct inhibited motility when arabinose was added (Fig. 2B). Based on a search using the protein domain database Pfam (15), most of EcnR is not homologous to any domains of known function. The C terminus of EcnR is homologous to helix-turn-helix DNA-binding domains. EcnR is thought to be the regulator of the entericidin genes *ecnA* and *ecnB*, which are near *ecnR*. The entericidin genes encode small, amphipathic α-helical lipoproteins that act as a toxin-antitoxin system. Entericidins may induce apoptosis in stationary-phase *E. coli* cells (3).

Other T-POP insertions were isolated in front of the *pefI* and *srgD* genes on the virulence plasmid of *Salmonella* (Fig. 1E). The *pefI* and *srgD* genes are a part of the *pef* locus, whose genes encode a second set of fimbriae (the *p*lasmid-*e*ncoded *f*imbriae). Although the *fim* locus encodes the major type of fimbriae expressed in *Salmonella enterica* serovar Typhimurium, there are 12 additional fimbrial operons in the genome. Some expression of *pef* fimbriae and other minor fimbriae during growth at low pH or after the infection of intestinal cells can be detected (14, 23, 39). PefI is a DNA-binding protein that affects DNA methylation patterns and inhibits the *pefA* fimbrial

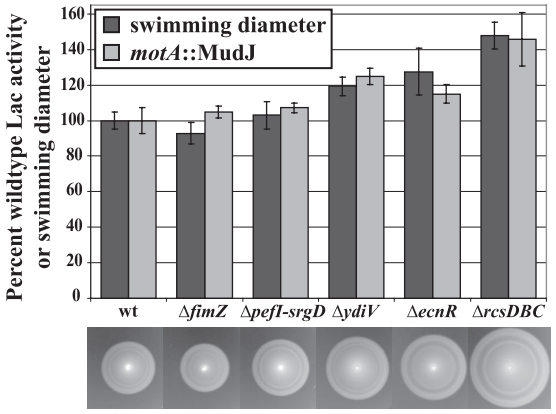

FIG. 3. Motility and class 3 flagellar transcription of strains with deletions of the negative regulators. Motility was determined by measuring the diameters in motility plates after 6 h. Each reported diameter is an average for six replicates and is normalized with respect to the diameter for the wild type (wt). A representative photo of each strain is shown beneath the bar graph. Class 3 transcription from the *motA*::MudJ transcriptional reporter was quantified through β-galactosidase assays.

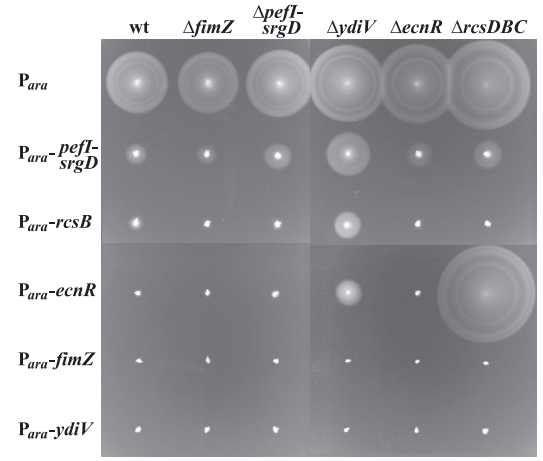

FIG. 4. Epistatic analysis of negative regulators of motility. A deletion of each regulator was introduced into strains in which one of the five negative regulators could be induced. Colonies picked from LB-Ara plates were poked into motility plates containing arabinose using toothpicks. Pictures were taken after 6 h. wt, wild type.

promoter. PefI is homologous to the *E. coli* fimbrial regulatory proteins FaeA and PapI (39). Through a Pfam search (15), SrgD appears to be a single-domain protein that contains a helix-turn-helix motif. When *pefI*, *srgD*, or *pefI-srgD* was cloned at the *ara* locus, no significant inhibition of motility was observed (data not shown). In order to further map the genes responsible, insertions of a large, polar transposon (MudJ) were isolated near one of the T-POP insertions. A MudJ element inside *srgD* no longer inhibited motility, and a MudJ element located about 200 bp after *srgD* continued to inhibit motility (Fig. 1E). This genetic analysis suggested that DNA between the T-POP upstream of *pefI* and the MudJ insertion downstream of *srgD* was important for the efficient expression of PefI-SrgD or for the inhibition of motility. When 250 bp of DNA upstream of *pefI* was included in the P_{ara}::*pefI*$^+$-*srgD*$^+$ construct, inhibition of motility was observed (Fig. 2B). This 250 bp of upstream DNA fused only to *srgD* was not sufficient to inhibit motility (data not shown). This finding suggests that both *pefI* and *srgD* are needed to inhibit motility. Adding the 200 bp of DNA downstream of *srgD* had no effect on inhibition by the *ara* constructs (data not shown).

One T-POP insertion was isolated inside the iron transport gene *feoB* (Fig. 1F). MudJ insertions linked to the T-POP were isolated, and DNA sequence analysis revealed that several MudJ insertions were located near *feoB* and several were inserted in the *E. coli* F plasmid sequence. PCR amplification and DNA sequence analysis of this region revealed that a 6-kb section of F plasmid DNA flanked by two T-POPs had transposed into *feoB*. This F plasmid DNA originated from the donor strain and was somehow coinherited along with the T-POP element during transposition. The junctions between the T-POP insertions and the F plasmid DNA on the chromosome were identical to the original insertion of the T-POP in the F plasmid in the donor strain. It is unclear whether the transfer of F plasmid DNA was a result of a duplication of the T-POP and a chromosomal rearrangement of F plasmid DNA that occurred before transposition or was due to the T-POP uncharacteristically undergoing a replicative transposition event. When this F plasmid DNA was recombined out, the T-POP did not significantly inhibit motility and only a small, 17% decrease in class 3 transcription was observed. The *feoB*::T-POP insertion was not analyzed further.

Effects on motility of deleting negative regulators. Deletions of *ecnR*, *fimZ*, *pefI-srgD*, *rcsDBC*, and *ydiV* were examined for their effects on motility and flagellar transcription. Deletions of *ecnR*, *rcsDBC*, and *ydiV* significantly increased motility and generated a corresponding increase in class 3 transcription (Fig. 3). Increases in motility for *rcsB* and *ydiV* mutants have been observed before (48, 57). A deletion of *pefI-srgD* did not significantly increase motility or class 3 transcription. If *pefI-srgD* is not highly expressed during growth in LB, this regulator may not have a large effect on motility when deleted. In particular, genes in the *pef* regulon are better expressed in cells grown in acidic media (23, 39). When the *pefI-srgD* deletion strain was inoculated into pH 5.1 motility plates, the deletion strain was not more motile than the wild type (data not shown). Therefore, the levels of PefI-SrgD in wild-type cells in motility plates containing a mixture of tryptone, salt, and agar were not enough to inhibit motility. Finally, the strain with *fimZ* deleted showed a small but significant decrease in motility, which was an unexpected result for the deletion of a negative regulator of motility. If there is coordination among the different fimbrial systems, perhaps a decrease in *fimZ* levels would upregulate another fimbrial inhibitor of motility like *pefI-srgD*.

Epistatic analysis of the negative regulators. To determine whether the regulators are in the same or independent pathways for inhibiting motility, a deletion of each regulator was introduced into strains in which one of the five negative regulators could be induced (Fig. 4). If an overexpressed regulator depends upon a second regulator for inhibition, there should not be any inhibition of motility when that second regulator is deleted. The columns in Fig. 4 were arranged in order of increasing motility of the deletion strains, and the rows were

organized in order of increasing flagellar inhibition by each regulator expressed from the arabinose promoter.

While motility generally increases as one moves up and to the right in Fig. 4, a number of irregularities in this pattern can be observed. First, the deletion of the *rcsDBC* genes (Fig. 4) or an insertion in *rcsB* (data not shown) completely prevented the inhibition of motility by P_{ara}-*ecnR*$^+$. This finding suggests that EcnR acts through RcsB. Second, when *rcsB* was overexpressed in a strain with the *rcsDBC* genes deleted, a further decrease in motility was seen. This effect was more noticeable after a full day's incubation (data not shown). This tighter inhibition has been observed before and is likely a result of RcsC's no longer being able to remove phosphates to inactivate the response regulator RcsB (36). Third, the deletion of *ydiV* relieved the inhibition of motility by all the regulators by more than what would be expected from the 20% increase in the motility of the *ydiV* deletion mutant over the wild-type strain. This potent effect on motility indicates that YdiV acts by a mechanism that is independent of the mechanism(s) of action of the other regulators. Overall, with the exception of EcnR, the regulators do not depend upon any of the other four to inhibit motility.

Effect of regulators on flagellar transcription. Transcriptional fusions to the class 1 promoter (*flhC*::MudJ), a class 2 promoter (*fliL*::MudJ), and a class 3 promoter (*motA*::MudJ) were utilized to determine which levels of the flagellar transcriptional hierarchy are affected by the negative regulators. PefI-SrgD, RcsB, EcnR, and FimZ inhibited all three classes of flagellar promoters (Fig. 5A). This result indicates that these four regulators act at the top of the hierarchy at the class 1 promoter. In the case of YdiV, only the class 2 and class 3 flagellar promoters were inhibited. These data suggest that some step after class 1 transcription is being affected by YdiV.

Since PefI-SrgD, RcsB, EcnR, and FimZ inhibit class 1 transcription, these regulators may act directly at the class 1 promoter. A T-POP insertion located after all known transcription start sites in the class 1 *flhDC* promoter and upstream of the coding sequence was tested for its ability to bypass the negative regulation (Fig. 5B). This T-POP insertion is 163 bp downstream of the first transcription start site in the class 1 promoter (48 bp upstream of the start codon) and expresses the *flhDC* genes from the *tetA* promoter (24). As judged by motility and transcriptional fusions to flagellar genes, PefI-SrgD and EcnR were no longer able to inhibit flagellar expression. As expected, YdiV still inhibited motility, which is consistent with YdiV's acting at a step after class 1 transcription. RcsB and FimZ, however, retained some capacity for negatively regulating motility. This partial inhibition by RcsB and FimZ might be an indirect effect through the upregulation of capsule and fimbrial synthesis. More of these extracellular structures may comprise a physical barrier to flagellar assembly. Therefore, null mutations of a capsular gene and fimbrial genes were used to test the effect of the absence of these extracellular structures on motility.

The assembly of fimbriae was disrupted using insertions in the genes for the filament subunit (*fimA*), the outer membrane usher (*fimD*), and the tip adhesin (*fimH*) (40). For each of the three insertion strains, the loss of fimbriae increased motility in the P_{ara}-*fimZ* T-POP-*flhDC* background to about 74% of the wild-type level (data not shown). While these insertions did not relieve all of the inhibition, the data indicate that fimbrial structures are themselves inhibitory to motility.

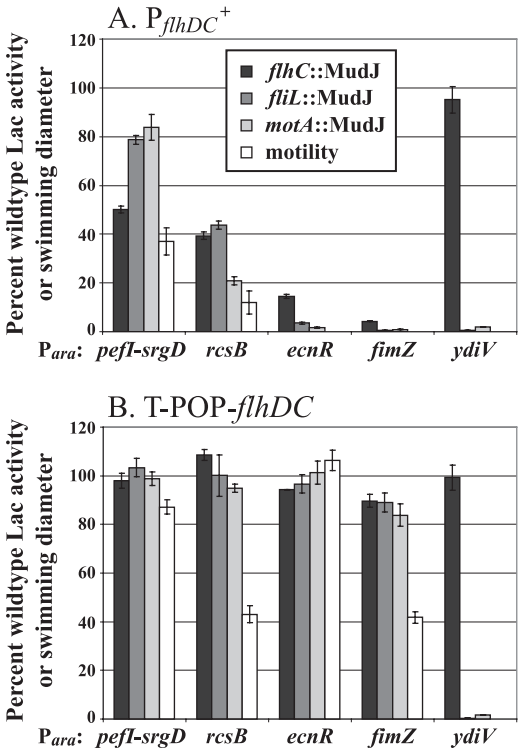

FIG. 5. Flagellar transcription and motility in strains expressing the negative regulators from the arabinose promoter. For both panels, the activities of MudJ transcriptional fusions to the *flhC* class 1 promoter, the *fliL* class 2 promoter, and the *motA* class 3 promoter were quantified through the β-galactosidase assay. Transcription activities and swimming-area diameters were normalized with respect to the wild-type levels. In the strains analyzed in panel A, the *flhDC* operon was expressed from its wild-type promoter. In those analyzed in panel B, the *flhDC* operon was expressed from a T-POP induced with Tet. The key in panel A also applies to panel B.

Capsule production was prevented by using a deletion of the promoter and first gene (*wza*) of the capsule synthesis operon. This *wza* deletion did not increase motility (data not shown). This result suggests that the capsule itself is not interfering with flagellar synthesis or function and that some other RcsB-regulated gene is responsible. In another study, however, this *wza* deletion did restore full motility in a strain exhibiting high RcsB activity and containing a mutated RcsAB box that prevented RcsB binding (57). The discrepancy between the findings of our studies regarding the effect of the *wza* deletion on motility may result from different approaches for generating increased RcsB activity. In the study by Wang et al. (57), an *igaA* mutation was used to induce RcsB activity, whereas we overexpressed RcsB directly from the arabinose promoter. Our different approaches may have induced different gene sets and inhibited motility through different mechanisms.

Spontaneous mutations that disrupt inhibition by EcnR and PefI-SrgD. To further map the pathways by which the novel regulators (EcnR and PefI-SrgD) inhibit motility, spontaneous Mot$^+$ mutants were isolated and characterized. Colonies of the T-POP-*pefI-srgD* strain (TH8774) or the P_{ara}-*ecnR*$^+$ strain (TH9386) were poked into motility plates containing tetracy-

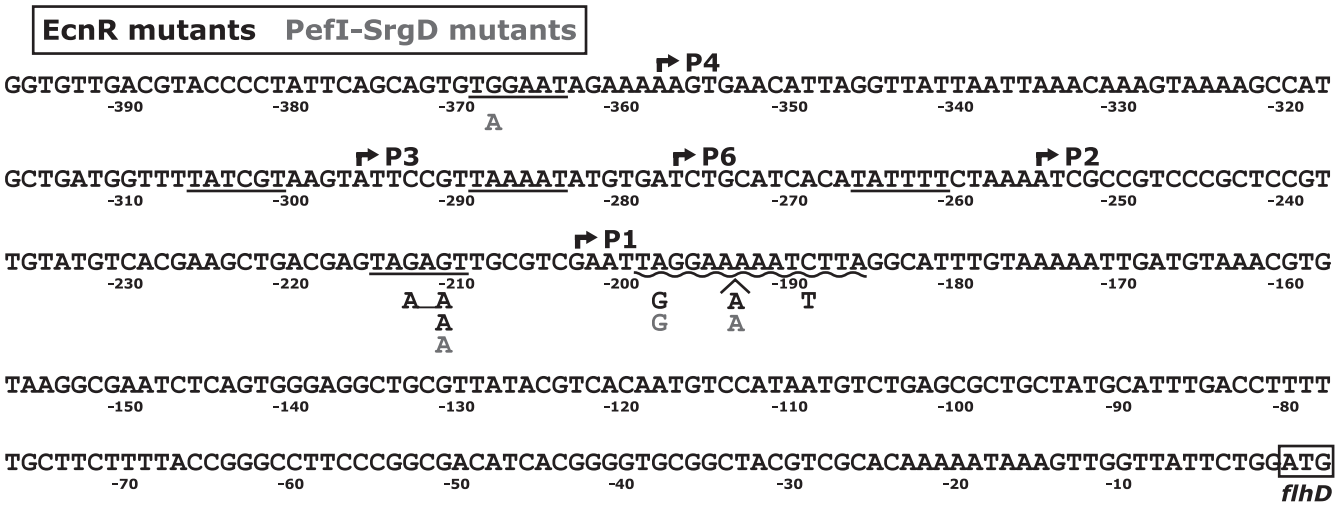

FIG. 6. Locations of spontaneous mutations in the *flhDC* promoter region that suppress the inhibition of motility by EcnR and PefI-SrgD overexpression. Transcription start sites (P1, P2, P3, P4, and P6) as determined by Yanagihara et al. (61) are marked with bent arrows, and −10 hexamers are underlined. The sequence that matches the consensus for the RcsAB box is identified by a wavy line (17, 36). The start codon of *flhD* is boxed. One double mutation was isolated and is indicated by a line connecting the two changes (G−211A and G−213A). The A−198G mutation was found in 6 of the 10 *flhDC* promoter mutants that suppressed PefI-SrgD inhibition and 20 of the 26 *flhDC* promoter mutants that suppressed EcnR inhibition.

cline or arabinose, respectively, to inhibit motility, and the plates were incubated overnight at 30°C. Mot^+ mutants were then picked. Only one motile flare from each inoculated colony was chosen to ensure that each mutation originated independently. Of 46 spontaneous Mot^+ mutations isolated in the T-POP-*pefI-srgD* strain, 36 were linked to the T-POP and 10 were linked to *flhDC*. Of 45 spontaneous Mot^+ mutations isolated in the P_{ara}-$ecnR^+$ strain, 13 were linked to the *ara* locus, 6 were linked to *rcsB*, and 26 were linked to *flhDC*. This spontaneous-mutation analysis suggests that no other nonessential genes are involved in these pathways. The *flhDC* promoter was sequenced for mutations linked to *flhDC* (Fig. 6). Similar *flhDC* promoter mutations were observed for EcnR and PefI-SrgD: mutations in the first 15 bp of the untranslated region for the P1 transcript and mutations that increased the match to the consensus sequence in the −10 hexamers for σ^{70}. Since EcnR and PefI-SrgD probably do not inhibit the *flhDC* promoter in the same way, these mutations are likely to be suppressors that generally increase *flhDC* transcription. It may be that single mutations do not disrupt EcnR and PefI-SrgD binding sites enough to restore motility.

Effect of flagellar synthesis on the transcription of negative regulators. While we have demonstrated that these five regulators inhibit flagellar transcription, these regulators are part of a regulatory network and might themselves be affected by flagellar proteins. To test this, *lac* fusions to *ecnR*, *ydiV*, *fimH*, *rcsB*, and *srgD* were isolated or constructed. The T-POP insertion in front of the class 1 *flhDC* promoter was moved into the resulting *lac* fusion strains. By inducing flagellar transcription using the T-POP, we could determine the effect of flagellar gene expression on the transcription of each of these regulatory genes (Fig. 7). The *ecnR* gene was turned off in the absence of flagellar gene expression and turned on following *flhDC* induction. This feedback on *ecnR* transcription probably moderates the level of expression of both *ecnR* and the flagellar genes. A computational search for $FlhD_4C_2$ or σ^{28} binding sites revealed a weak match to the $FlhD_4C_2$ consensus sequence 80 bp upstream of the *ecnR* start codon. Transcription of the *ydiV* gene, on the other hand, increased in the absence of flagellar gene expression. Since EAL domain proteins like YdiV are involved in the switch between swimming and sticking to surfaces, this competition between *ydiV* and flagellar genes for transcription may contribute to tighter regulation. The *lac* fusions to *fimH*, *rcsB*, and *srgD* were not affected by the induction of the flagellar regulon.

DISCUSSION

A screen for transposon insertions that inducibly affected flagellar transcription enabled us to identify two new regula-

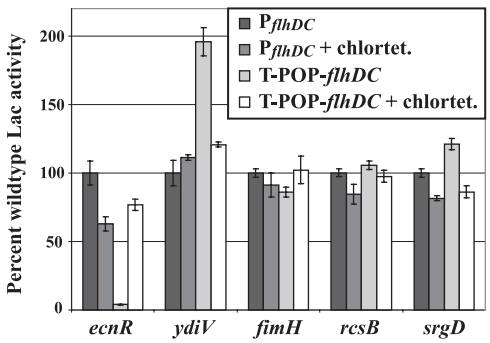

FIG. 7. Effect of the flagellar system on the transcription of the negative regulator genes. The fusion of *lac* to each negative regulator gene was used to quantify changes in transcription. The *flhDC* operon was expressed from its wild-type promoter or from the *tetA* promoter in the T-POP. When under the control of the T-POP, the *flhDC* operon is not expressed without the inducer chlortetracycline (chlortet.).

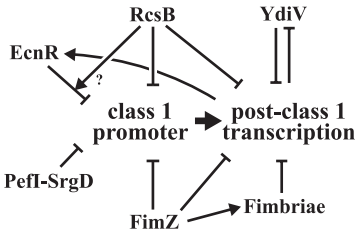

FIG. 8. Diagram of interactions for the five negative regulators identified in this study. While arrows show interactions between the regulators and the flagellar system, it is not known whether the majority of the interactions are direct or are mediated by regulators other than the five that were studied. A binding site in the class 1 promoter has been identified only for RcsB (57). It is also not clear how RcsB helps EcnR to inhibit class 1 transcription, and this interaction is labeled with a question mark. Since part of RcsB's inhibition of motility occurs after class 1 transcription, EcnR may not inhibit flagellar transcription by directly activating RcsB.

tors (EcnR and PefI-SrgD) in addition to three known regulators (FimZ, RcsB, and YdiV) of flagellar synthesis. Other T-POP insertions upstream of flagellar genes were isolated. Deletions and *lac* fusions were used to separate the regulators into pathways and map interactions. Even though all five regulators inhibited flagellar transcription, these regulatory proteins exhibited little cross talk (Fig. 8). Only EcnR was dependent upon another regulator (RcsB) for the inhibition of motility. These proteins provide further evidence that other regulatory systems act independently and in parallel to control the flagellar regulon.

Four of the negative regulators that were analyzed in this study may contribute to a regulatory network for external structures. RcsB is an activator of capsule synthesis, FimZ upregulates type 1 fimbriae, PefI controls the synthesis of virulence plasmid-encoded fimbriae, and YdiV has weak homology to EAL domain proteins which are known to decrease c-di-GMP levels, enhance swimming, and decrease cell adhesion to surfaces. Since RcsB, FimZ, and PefI-SrgD are associated with specific structures involved in sticking to surfaces, it makes sense that these regulators would inhibit flagellar transcription. As a c-di-GMP-related protein, YdiV may coordinate the sticking and swimming strategies more generally. YdiV's role in shutting off flagellar expression may be more important during the infection of host cells, since YdiV has been implicated previously in the level of macrophage killing during pathogenesis (20).

The function of EcnR is less clear. Because the *ecnR* gene is located next to the entericidin genes, EcnR may be involved in their regulation. It has been proposed previously that the entericidins in *E. coli* are produced in stationary phase to kill some cells in order to feed the rest of the population (3). If this hypothesis is correct, entericidins and flagella would represent two different strategies for survival. Flagella are used to find more nutrients, and entericidins would enable survival without nutrients. EcnR may help coordinate these two strategies. Moreover, the activation of *ecnR* transcription by flagellar genes may provide the entericidin system with a readout on the metabolic state of the cell.

Part of the flagellar inhibition by FimZ was determined to occur at a step after class 1 transcription. Mutations in the structural genes for fimbriae removed some of this secondary inhibition of motility by FimZ. These data suggest that the fimbrial structure can be inhibitory to flagellar synthesis or function, as has been observed before in *E. coli* (19, 31, 48). The inhibition of flagellar synthesis may occur through fimbrial proteins acting as a physical barrier to the assembly of the flagellum past the outer membrane. An example of this sort of inhibition may exist in *Salmonella enterica* serovar Typhi, in which the Vi capsular antigen is able to inhibit flagellar secretion (1). Alternatively, inhibition may occur through the proton motive force (PMF). If excess fimbrial production depletes the PMF, the result will be the inhibition of flagellar assembly since flagellar secretion utilizes the PMF (41). Finally, the inhibition may occur through competition for secretion systems or other resources to build the extracellular structures. The presence of fewer functional flagella may provide feedback to reduce flagellar transcription through flagellar regulatory proteins like FlgM and FliT.

While two new regulators of flagellar transcription were identified in this study, our screen probably did not saturate the genome. Insertions in front of the genes for known regulators like AdrA, CAP, ClpXP, CsrA, Fur, H-NS, and RtsB were not isolated. Regulators that have a more subtle effect on flagellar transcription might not have been detected on the Mac-Lac indicator medium for the *fliC-lac* reporter fusion that was used. Since we did not saturate our screen for low-level effects, there are likely to be other parts of the flagellar regulatory network that have not been identified. Further characterization of these pathways should improve our understanding of the complex regulatory mechanisms involved in adapting to different environments.

ACKNOWLEDGMENTS

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