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Genetic Transplantation: *Salmonella enterica* Serovar Typhimurium as a Host To Study Sigma Factor and Anti-Sigma Factor Interactions in Genetically Intractable Systems

Joyce E. Karlinsey* and Kelly T. Hughes†

Department of Microbiology, University of Washington, Seattle, Washington 98195

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In *Salmonella enterica* serovar Typhimurium, σ^{28} and anti-sigma factor FlgM are regulatory proteins crucial for flagellar biogenesis and motility. In this study, we used *S. enterica* serovar Typhimurium as an in vivo heterologous system to study σ^{28} and anti-σ^{28} interactions in organisms where genetic manipulation poses a significant challenge due to special growth requirements. The chromosomal copy of the *S. enterica* serovar Typhimurium σ^{28} structural gene *fliA* was exchanged with homologs of *Aquifex aeolicus* (an extreme thermophile) and *Chlamydia trachomatis* (an obligate intracellular pathogen) by targeted replacement of a *tetRA* element in the *fliA* gene location using λ-Red-mediated recombination. The *S. enterica* serovar Typhimurium hybrid strains showed σ^{28}-dependent gene expression, suggesting that σ^{28} activities from diverse species are preserved in the heterologous host system. *A. aeolicus* mutants defective for σ^{28}/FlgM interactions were also isolated in *S. enterica* serovar Typhimurium. These studies highlight a general strategy for analysis of protein function in species that are otherwise genetically intractable and a straightforward method of chromosome restructuring using λ-Red-mediated recombination.

Many organisms possess flagellar organelles that enable them to move about in liquid environments. The cells are able to direct their movement through chemical gradients as a result of intricate interactions between individual flagella and a chemosensory system, a process known as chemotaxis (3, 46). Motility is advantageous, since it allows cells to seek and acquire nutrients and to escape from adverse environmental conditions (3, 46). In addition, in many pathogenic organisms, flagella are important for adhesion, invasion, colonization, and biofilm formation on host cells and thereby contribute to virulence (17, 19). Flagellin monomers that constitute the flagella have also been shown to induce immune responses in mammalian hosts and the activation of plant defense mechanisms (33).

Flagellar gene regulation and assembly have been extensively characterized in *Salmonella enterica* serovar Typhimurium (1, 25). These studies revealed important regulatory mechanisms evolved by the organism to build these complex supramolecular structures. The assembly of the flagellum coincides with the cocoordinated regulation and expression of over 50 genes (25). The flagellar genes are expressed in a transcriptional hierarchy involving three promoter classes, classes 1, 2, and 3 (21). The class 1 operon responds to global regulatory signals and encodes transcriptional activators that interact with the housekeeping sigma factor σ^{70} and RNA polymerase (RNAP) to transcribe class 2 promoters. The class 2 genes encode proteins required for the structure and assembly of the hook-basal body (HBB) and regulatory proteins including FliA (σ^{28}), required for class 3 promoter transcription, and the anti-σ^{28} factor FlgM. The class 3 genes encode proteins that are required for filament assembly, motor force generation, and chemotaxis. The HBB anchors the flagellum to the bacterial membranes and includes a channel that enables the flagellar type III secretion system to secrete flagellar protein subunits out of the cytoplasm (26). Prior to completion of the HBB structure, FlgM binds σ^{28}, and class 3 promoters are not transcribed. When the HBB is completed, FlgM is secreted from the cell; this frees σ^{28}, and class 3 genes are expressed (18). This σ^{28}/FlgM regulatory checkpoint is thought to prevent production of class 3 gene products until they can be assembled into the flagellar structure (15).

Genetic and biochemical studies of σ^{28}/FlgM interactions have contributed to the understanding of flagellar regulation. Nuclear magnetic resonance studies with FlgM revealed that the protein was unstructured in solution but that, upon binding to σ^{28}, the C-terminal domain gained structure (9). These studies showed that the C-terminal domain of FlgM is important for interactions with σ^{28} (9). Mutations in σ^{28} that were insensitive to FlgM inhibition mapped throughout the protein. Based on these studies, a model suggesting that FlgM binds to σ^{28} in a multipartite manner to inhibit σ^{28} interaction with RNAP was proposed (4). In addition, it was shown that FlgM could inhibit σ^{28} activity by destabilizing the σ^{28}-RNAP holoenzyme complex (4, 5).

Recently, a cocrystal structure of *A. aeolicus* σ^{28}/FlgM has been solved, and these studies defined the important domains in σ^{28} and FlgM interactions (41). Further, these studies provide insights into the mechanism of FlgM inhibition through destabilization of the σ^{28}-RNAP holoenzyme complex (41). Because the overall amino acid homologies of *A. aeolicus* σ^{28} and FlgM to *S. enterica* serovar Typhimurium are low, it is difficult to extrapolate whether the important interacting amino acids identified in *S. enterica* serovar Typhimurium are significant in *A. aeolicus* σ^{28}/FlgM interactions. Hence, we

* Corresponding author. Mailing address: Department of Microbiology, University of Washington, Seattle, WA 98195. Phone: (206) 616-9925. Fax: (206) 543-8297. E-mail: joya@u.washington.edu.

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

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype description, or sequence (5′→3′)[a]	Source or reference[b]
Strains		
S. enterica serovar Typhimurium		
TH6238	*motA5461*::Mu*dJ*	
TH8059	*motA5461*::Mu*dJ fliA6088* (*A. aeolicus* GTG-*fliA*$^+$)	
TH8120	*motA5461*::Mu*dJ flgM6095* (*A. aeolicus flgM*$^+$)	
TH8122	*motA5461*::Mu*dJ fliA6088 flgM6095*	
TH8194	*motA5461*::Mu*dJ* Δ*flgM5794*::FRT[c]	
TH8196	*motA5461*::Mu*dJ fliA6088* Δ*flgM5794*::FRT	
TH8197	*motA5461*::Mu*dJ* Δ*flgM5794*::FRT Δ*fliA5805*::*tetRA*	
TH4098	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2*	
TH8061	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090* (*A. aeolicus* GTG-*fliA*$^+$)	
TH8123	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095*	
TH8125	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6095*	
TH8198	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2* Δ*flgM5794*::FRT	
TH8200	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090* Δ*flgM5794*::FRT	
TH8201	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2* Δ*flgM5794*::FRT Δ*fliA5805*::*tetRA*	
MST4190	*leuBCD485 trp*::[Spc^r P$_{lac}$T7-RNAP *lacI*]	S. Maloy
TH9031	*leuBCD485 trp*::[Spc^r P$_{lac}$T7-RNAP *lacI*] *motA5461*::Mu*dJ fliA6325* (*C. trachomatis* L2 GTG-*fliA*$^+$) Δ*flgM5794*::FRT/pJK627	
TH9032	*leuBCD485 trp*::[Spc^r P$_{lac}$T7-RNAP *lacI*] *motA5461*::Mu*dJ fliA6325* Δ*flgM5794*::FRT/pET15b	
TH9033	*leuBCD485 trp*::[Spc^r P$_{lac}$T7-RNAP *lacI*] *motA5461*::Mu*dJ fliA6325*/pET15b	
TH9040	*leuBCD485 trp*::[Spc^r P$_{lac}$T7-RNAP *lacI*] *fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6325* Δ*flgM5794*::FRT/pJK627	
TH9041	*leuBCD485 trp*::[Spc^r P$_{lac}$T7-RNAP *lacI*] *fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6325* Δ*flgM5794*::FRT/pET15b	
TH9042	*leuBCD485 trp*::[Spc^r P$_{lac}$T7-RNAP *lacI*] *fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6325*/pET15b	
TH8604	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6237* (*A. aeolicus flgM* A→G −6 bp from ATG)	
TH8605	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6238* (*A. aeolicus flgM* A→C −5 bp from ATG)	
TH8606	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6239* (*A. aeolicus flgM* A→G −4 bp from ATG)	
TH8607	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6240* (*A. aeolicus flgM* ATG→GTG M1V)	
TH8608	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6241* (*A. aeolicus flgM* AAG→AGG K19R)	
TH8609	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6242* (*A. aeolicus flgM* Δ bp +55–56)	
TH8610	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6243* (*A. aeolicus flgM* ΔA bp +177 from ATG)	
TH8611	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6244* (*A. aeolicus flgM* AAA→AAG K64K, ΔA bp +199 from ATG)	
TH8612	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6245* (*A. aeolicus fliA* CCT→TCT P4S)	
TH8613	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6246* (*A. aeolicus fliA* ACA→TCA T27S)	
TH8614	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6247* (*A. aeolicus fliA* ATA→ATG 129M)	
TH8615	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6248* (*A. aeolicus fliA* AGA→GGA R149G)	
TH8616	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6249* (*A. aeolicus fliA* ACG→ATG 1175M)	
TH8617	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6250* (*A. aeolicus fliA* GAA→GGA E189G)	
TH8618	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6251* (*A. aeolicus fliA* GAA→GGA E199G)	
TH9607	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6245* Δ*flgM5794*::FRT	
TH9608	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6246* Δ*flgM5794*::FRT	
TH9609	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6247* Δ*flgM5794*::FRT	
TH9610	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6248* Δ*flgM5794*::FRT	
TH9611	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6249* Δ*flgM5794*::FRT	
TH9612	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6250* Δ*flgM5794*::FRT	
TH9647	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095 fliA6251* Δ*flgM5794*::FRT	
TH3467	proAB47/F′128 (pro-lac) zzf-3833::Tn*10d*Tc[del-25] (T-POP)	34
TH4387	*fliA5059*::Tn*10d*Tc *fljB*^{e,n,x} *vh2*	
TH7953	*flgM6085*::*tetRA*/pKD46	
TH8009	Δ*fliA5805*::*tetRA*/pKD46	
TH8010	Δ*fliA5805*::*tetRA motA5461*::Mu*dJ*/pKD46	
TH8011	Δ*fliA5805*::*tetRA fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2*/pKD46	
TH8349	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 fliA6090 flgM6085*::*tetRA*/pKD46	
TH8350	*fliC5050*::Mu*dJ fljB*^{e,n,x} *vh2 flgM6095* Δ*fliA5805*::*tetRA*/pKD46	
TH8142	*fliA6088* (*A. aeolicus* GTG-*fliA*$^+$)	
TH8982	*fliA6325* (*C. trachomatis* L2 GTG *fliA*$^+$)	
TH9065	Δ*fliA5647*::FRT/pBAD24	
TH9066	Δ*fliA5647*::FRT/pMC147	
TH9067	Δ*fliA5647*::FRT/pJK558	
TH9068	Δ*fliA5647*::FRT/pJK629	
E. coli		
DH5α	ϕ80d*lacZ*Δ*M15 endA1 hsdR17* ($r_K^- m_K^-$) *supE44 thi-1 recA1 gyrA* (Nal^r (*lacZYA-argF*) *U169*)	New England Biolabs

Continued on following page

TABLE 1—*Continued*

Strain, plasmid, or primer	Genotype description, or sequence (5′→3′)[a]	Source or reference[b]
Plasmids		
pMC147	(pBAD24-*fliA S. enterica* serovar Typhimurium)	
pJK558	(pBAD24-*fliA A. aeolicus*)	
pJK629	(pBAD24-*rspD C. trachomatis*)	
pJK627	(pET15b-*rsbW C. trachomatis* L2)	
pKD46	(pBAD- λ-Red (γ β *exo*)	8
pBAD24	(P_{ara} cloning vector pBR322 ori, Ap^r)	14
pET15b		Novagen
pMS531	(*S. enterica* serovar Typhimurium *fliA*)	42
pLF28	(P_{BAD}-*rspD C. trachomatis*)	39
pET28a-*rsbW*	*C. trachomatis* L2	T. P. Hatch
Primers		
FliAdeltetR	CTCATTTAACGCAGGGCTGTTTATCGTGAATATAGGTCGCGCATGATCGCACCC GAAAAGTttaagacccactttcaca	
FliAdeltetA	CTTTTCGGGTGCGATCATGCGCGACCTATATTCACGATAAACAGCCCTGCGTTA AATGAGTctaagcacttgtctcctg	
FlgMAUGtetR	AGCTGGCCGCTACAACGTAACCCTCGATGAGGATAAATAAttaagacccactttcacatt	
FlgMAUGtetA	TGCTAACGGGTTTCAAAGGTGAGGTACGGTCAATGCTCATctaagcacttgtctcctg	
AAGTG	TAATCATGCCGATAACTCATTTAACGCAGGGCTGTTTATCgtgaaaaaccttacagcaacc	
AA28R	CGTTGTGCGGCACTTTTCGGGTGCGATCATGCGCGACCTAtagaggattagagagcatttc	
AAFLGM1	AGCTGGCCGCTACAACGTAACCCTCGATGAGGATAAATAAatggtgaacaggattcaactc	
AAFLGM2	CATCTGGTCAAGTATTTCTGACAAACGAGTCATACGCTTAcgtaaaaaactctatcagtcc	
CTFLIAGTG	TAATCATGCCGATAACTCATTTAACGCAGGGCTGTTTATCgtgaagactcacgatctcgc	
CTFLIASTOP	CGTTGTGCGGCACTTTTCGGGTGCGATCATGCGCGACCTAaagcagactggacaatgtac	
AQAA1	AAGAATTCATGAAAAACCCTTACAGCAACC	
AQAA2	ACAAAGCTTATAGAGGATTAGAGAGCATTTC	
FliA#3	GGCGCTACAGGTTACATAAG	
FliA#4	TAGTCTATACGTTGTGCGGC	
FlgM5′UP	GAACCGTCGATTCTGATG	
FlgN reverse	GTCTTCAGGTCATTCAG	

[a] In primer sequences, lowercase indicates homology to the *tetRA* element.
[b] All strains were constructed for this study unless otherwise noted.
[c] FRT, FLP recognition target scar (8).

sought to isolate mutants that specifically affect *A. aeolicus* σ^{28}/FlgM interactions. *A. aeolicus* is a motile extreme thermophile, and unlike *S. enterica* serovar Typhimurium, it is difficult to propagate and requires special growth conditions (11). As a result, genetic studies with *A. aeolicus* are challenging. To corroborate the structural studies of *A. aeolicus* σ^{28}/FlgM interaction with an in vivo analysis, we utilized *S. enterica* serovar Typhimurium as a host cell system to genetically characterize the flagellar regulatory proteins of *A. aeolicus*. The availability of genetic and molecular tools and the extensive characterization of the flagellar regulon in *S. enterica* serovar Typhimurium present this organism as an ideal model system.

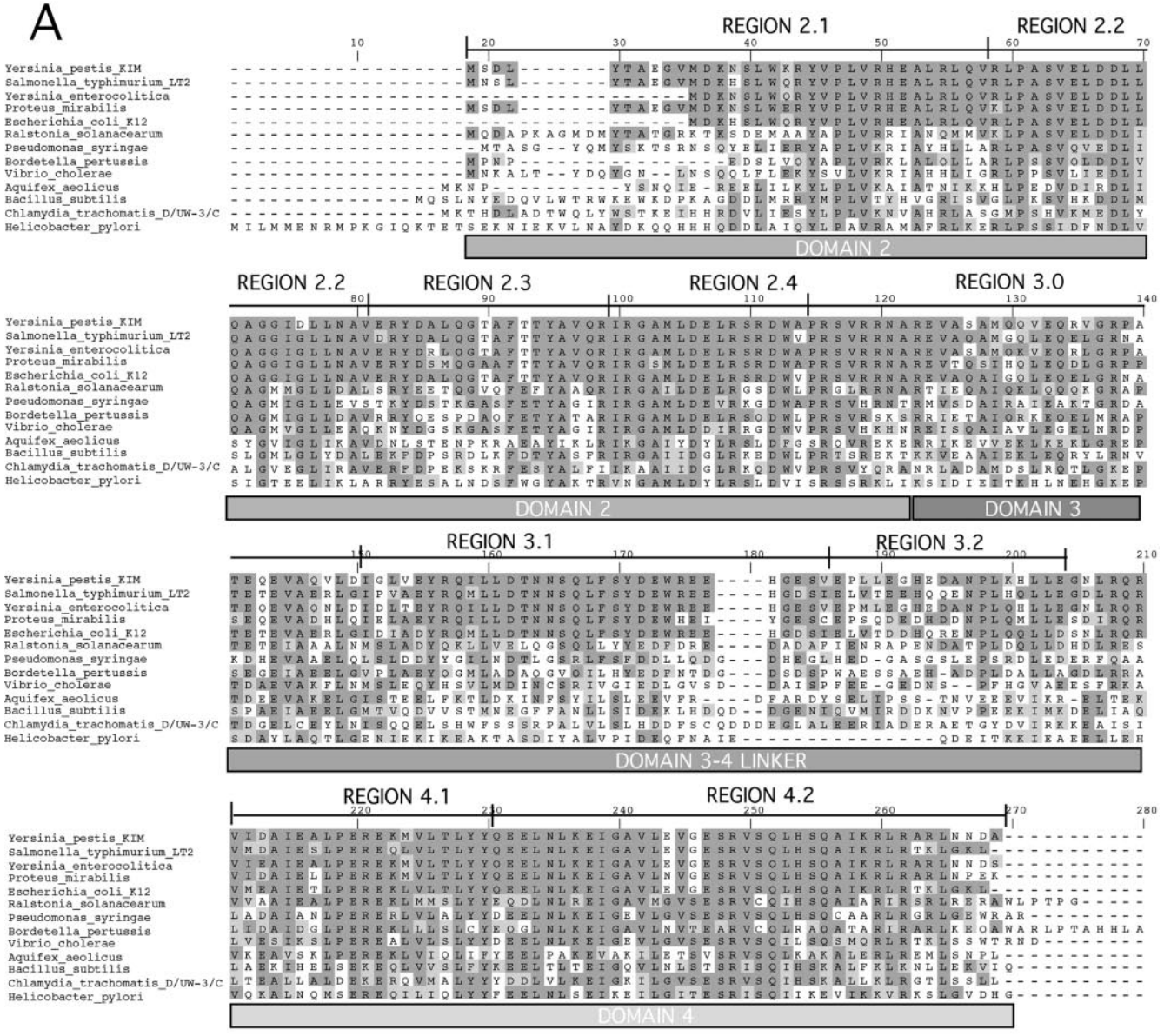
We also sought to test whether *S. enterica* serovar Typhimurium could be used as a general host to study σ^{28} proteins from organisms that are genetically intractable. For this reason, we included the σ^{28} homolog *rspD* from *Chlamydia trachomatis* in these studies. *C. trachomatis* is an obligate intracellular pathogen and is the causal agent of different human diseases such as trachoma, genital infections, and sexually transmitted diseases (37). *Chlamydia* are also difficult to propagate, and construction of stable transformants has been difficult (43). Although *C. trachomatis* is nonmotile, its σ^{28} homolog was shown to recognize the *fliC* promoter of *Escherichia coli* in vitro (39). In this study, we present a genetic characterization of σ^{28} from *A. aeolicus* and *C. trachomatis* in *S. enterica* serovar Typhimurium and an isolation of *A. aeolicus* σ^{28}/FlgM through complementation and mutagenesis experiments.

MATERIALS AND METHODS

Strains. Bacterial strains, plasmids, and primers used in this study are listed in Table 1.

Media and standard genetic and molecular techniques. Media, growth conditions, phage P22 transduction methods, and motility assays were as described previously (10, 13). Tetracycline-sensitive (Tc^s) plates were made as described by Maloy (28). Antibiotics in rich media were used at the following concentrations (in μg/ml): ampicillin (Ap; 100), kanamycin (50), tetracycline (15), and spectinomycin (100). PCR and DNA cloning were performed as described previously (35). DNA primers (Integrated DNA Technologies, Coralville, IA) used in this study are listed in Table 1. PCRs were performed with *Taq* (Promega, Madison, WI), Accuzyme (Bioline, Randolph, MA), or ThermalAce (Invitrogen, Carlsbad, CA). All enzymes used for DNA cloning were from New England Biolabs (Beverly, MA). DNA sequencing was performed using BigDye v3.1 (Applied Biosystems, Foster City, CA), and the reactions were analyzed using a 3730XL sequencer at the DNA sequencing facility of the Department of Biochemistry, University of Washington, Seattle, WA.

λ-Red-mediated insertion of the *tetRA element*. The *fliA* coding region in *S. enterica* serovar Typhimurium was replaced by allelic exchange with a *tetRA* element using λ-Red-mediated recombination (8, 32, 48). In addition, a *tetRA* element was inserted just before the ATG start codon of *flgM*. This *tetRA* element includes the coding sequences of *tetR* and *tetA* from transposon Tn*10d*Tc and confers tetracycline resistance (47). The PCR primers for amplification of the *tetRA* element were flanked by 40-bp sequences of homology for recombination on the chromosome. Primers FliAdeltetR, FliAdeltetA, FlgMAUGtetR, and FlgMAUGtetA were used for the construction of Δ*fliA5805*::*tetRA* and


A
REGION 2.1
REGION 2.2
REGION 2.3
REGION 2.4
REGION 3.0
REGION 3.1
REGION 3.2
REGION 4.1
REGION 4.2
Yersinia_pestis_KIM
Salmonella_typhimurium_LT2
Yersinia_enterocolitica
Proteus_mirabilis
Escherichia_coli_K12
Ralstonia_solanacearum
Pseudomonas_syringae
Bordetella_pertussis
Vibrio_cholerae
Aquifex_aeolicus
Bacillus_subtilis
Chlamydia_trachomatis_D/UW-3/C
Helicobacter_pylori
DOMAIN 2
DOMAIN 3
DOMAIN 3-4 LINKER
DOMAIN 4
B
Yersinia_enterocolitica
Escherichia_coli_K12
Yersinia_pestis_KI
Salmonella_typhimurium_LT2
Proteus_mirabilis
Bordetella_pertussis
Bacillus_subtilis
Vibrio_cholerae
Pseudomonas_syringae
Helicobacter_pylori
Ralstonia_solanacearum
Aquifex_aeolicus
σ28 BINDING DOMAIN

flgM6085::*tetRA*, respectively. The PCRs containing genomic DNA of TH3467, DNA primers, dNTPs, and *Taq* polymerase were amplified as follows; 3 min at 95°C for 1 cycle, 30 s at 95°C, 30 s at 49°C, 2 min at 72°C for 30 cycles, and 10 min at 72°C for 1 cycle. A 1,990-bp product was purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA). *S. enterica* serovar Typhimurium strain TH4702 containing a λ-Red plasmid pKD46 that has a temperature-sensitive replicon (8) was grown under inducing conditions in LB plus Ap (100 μg/ml) plus arabinose at 0.2% at 30°C until the optical density at 600 nm reached 0.6 to 0.8. The cells were washed twice in equal volumes of cold sterile water and concentrated 250-fold. Fifty μl of cells was electroporated with 100 to 200 ng of purified PCR fragment using 0.1-cm cuvettes at 200 Ω, 1.6 kV, and 25 μF. Subsequently, one ml of LB was added, and cells were incubated for 1 hour at 37°C. Approximately 0.5 ml of cells was plated on LB plates containing tetracycline and incubated at 37°C. Tc^r colonies were purified on LB plates without antibiotics and incubated at 42°C. Tc^r colonies were screened for Ap^s to confirm the loss of the pKD46 plasmid. To confirm that the *tetRA* integrated into the correct region on the chromosome, Tc^r and Ap^s colonies were screened by PCR using primers within the *tetRA* element and in sequences flanking *fliA* or *flgM*, respectively.

λ-Red-mediated replacement of the *tetRA* element. The *fliA* and *flgM* genes were replaced in *S. enterica* serovar Typhimurium with homologs from *A. aeolicus* and *C. trachomatis* using λ-Red-mediated replacement of the *tetRA* element as described below. The *fliA* homologs from *A. aeolicus* (*fliA*) and *C. trachomatis* (*rspD*) were PCR amplified using genomic DNA from *A. aeolicus* (kind gift from K. O. Stetter) and pLF28 (39), respectively. Primer sets to amplify the *fliA* homologs are as follows: for *A. aeolicus*, AAGTG and AA28R; for *C. trachomatis*, CTFLIAGTG and CTFLIASTOP. PCR amplification was performed as described above, except the annealing temperature used was 45°C. The PCR products were purified, electroporated into strain TH8350 (Δ*fliA*::*tetRA*) as described above except for subsequent plating of the cells onto Tc^s medium (28), and incubated at 42°C overnight. Colonies were purified once on Tc^s medium at 42°C and then on LB plates at 37°C. The constructs were screened for the loss of the *tetRA* element by PCR, and the region was subsequently PCR amplified for DNA sequencing to confirm the replacement of *S. enterica* serovar Typhimurium *fliA* with the *fliA* homologs of *A. aeolicus* and *C. trachomatis*. The coding region of *flgM* in *S. enterica* serovar Typhimurium was replaced with the homolog from *A. aeolicus* as described for *fliA* using *A. aeolicus* genomic DNA, primers AAF LGM1 and AAFLGM2, and TH8349.

Isolation of *A. aeolicus fliA* and *flgM* mutants using error-prone PCR and λ-Red-mediated replacement of the *tetRA* element. The coding regions of *fliA* and *flgM* from *A. aeolicus* were mutagenized by error-prone PCR with genomic DNA from TH8125, primers FliA#3 and FliA#4 (for σ^{28}), FlgM5′UP and FlgN reverse (for *flgM*), dNTPs, and 5 units of Promega *Taq* per reaction. The PCR amplifications were performed as described above. Electrocompetent cells of strains TH8350 (for *fliA*) and TH8349 (for *flgM*) were prepared as described above, and transformants were selected on Tc^s medium and incubated at 42°C. Colonies were further purified on Tc^s medium at 42°C and then on LB medium at 37°C. The colonies were screened for Lac^+ on MacConkey medium. The *fliA* or *flgM* regions from the Lac^+ isolates were DNA sequenced.

Plasmid constructions. The *fliA* coding sequences from *S. enterica* serovar Typhimurium, *A. aeolicus*, and *C. trachomatis* were cloned under the arabinose promoter to construct pMC147, pJK558, and pJK629 as follows. A 770-bp EcoRI fragment blunted with Klenow fragment containing *S. enterica* serovar Typhimurium *fliA* from pMS531 was ligated into pBAD24 digested with NcoI and blunted with Klenow fragment to generate pMC147. *A. aeolicus fliA* was PCR amplified using *A. aeolicus* genomic DNA and primers AQAA1 and AQAA2. The PCR product was digested with BspHI and HindIII and ligated into pBAD24 digested with NcoI and HindIII to generate pJK588. A 515-bp NcoI-PstI fragment from pLF28 containing *C. trachomatis rspD* was ligated into pBAD24 digested with NcoI-PstI to generate pJK629. A 500-bp NcoI-HindIII fragment from pET-*rsbW C. trachomatis* L2 containing His-tagged *rsbW* from *C. trachomatis* (gift from T. P. Hatch) was ligated into pET15b digested with NcoI-XhoI to generate pJK627.

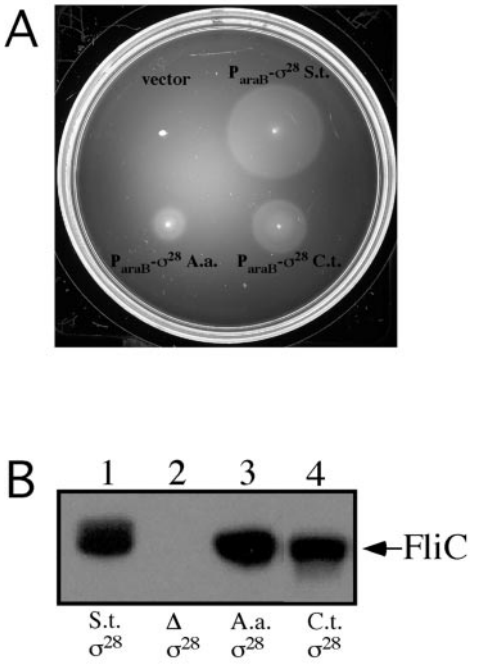


FIG. 2. (A) Motility assay of *S. enterica* serovar Typhimurium *fliA*-defective strain TH4387 with the following plasmids: vector (pBAD24), P_{araB}-σ^{28} *S. enterica* serovar Typhimurium (S.t.) (pMC147), P_{araB}-σ^{28} *A. aeolicus* (A.a.) (pJK558), and P_{araB}-σ^{28} *C. trachomatis* (C.t.) (pJK629). (B) Western analysis of total cellular proteins from *S. enterica* serovar Typhimurium hybrid strains *fliA* (σ^{28}) from *A. aeolicus* and C. *trachomatis* probing with anti-FliC antiserum. Lane 1, TH437 (*S. enterica* serovar Typhimurium wild-type strain LT2); lane 2, TH6827 (Δ*fliA5805*::*tetRA*); lane 3, TH8142 ((*fliA6068* [*A. aeolicus fliA*]); lane 4, TH8982 ((*fliA6325* [*C. trachomatis rspD*]). Fourfold more cellular lysate was loaded in lanes 2 to 4 than in lane 1.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Maloy (27). Cells were grown in LB or LB plus Ap (100 μg/ml) plus 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (in strains containing plasmids) at 37°C until mid-log phase. Assays were performed in triplicate, and the values were normalized as percentages of the wild-type (WT) value.

Western blot analysis. Cells were grown in LB until mid-log phase, and 1 ml of cells was centrifuged and resuspended in sodium dodecyl sulfate sample buffer. The samples were run on a 10% Tricine-sodium dodecyl sulfate polyacrylamide gel and electrotransferred in CAPS buffer onto polyvinylidene diflu-

FIG. 1. (A) Clustal W amino acid alignments of σ^{28} homologs. The GenInfo Identifier numbers of the σ^{28}s used in the alignments are as follows: *Yersinia pestis* KIM (gi22126347), *Salmonella enterica* serovar Typhimurium LT2 (gi16765294), *Yersinia enterocolitica* (gi1706832), *Proteus mirabilis* (gi6959882), *Escherichia coli* K-12 (gi33347603), *Ralstonia solanacearum* (gi17549609), *Pseudomonas syringae* (gi28869183), *Bordetella pertussis* (gi33571813), *Vibrio cholerae* (gi15642066), *Aquifex aeolicus* (gi15606452), *Bacillus subtilis* (gi1350863), *Chlamydia trachomatis* (gi15604780), and *Helicobacter pylori* (gi15645646). Conserved amino acid regions as described by Lonetto et al. (24) are marked in black at the top of the alignments. The σ^{28} domains from previous structural studies by Sorenson et al. (41) are noted below. (B) Clustal W amino acid alignments of FlgM homologs. The GenInfo Identifier numbers of the FlgM used in the alignments are as follows: *Yersinia enterocolitica* (gi6166171), *Escherichia coli* K-12 (gi1651526), *Yersinia pestis* KIM (gi22126396), *Salmonella enterica* serovar Typhimurium LT2 (gi16764528), *Proteus mirabilis* (gi1857441), *Bordetella pertussis* (gi33572120), *Bacillus subtilis* (gi729520), *Vibrio cholerae* (gi15642203), *Pseudomonas syringae* (gi28869129), *Ralstonia solanacearum* (gi17548561), *Helicobacter pylori* (gi18075990), and *Aquifex aeolicus* (gi15605866). The σ^{28} binding domain in *S. enterica* serovar Typhimurium FlgM is highlighted at the bottom of the alignments. The shading scheme for amino acid homologies was determined using Blosum62 (7).

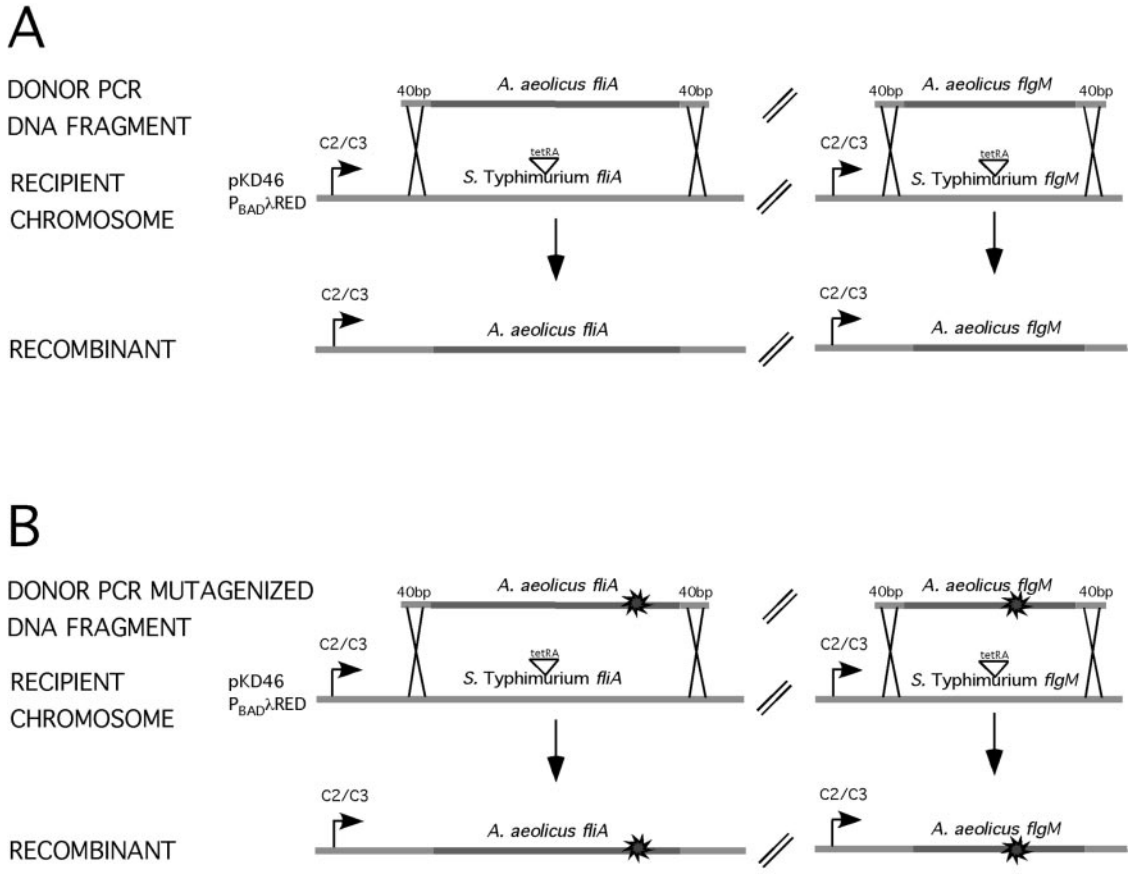


FIG. 3. (A) λ-Red-mediated replacement of the *tetRA* element. Donor PCR products from coding sequences of *fliA* (σ^{28}) or *flgM* from *A. aeolicus* were flanked by 40-bp sequences of homology for recombination in the chromosome. The donor PCR products were electroporated into *S. enterica* serovar Typhimurium strains with *tetRA* elements inserted in the *fliA* or *flgM* genes. The *tetRA* element includes the coding sequences of *tetR* and *tetA* from transposon Tn*10d*Tc and confers tetracycline resistance. The recombination was mediated by λ-Red (plasmid pKD46) (8), resulting in the loss of the *tetRA* element and replacement with the coding sequences of *fliA* or *flgM* from *A. aeolicus* by selection on tetracycline-sensitive medium (28). (B) Isolation of *A. aeolicus fliA* (σ^{28}) and *flgM* mutants using error-prone PCR and λ-Red-mediated replacement of the *tetRA* element. Replacement of the *tetRA* element were performed as described for panel A, except the donor PCR products of coding sequences from *fliA* (σ^{28}) or *flgM* were generated by error-prone PCR (as indicated by the sunburst symbol).

oride membranes (29, 38). The membrane was probed with rabbit anti-FliC antibodies (Salmonella H antiserum i rabbit, catalog number 228241; Difco) purified as described previously (15) and developed using an ECL-Plus kit (Amersham Biosciences, Piscataway, NJ).

Software programs. Amino acid alignments were performed using Clustal W (6). The sequence alignments were edited using Jalview alignment editor (7). *Aquifex aeolicus* σ^{28}/FlgM cocrystal analysis used coordinates PDB ID 1SC5 from the RCSB protein database (www.rcsb.org/pdb/) and RasMol v. 2.7 (2, 36). The analysis of helix interactions was done with PDBsum (23). The analysis of residue contacts was determined on PDB ID 1SC5 using CSU (contact of structural units) software (40).

RESULTS

***A. aeolicus* and *C. trachomatis* σ^{28} restore motility to an *S. enterica* serovar Typhimurium σ^{28} mutant.** Given the similarities in the critical domains of σ^{28} (see alignments in Fig. 1), we tested whether σ^{28} homologs of *A. aeolicus* and *C. trachomatis* would complement an *S. enterica* serovar Typhimurium σ^{28} mutant. The genes encoding σ^{28} of *S. enterica* serovar Typhimurium ($fliA_{Se\text{-}T}$), *A. aeolicus* ($fliA_{Aa}$), and *C. trachomatis* ($rspD_{Ct}$) were cloned under the arabinose-inducible promoter (P_{araB}) and introduced into the *S. enterica* serovar Typhimurium *fliA* mutant strain TH4387. The plasmid-containing strains were assessed for motility on plates containing 0.2% arabinose (Fig. 2A). Levels of complementation for motility by P_{araB}-*A. aeolicus* $fliA^+$ and P_{araB}-*C. trachomatis* $rspD^+$ were 39% and 58%, respectively, of the level for P_{araB}-*S. enterica* serovar Typhimurium $fliA^+$ (Fig. 2A). Similar results were previously observed with *A. aeolicus* $fliA^+$ from *E. coli.* (44). Interestingly, a P_{araB}-*C. trachomatis* $rspD^+$ plasmid did not restore motility in *E. coli* with a *fliA* deletion (39). Although *E. coli* and *S. enterica* serovar Typhimurium possess nearly identical flagellar systems (25), the dramatic differences in complementation for motility with P_{araB}-*C. trachomatis* $rspD^+$ demonstrates that differences in the flagellar regulons between the two species can be uncovered.

The coding region of chromosomal *S. enterica* serovar Typhimurium *fliA* was replaced with either the *fliA* from *A. aeolicus* or the *rspD* from *C. trachomatis* by targeted replacement of a *tetRA* element using a λ-Red recombination system (Fig. 3A; also see reference 8 and Materials and Methods). Therefore, σ^{28}-mediated gene expression in these hybrid strains remained

under the regulation of the *S. enterica* serovar Typhimurium flagellar regulon. These σ^{28} hybrid strains were weakly motile (data not shown). Because motility was weak, we analyzed whether the hybrid strains expressed the σ^{28}-dependent flagellar filament protein FliC. Western blot analysis using anti-FliC serum on cellular lysates showed the presence of FliC protein in *S. enterica* serovar Typhimurium hybrid strains with *A. aeolicus* σ^{28} or *C. trachomatis* σ^{28} (Fig. 2B, lanes 3 and 4, respectively). However, the levels of FliC protein were reduced in the σ^{28} hybrid strains by fourfold relative to the level for WT *S. enterica* serovar Typhimurium (Fig. 2B). Although motility was reduced in the σ^{28} hybrid strains, the presence of FliC protein and the ability to transcribe class 3 flagellar promoters (see below) indicate the conservation of σ^{28} function. Together, these results indicate that σ^{28} from diverse species can interact with the heterologous host transcriptional machinery for flagellar biogenesis.

Regulation of σ^{28} activity by the cognate negative regulator FlgM is species specific. To investigate the interactions of cognate sigma factor and anti-sigma factor activities between species, hybrid *S. enterica* serovar Typhimurium strains were constructed by use of λ-Red (Fig. 3A; also see Materials and Methods). The *fliA*$^+$ gene (σ^{28}) of *S. enterica* serovar Typhimurium was replaced with the σ^{28} homologs from *A. aeolicus* and *C. trachomatis*, *fliA*$^+$ and *rspD*$^+$, respectively. Further, the negative regulatory gene *flgM*$^+$ of *S. enterica* serovar Typhimurium was replaced with *A. aeolicus flgM*$^+$. We assayed σ^{28}-mediated gene expression in the hybrid strains using *lac* gene transcriptional fusion reporters to two σ^{28}-dependent promoters, *motA* and *fliC*. The *motA* and *fliC* genes encode vital components of the flagellum; MotA is required for flagellar rotation, and FliC is the main structural component of the flagellar filament (25).

Relative β-galactosidase levels of *motA-lac* and *fliC-lac* expression in WT *S. enterica* serovar Typhimurium increased 3- and 1.7-fold, respectively, in the absence of the *S. enterica* serovar Typhimurium FlgM (Fig. 4, compare lanes 2 and 3 in both panels). Likewise, in *S. enterica* serovar Typhimurium strains with *A. aeolicus* σ^{28}, in the absence of *A. aeolicus* FlgM, *motA-lac* and *fliC-lac* expression was increased around 2.5-fold (Fig. 4, compare lanes 6 and 7). This is in support of structure studies on *A. aeolicus* σ^{28}/FlgM interaction which suggest that FlgM binding to σ^{28} inhibits its interaction with core RNAP (41). Interestingly, in the presence of *S. enterica* serovar Typhimurium FlgM, *A. aeolicus* σ^{28}-mediated levels of *motA-lac* and *fliC-lac* expression were similar to that of an *S. enterica* serovar Typhimurium *flgM* deletion strain, indicating the absence of negative regulation on *A. aeolicus* σ^{28} activity (Fig. 4, compare lanes 7 and 8). In *S. enterica* serovar Typhimurium σ^{28} strains, the relative levels of β-galactosidase activity in the *S. enterica* serovar Typhimurium *flgM* deletion strain were similar to those of strains containing *A. aeolicus* FlgM (Fig. 4, compare lanes 3 and 4), suggesting that *A. aeolicus* FlgM has no effect on *S. enterica* serovar Typhimurium σ^{28} activity. These findings show inhibition of *A. aeolicus* σ^{28} activity by its cognate negative regulator FlgM but not by *S. enterica* serovar Typhimurium FlgM. Similarly, *S. enterica* serovar Typhimurium σ^{28} activity is inhibited by its cognate negative regulator FlgM but not by *A. aeolicus* FlgM. Collectively, these studies indicate that the anti-σ^{28} FlgM factors interactions with σ^{28}, and their negative regulation on σ^{28} activities are species specific.

Hybrid strains of *S. enterica* serovar Typhimurium containing *C. trachomatis* rspD$^+$ (*C. trachomatis* σ^{28}) were constructed in *motA-lac* and *fliC-lac* backgrounds. *C. trachomatis* RsbW, the putative negative regulator of RspD (39), was encoded in *trans* by plasmid pJK627. However, there was no difference in the relative β-galactosidase levels in the presence of RsbW or in its absence (Fig. 4, lanes 10 and 11, respectively). *S. enterica* serovar Typhimurium FlgM also did not negatively regulate *C. trachomatis* σ^{28} activity (Fig. 4, lanes 12). *C. trachomatis* RsbW is 36% identical and 58% similar to homolog RsbW in *Bacillus subtilis*. In *B. subtilis*, anti-sigma factor RsbW and anti-anti-sigma factor RsbV, as well as other regulatory proteins, regulate σ^{B} activity but differ mechanistically from anti-σ^{28} FlgM homologs (16). The lack of anti-σ^{28} activity by *C. trachomatis* RsbW in *S. enterica* serovar Typhimurium suggests that additional factors for *C. trachomatis* σ^{28} inhibition may be required or that σ^{28} inhibition may be independent of RsbW.

Isolation of *fliA* mutants defective in *A. aeolicus* σ^{28}/FlgM interactions. We sought to isolate mutants defective in *A. aeolicus* σ^{28}/FlgM interactions using a genetic screen. *S. enterica* serovar Typhimurium strain TH8125 contains *A. aeolicus fliA*$^+$ and *A. aeolicus flgM*$^+$ genes as well as a σ^{28}-dependent *fliC-lac* reporter construct. TH8125 is white (Lac$^-$) on MacConkey lactose (Mac-lac) medium, and the same strain deleted for *A. aeolicus flgM* is red (Lac$^+$). Therefore, a phenotypic screen was used to identify mutants of TH8125 defective in *A. aeolicus* σ^{28}/FlgM interactions (red on Mac-lac). Error-prone PCR was used to mutagenize *A. aeolicus fliA* (σ^{28}) and *A. aeolicus flgM* genes. The mutagenized gene products were electroporated into *S. enterica* serovar Typhimurium strain TH8125 that had a *tetRA* insertion in either *A. aeolicus fliA* or *A. aeolicus flgM*. Subsequent recombination of *fliA* and *flgM* was achieved by use of λ-Red (Fig. 3B). The loss of tetracycline resistance, indicative of replacement with the mutagenized gene products, was selected on tetracycline-sensitive medium (28). The transformants were subsequently screened on Mac-lac medium, and red (Lac$^+$) colonies were sequenced for putative mutations in either *A. aeolicus fliA* or *A. aeolicus flgM.*

In the *A. aeolicus fliA* mutagenesis screen, 300 Tc^s colonies were isolated, of which 28 were Lac$^+$ on Mac-lac medium. Twelve Lac$^+$ mutants were sequenced, and seven contained missense mutations in *A. aeolicus fliA* (Fig. 5A and Table 2). Since the remaining five had multiple mutations in *A. aeolicus fliA* and are difficult to interpret, they were excluded from the study. β-Galactosidase assay results for *A. aeolicus* σ^{28} mutants compared to those for WT (TH8125) demonstrated an increase in activity in the presence of *A. aeolicus* FlgM (Fig. 5B). The relative activity of *A. aeolicus* σ^{28} mutants ranged from 1.3- to 2.9-fold higher than that for WT (TH8125) (Table 2).

To examine whether the mutants were more active due to increased activity of σ^{28}, β-galactosidase assays were performed in the absence of *A. aeolicus* FlgM (TH8200) (Fig. 5B). The relative activity of *A. aeolicus* σ^{28} mutants compared to WT (TH8200) ranged from 0.8- to 1.6-fold higher (Table 2). Mutants T27S, I29M, and T175M had more σ^{28} activity than WT (TH8200) in the absence *A. aeolicus* FlgM, suggesting that their ability to bypass FlgM inhibition was due to increased σ^{28}

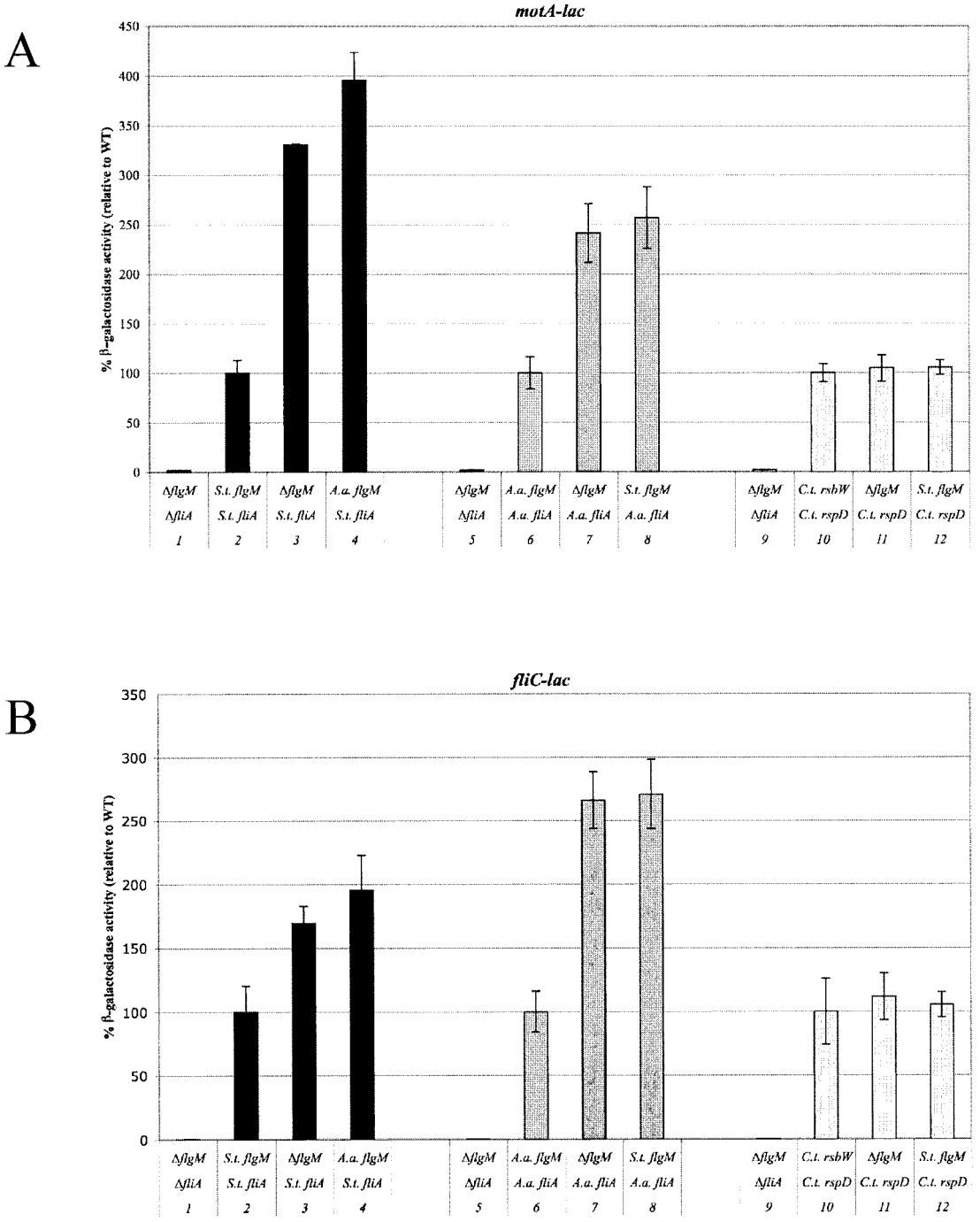


FIG. 4. β-Galactosidase activities of *lac* gene transcriptional fusion reporters to σ^{28}-dependent genes *motA* and *fliC* in *S. enterica* serovar Typhimurium. The σ^{28} coding sequences were replaced by λ-Red recombination with coding sequences from either σ^{28} from *A. aeolicus* (*fliA*) or *C. trachomatis* (*rspD*). The *flgM* coding sequence was replaced with *A. aeolicus flgM*. *C. trachomatis* RsbW, putative negative regulator of RspD, was encoded in *trans* by plasmid pJK627. (A) MotA-lac β-galactosidase activities were compared relative to those of the following isogenic WT σ^{28} strains (WT = 100%): TH6238, black bars; TH8196, gray bars; TH9033, white bars. Lane 1, Δ*fliA*, Δ*flgM* (TH8197); lane 2, *S. enterica* serovar Typhimurium (*S.t.*) *fliA*, *S. enterica* serovar Typhimurium *flgM* (TH6238); lane 3, *S. enterica* serovar Typhimurium *fliA*, Δ*flgM* (TH8194); lane 4, *S. enterica* serovar Typhimurium *fliA*, *A. aeolicus* (*A.a.*) *flgM* (TH8120); lane 5, Δ*fliA*, Δ*flgM* (TH8197); lane 6, *A. aeolicus fliA*, *A. aeolicus flgM* (TH8122); lane 7, *A. aeolicus fliA*, Δ*flgM* (TH8196); lane 8, *A. aeolicus fliA*, *S. enterica* serovar Typhimurium *flgM* (TH8059,); lane 9, Δ*fliA*, Δ*flgM* (TH8197); lane 10, *C. trachomatis* (*C.t.*) *rspD*, *C. trachomatis rsbW* (TH9031); lane 11, *C. trachomatis rspD*, Δ*flgM* (TH9032); lane 12, *C. trachomatis rspD*, *S. enterica* serovar Typhimurium *flgM* (TH9033). (B) FliC-lac β-galactosidase activities were compared relative to those of the following isogenic WT σ^{28} strains (WT = 100%): TH4098, black bars; TH8125, gray bars; TH9040, white bars. Lane 1, Δ*fliA*, Δ*flgM* (TH8201); lane 2, *S. enterica* serovar Typhimurium *fliA*, *S. enterica* serovar Typhimurium *flgM* (TH4098); lane 3, *S. enterica* serovar Typhimurium *fliA*, Δ*flgM* (TH8198); lane 4, *S. enterica* serovar Typhimurium *fliA*, *A. aeolicus flgM* (TH8123); lane 5, Δ*fliA*, Δ*flgM* (TH8201); lane 6, *A. aeolicus fliA*, *A. aeolicus flgM* (TH8125); lane 7, *A. aeolicus fliA*, Δ*flgM* (TH8200); lane 8, *A. aeolicus fliA*, *S. enterica* serovar Typhimurium *flgM* (TH8061); lane 9, Δ*fliA*, Δ*flgM* (TH8201); lane 10, *C. trachomatis rspD*, *C trachomatis. rsbW* (TH9040); lane 11, *C. trachomatis rspD*, Δ*flgM* (TH9041); lane 12, *C. trachomatis rspD*, *S. enterica* serovar Typhimurium *flgM* (TH9042).

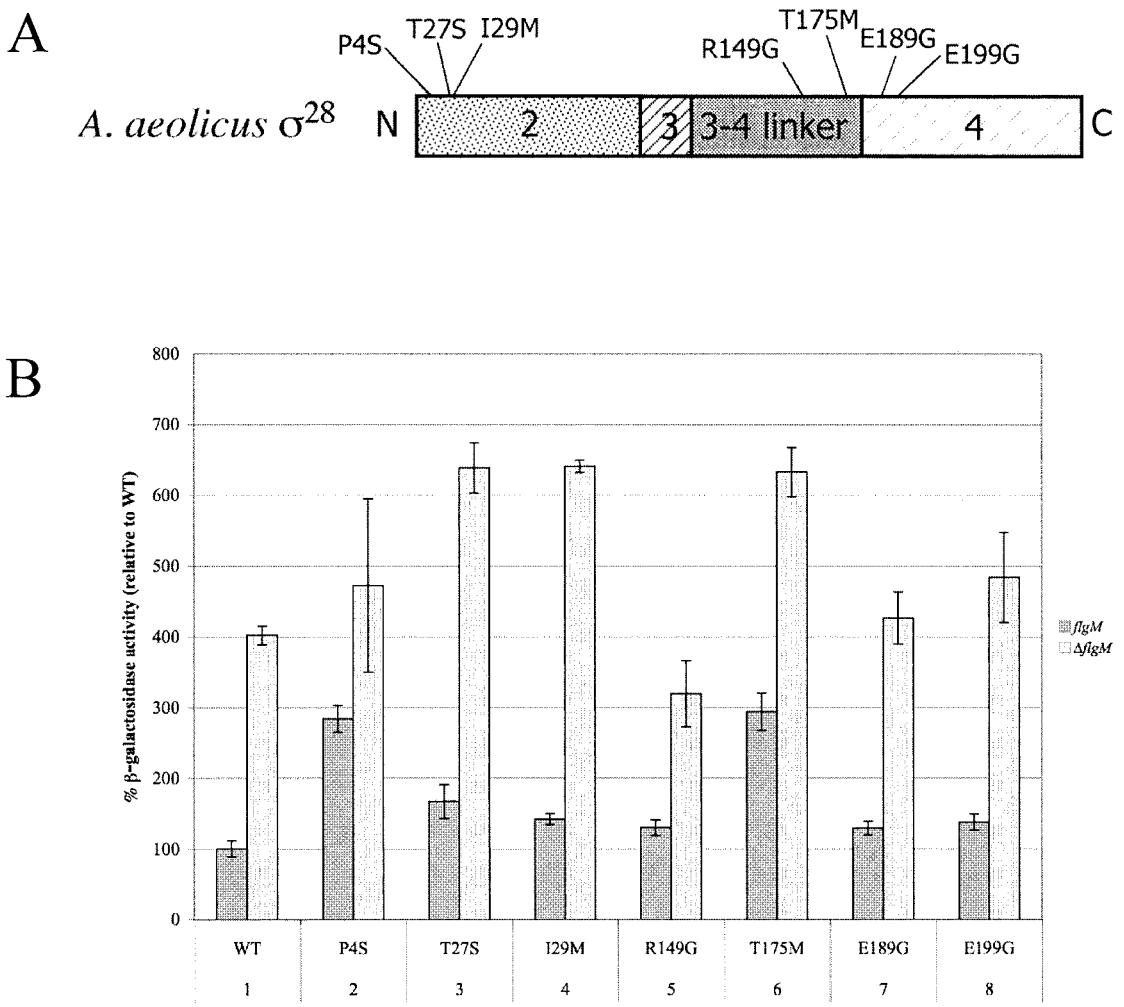


FIG. 5. (A) Summary of mutations isolated in *A. aeolicus fliA*. Domain regions correspond to regions shown in Fig. 1. Mutations resulting in single amino acid changes are listed in Table 2. (B) β-Galactosidase activities of *fliC-lac* in hybrid *S. enterica* serovar Typhimurium strains with *A. aeolicus fliA* mutants isolated defective for σ^{28}/FlgM interactions. β-Galactosidase activities were compared to those of TH8125. Dark gray bars indicate the presence of FlgM; light gray bars indicate the absence of FlgM. Lane 1, WT (TH8125, TH8200); lane 2, P4S (TH8612, TH9607); lane 3, T27S (TH8613, TH9608); lane 4, I29M (TH8614, TH9609); lane 5, R149G (TH8615, TH9610); lane 6, T175M (TH8616, TH9611); lane 7, E189G (TH8617, TH9612); lane 8, E199G (TH8618, TH9647).

activity and that they are not defective for FlgM interaction. Mutants I29M and T175M had changes in codon frequency that may have resulted in increased levels of σ^{28} expression (Table 2). The codon frequency change of mutant T27S was nominal (Table 2), suggesting that an increased σ^{28} activity is not due to increased translation. The increased activity could be attributed to increased stability, as was found for the H14D mutants of *S. enterica* serovar Typhimurium σ^{28} that were able to bypass FlgM inhibition (4).

Mutation R149G had more σ^{28} activity than WT (TH8125) in the presence of FlgM but had less σ^{28} activity in the absence of FlgM (TH8200) (Fig. 5B and Table 2). This class of mutant was also found in the *S. enterica* serovar Typhimurium σ^{28} defective for FlgM interaction (4). Although they had more σ^{28} activity in the presence of FlgM, they were implied to have less σ^{28} activity in the absence of FlgM, due to amino acid substitutions that increased the turnover of σ^{28} (4).

Mutants P4S, E189G, and E199G have σ^{28} activity levels similar to that of WT (TH8200) in the absence *A. aeolicus* FlgM and more σ^{28} activity than WT (TH8125) in the presence of *A. aeolicus* FlgM, suggesting they are defective for FlgM interaction (Fig. 5B and Table 2). Residue E189 is buried within the σ^{28} structure, where there are extensive contacts with the other interacting domains (41). This mutation may be involved in stabilizing the σ^{28} structure in a manner that renders it resistant to FlgM inhibition through allosteric effects. In contrast, P4 lies at the N terminus of σ^{28}, is not buried within the σ^{28}, and appears too distant to make contacts with FlgM (41). Nonetheless, the P4S mutation could involve a conformational change to alter inhibition by FlgM or modify σ^{28} protein stability or interaction with RNAP.

The E199G mutation found in *A. aeolicus* σ^{28} corresponds to location E203 of *S. enterica* serovar Typhimurium (4). An *S. enterica* serovar Typhimurium σ^{28} E203D mutant exhibited 10-fold less affinity to *S. enterica* serovar Typhimurium FlgM and was the most attenuated for the destabilizing of the *S.*

TABLE 2. Summary of mutants isolated in *A. aeolicus fliA*

Strain	Mutation relative to the start codon	Comments	Change in % of codon frequency from WT to mutant	Relative activity (*P* value) compared to[a]:	
				TH8125 in the presence of FlgM[b]	TH8200 in the absence of FlgM[c]
TH8125		WT		1	
TH8200		Δ*flgM*			1
TH8612	C→T +10 bp	CCT→TCT P4S, domain σ2	7.8 to 8.5	2.8 (<0.001)	1.2 (>0.05)
TH8613	A→T +79 bp	ACA→TCA T27S, domain σ2	4.7 to 5.9	1.7 (0.012)	1.6 (<0.001)
TH8614	A→G +87 bp	ATA→ATG, 129M, domain σ2	4.2 to 23.6	1.4 (0.006)	1.6 (0.003)
TH8615	A→G +445 bp	AGA→GGA, R149G, domain σ3-4 linker	2.2 to 6.3	1.3 (0.032)	0.8 (0.042)
TH8616	C→T +524 bp	ACG→ATG, T175M, domain σ3-4 linker	17.1 to 23.6	2.9 (<0.001)	1.6 (0.013)
TH8617	A→G +566 bp	GAA→GGA, E189G, domain σ4	55 to 14.5	1.3 (0.030)	1.1 (>0.05)
TH8618	A→G +596 bp	GAA→GGA, E199G, domain σ4	55 to 14.5	1.4 (0.016)	1.2 (>0.05)

[a] Relative β-galactosidase activities were calculated from Fig. 5B.
[b] *P* values were determined using two-tailed *t* tests on β-galactosidase activities compared to TH8125.
[c] *P* values were determined using two-tailed *t* tests on β-galactosidase activities compared to TH8200.

enterica serovar Typhimurium σ^{28}-RNAP holoenzyme by *S. enterica* serovar Typhimurium FlgM (4). By analogy to *S. enterica* serovar Typhimurium σ^{28}, the *A. aeolicus* σ^{28} E199 position is an important contact for FlgM in inhibition of σ^{28} interaction, with RNAP likely to be involved in the "stripping" of σ^{28} from the σ^{28}-RNAP holoenzyme complex by FlgM (4, 41). The E199G mutation is located in the conserved region 4 of the σ^{70} family of transcription factors (31). Interestingly, many mutations isolated in *S. enterica* serovar Typhimurium defective in σ^{28}/FlgM interactions mapped to σ^{28} region 4 (4, 20). This domain is also a target for transcriptional regulation in many other systems (12). Anti-sigma factors Rsd, SpoIIAB, and FlgM bind to region 4 of the sigma factors to inhibit its interaction with RNAP (12).

Previous structural studies showed that when FlgM is bound to σ^{28}, it could occlude the interaction of this domain with the β-flap of RNAP that is important for proper recognition of the −35 promoter element (22, 30, 41, 45). Further, studies suggest FlgM helix H3′ interacts with region 4 (where E199 of *A. aeolicus* σ^{28} is located) on the σ^{28}-RNAP holoenzyme complex, and either by a conformational change in the interaction with FlgM or through enzyme "breathing," region 4 is released for binding by helix H4′ of FlgM. This inhibits region 4 interaction with the RNAP β-flap and the subsequent dissociation of σ^{28} from RNAP (4, 41). E199 in σ^{28} lies within the face of the helix in s_4, where it interacts with H3′ helix of FlgM (41). Isolation of E199G in *A. aeolicus* σ^{28} that is resistant to FlgM inhibition supports this model.

Isolation of *flgM* mutants defective in *A. aeolicus* σ^{28}/FlgM interactions. Three hundred Tc^s clones were isolated from the *A. aeolicus flgM* mutagenesis screen, of which 25 were Lac^+ on Mac-lac indicator medium. Twelve Lac^+ colonies were sequenced, and eight had either single base pair changes or deletions in FlgM (Fig. 6 and Table 3). The remaining four had multiple mutations, and they were excluded from the study. All strains with *A. aeolicus flgM* mutations were assayed for σ^{28} activity by use of β-galactosidase assays to compare the levels of *fliC-lac* expression with a strain harboring wild-type *A. aeolicus flgM* (Table 3).

Of the mutations characterized, several were found in the ribosome binding site, one altered in the start codon, and another introduced a rare codon; all mutations presumably lowered the levels of FlgM, thereby enabling increased σ^{28} activity (Fig. 6 and Table 3). Three mutations resulted in frameshifts in FlgM after codon positions 18, 59, and 67. Frameshift mutation after codon 67 suggests that the last 21 amino acids in FlgM are important for inhibition of σ^{28} activity. Previous studies of FlgM show the C terminus is important for σ^{28} interaction (9, 41). Alternatively, the resulting frameshift mutation could destabilize FlgM protein; therefore, additional biochemical methods are needed to support the in vivo findings.

We found a bias in favor of *A. aeolicus flgM* null mutant isolation, because the screen was based on the loss of anti-σ^{28} activity. Previous isolations of FlgM mutants of *S. enterica* serovar Typhimurium defective in interaction with *S. enterica* serovar Typhimurium σ^{28} indicated a bias against *flgM* null alleles. This is because *S. enterica* serovar Typhimurium strains carrying *flgM* null alleles and remaining *fliA*$^+$ are sick, and excess σ^{28} activity is lethal in *S. enterica* serovar Typhimurium. The FlgM mutants obtained were predominantly single amino acid substitution mutants that retained some anti-σ^{28} activity (5, 9). Since it was easy to isolate *flgM* null alleles of *A. aeolicus* in a strain expressing *A. aeolicus* σ^{28}, we conclude that excess *A. aeolicus* σ^{28} activity, unlike excess *S. enterica* serovar Typhimurium σ^{28} activity, does not inhibit cell growth.

DISCUSSION

We have demonstrated the use of *S. enterica* serovar Typhimurium as an in vivo heterologous system to assess the sigma factor activities of homologs from other genetically intractable species, such as *A. aeolicus* and *C. trachomatis*. We demonstrate that the λ-Red technology provides a simple method to replace *S. enterica* serovar Typhimurium genes with functional homologues from bacterial species that are normally geneti-

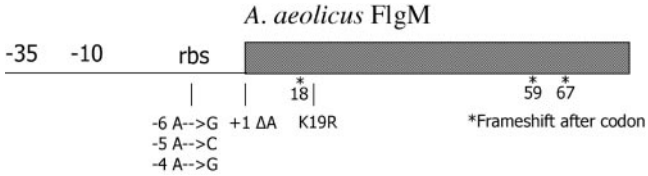


FIG. 6. Summary of mutations isolated in *A. aeolicus flgM*. rbs, ribosome binding site.

TABLE 3. Summary of mutations isolated in *A. aeolicus flgM*

Strain	Mutation relative to the start codon	Comments[a]	Change in % of codon frequency from WT to mutant	% *fliC-lac* β-galactosidase activity relative to WT	Relative activity[b]
TH8125		WT		100	1
TH8200		Δ*flgM*		395	4.0
TH8604	A→G −6 bp	RBS		116	1.2
TH8605	A→C −5 bp	RBS		316	3.2
TH8606	A→G −4 bp	RBS		95	0.95
TH8607	A→G +1 bp	ATG→GTG M1V	26.3 to 26.4	168	1.7
TH8608	A→G +56 bp	AAG→AGG K19R, rare codon	11.7 to 1.2	116	1.2
TH8609	ΔA +55–56 bp	Frameshift after codon 18		395	4.0
TH8610	ΔA +177 bp	Frameshift after codon 59		374	3.7
TH8611	ΔA +199 bp	Frameshift after codon 67		437	4.4

[a] RBS, ribosome binding site.
[b] Relative activity calculated from β-galactosidase activities in column 5.

cally intractable. Chromosomal replacement of both the *A. aeolicus* and *C. trachomatis fliA*$^+$ genes complemented an *S. enterica* serovar Typhimurium *fliA* mutant for motility and production of FliC flagellin. While complementation by *A. aeolicus fliA*$^+$ was not as robust as that with *C. trachomatis fliA*$^+$, these assays were done without the aid of a rare-codon tRNA plasmid that was required to obtain large amounts of *A. aeolicus* FliA from expression in *E. coli* for crystallization (41). Indeed, by not using a rare-codon tRNA plasmid in these studies, the sensitivity of our genetic selection allowed for the isolation of a class of *A. aeolicus fliA* mutants with increased efficiency of translation. In addition, we isolated mutations in *A. aeolicus* of the class expected to be defective for σ^{28}/FlgM interactions: the increase in class 3 transcription was only in the presence of FlgM and not in the absence of FlgM.

We found that FlgM inhibition of *S. enterica* serovar Typhimurium and *A. aeolicus* σ^{28}-dependent class 3 transcription was species specific. *A. aeolicus* FlgM would inhibit only *A. aeolicus* σ^{28} activity and not *S. enterica* serovar Typhimurium σ^{28} activity; *S. enterica* serovar Typhimurium FlgM would inhibit only *S. enterica* serovar Typhimurium σ^{28} activity and not *A. aeolicus* σ^{28} activity. We also tested the possibility that RsbW was a negative regulator of *C. trachomatis* σ^{28}, as had been suggested previously (39). However, we found that RsbW did not inhibit *C. trachomatis* σ^{28} in *S. enterica* serovar Typhimurium. Either RsbW does not inhibit *C. trachomatis* σ^{28} activity or other factors are required for such an activity to be observed.

The ability to replace the *S. enterica* serovar Typhimurium *fliA* and *flgM* genes with homologous genes of genetically intractable organisms allowed us to use standard *Salmonella* genetic methods to study interactions of sigma and anti-sigma factors from bacterial species that are otherwise difficult to analyze genetically. It also provided an additional benefit in that plasmid artifacts are avoided when such genes are expressed from the normal *S. enterica* serovar Typhimurium chromosomal location. We also demonstrate the ability to use λ-Red recombination to replace any gene to be targeted for mutagenesis with the *tetRA* element. The *tetRA* element is an excellent tool for targeted mutagenesis because of the ability to select for inheritance of (Tc^r) and replacement of (Tc^s) the *tetRA* element. This general strategy could be employed with any bacterial species where the λ-Red system can be expressed and is functional, and a selectable/counterselectable marker(s), such as *tetRA* or *sacB* linked to a drug-resistance gene, can be used to perform targeted mutagenesis.

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