

A Little Gene with Big Effects: a *serT* Mutant Is Defective in *flgM* Gene Translation

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A conditional-lethal mutant was isolated as having a flagellar regulatory phenotype at 30°C and being unable to grow at 42°C. Chromosomal mapping localized the mutation to the *serT* gene, which encodes an essential serine tRNA species (tRNA^{Ser}_{cmo5UGA}). DNA sequence analysis revealed the mutation to be a single base change in G:A at position 10 of the *serT* gene that lies within the D-stem of the essential tRNA^{Ser}_{cmo5UGA} species. tRNA^{Ser}_{cmo5UGA} recognizes UCA, UCG, and UCU codons, but UCU is also recognized by tRNA^{Ser}_{GGA} and UCG by tRNA^{Ser}_{CGA}. No other tRNAs are known to read the UCA codon. Thus, the UCA codon is specifically recognized by tRNA^{Ser}_{cmo5UGA}. We show that the anti- σ^{28} activity of FlgM is defective in the *serT* mutant strain. The *serT* allele causes a 10-fold increase in σ^{28} -dependent *fliC* promoter transcription, indicating a defect in FlgM anti- σ^{28} activity in the presence of the *serT* mutation. The *flgM* gene contains only one UCA codon. Changing the UCA of *flgM* to ACG reversed the effect of the *serT* allele. Implications for context effects in regulation of gene expression are discussed.

The flagellar regulon of *Salmonella enterica* serovar Typhimurium includes over 60 genes (reviewed in references 11 and 36). The transcripts of the flagellar regulon are organized into a transcriptional hierarchy based on three promoter classes that are temporally regulated in response to assembly (28) (Fig. 1). At the top of this hierarchy lies the flagellar master operon, *flhDC*, where the fundamental decision to produce flagella is controlled. The *flhDC* operon is expressed from what is defined as the class 1 promoter (58). The FlhD and FlhC proteins form a heteromultimeric complex (FlhD₂C₂) that acts as a transcriptional activator to promote σ^{70} -dependent transcription from the class 2 flagellar promoters (34, 35). The class 2 promoters direct transcription of genes needed for the structure and assembly of the flagellar motor structure, also known as the hook-basal body (HBB). Upon HBB completion, class 3 promoters are transcribed by σ^{28} -RNA polymerase, which is specific for flagellar class 3 promoters. Prior to HBB completion, σ^{28} -RNA polymerase is inhibited by the anti- σ^{28} factor, FlgM. Upon HBB completion, FlgM is secreted from the cell, presumably through the completed HBB structure, and σ^{28} -dependent transcription ensues. In this way, genes whose products are needed only after HBB formation, such as the flagellin filament genes, are transcribed only when there is a functional motor onto which they may be assembled.

FlgM secretion does not occur in strains defective in HBB formation (21, 22). FlgM secretion also can be prevented in strains with a functional HBB, either by mutation in the FlgM secretion signal (12) or by fusion of FlgM to a bulky protein such as β -galactosidase (LacZ) that retains anti- σ^{28} activity (25). A fusion of LacZ to the N-terminal region of FlgM is

nonmotile, presumably because the fusion fails to be secreted, resulting in constitutive inhibition of σ^{28} activity (25). Motile revertants in strains expressing the FlgM-LacZ fusion were isolated and found to be specifically defective in FlgM- σ^{28} interactions (9, 25). We decided to use this positive selection in a screen for conditional-lethal mutants specific to FlgM- σ^{28} interactions in an attempt to identify any essential genes that might be involved in the regulation of σ^{28} -dependent transcription. Our definition of a conditional-lethal mutant is one that grows at 30°C, but not at 42°C, on rich (LB) medium. This resulted in the isolation in a mutant with a single nucleotide substitution in the tRNA^{Ser}_{cmo5UGA} that is defective in FlgM inhibition of σ^{28} -dependent transcription at 30°C and lethal at 42°C.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this study and their origins are listed in Table 1. Mud vectors were used for *lac* operon and *lacZ* gene fusion constructions (24).

Media and standard genetic manipulations. Media, growth conditions, transductional methods, and motility assays were as described previously (21, 22). The generalized transducing phage of *Salmonella* serovar Typhimurium, P22 HT105/1 *int-201*, was used in all transductional crosses (48).

Mapping regulatory mutants to *flgM* and *fliA*. Mutants that allowed P_{fliC-cat} expression in the presence of an FlgM-LacZ fusion (*flgM5208::MudB*) were isolated by their ability to grow on L medium with 12.5 μ g/ml chloramphenicol (Cm) (see Results). Phage P22 was grown on the Cm^r revertants of strain TH3228 (FlgM-LacZ P_{fliC-cat}) and used to check for linkage of the mutation to the *flgM-lacZ* fusion or the σ^{28} structural gene *fliA*. For linkage to the *flgM* region, the phage lysates were used to transduce strain TH3227 that carries the P_{fliC-cat} construct to MudB-encoded Ap^r and screened for resistance or sensitivity to chloramphenicol. Any mutation in the *flgM-lacZ* fusion will show 100% linkage to Cm^r. For linkage to the *fliA* region, phage lysates were used to transduce strain TH2893 (Δ *flgG-L2157* [HBB⁻], *fliC5050::MudJ* [P_{fliC-lac}], *fliA5059::Tn10dTc* [σ^{28} defective]) to Lac⁺. Since TH2893 carries an insertion in the *fliA* gene, the *fliC-lac* transcriptional fusion (*fliC5050::MudJ*) cannot be expressed. However, transduction to *fliA*⁺ will not result in a Lac⁺ phenotype because the strain carries an HBB mutation (Δ *flgG-L2157*), so FlgM is not

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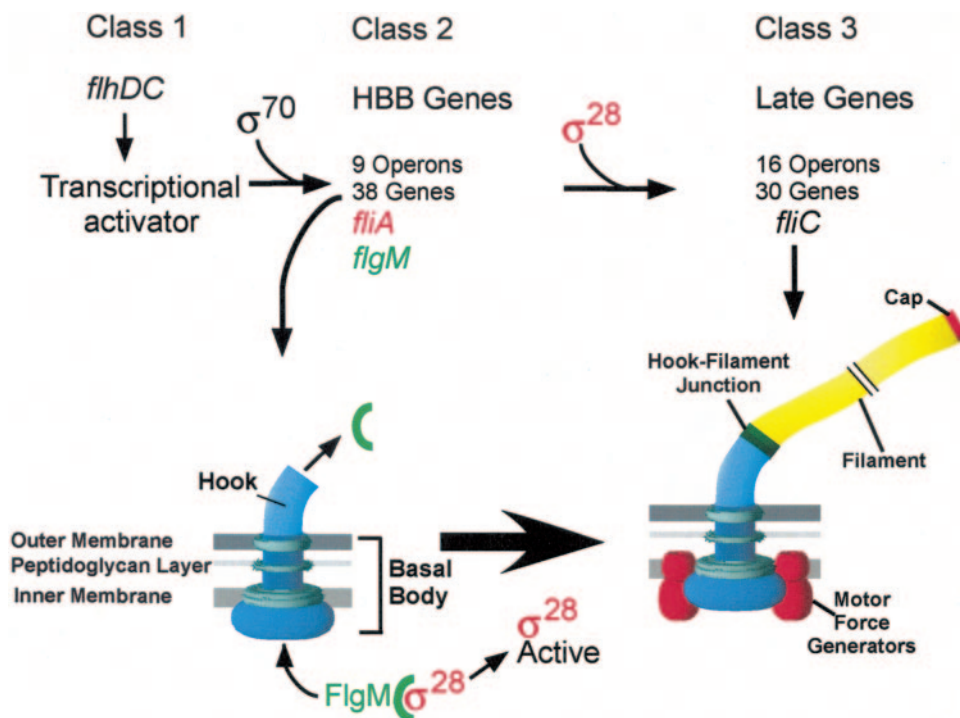


FIG. 1. The coupling of flagellar gene regulation to flagellum assembly. The gene products of the *flhDC* operon activate σ^{70} -dependent transcription from flagellar class 2 promoters, which include everything that is needed for the structure and assembly of the HBB, the ion-powered flagellar rotary motor that transverse from the cytoplasm through the membranes and cell wall to the outside of the cell. Also transcribed from a class 2 promoter is the *flhA* gene, which encodes the alternative σ transcription factor, σ^{28} . Transcription of class 3 promoters by σ^{28} -RNA polymerase produces all the gene products needed after the HBB motor is constructed. The genes expressed from class 3 promoters include everything that is needed late in the assembly process: flagellin, motor force generators, and a brain, known as the chemosensory system, which allows the flagellum to direct movement of the bacterium up a chemical gradient. The anti- σ^{28} factor FlgM couples class 3 promoter transcription to HBB completion. Upon HBB completion, the secretion specificity of the flagellar type III secretion system changes from hook/rod-type substrates to late substrates. FlgM, a late secretion substrate, is secreted, and σ^{28} is free, timed to HBB completion, to transcribe the late flagellar genes and complete flagellum assembly and the chemosensory network.

secreted. However, if the donor lysate carries an FlgM bypass mutation in *flhA*, then this lysate will transduce TH2893 to growth on minimal lactose at high efficiency (9, 32).

Isolation of Tn10dTc and Tn10dCm insertions near the *serT* gene. P22 was grown separately on strains TT10423 and TT10604, which carry the Tn10dTc and Tn10dCm insertions, respectively, in the F plasmid and used to transduce strain TT10427 to either tetracycline (Tc) or chloramphenicol resistance, respectively. TT10427 carries plasmid pNK972, which constitutively expresses Tn10 transposase. Because there is no F plasmid in strain TT10427, it is not possible to inherit the Tn10dTc and Tn10dCm transposons by homologous recombination. The Tc^r and Cm^r transductants are inherited by transposition into the recipient chromosome. More than 50,000 Tc^r or Cm^r transductants were pooled together, and P22 lysates were prepared on the pooled cells carrying insertions located at positions throughout the chromosome. In order to isolate Tn10dTc and Tn10dCm insertions in the *serT* region, the pooled Tn10dTc lysate was used to transduce TH3238 (*serT* [42°C-lethal]) to Tc^r on L-Tc plates, and the colonies were screened for growth at 42°C (*serT*⁺). Two Tn10dTc insertions that were 50% (*hpaB*) and 82% (*ycwW*) linked to the *serT* temperature-sensitive (ts) phenotype were isolated. A P22 lysate grown on the pooled Tn10dCm insertions was used to transduce the strain carrying the 82%-linked (*ycwW*) Tn10dTc insertion to Cm^r, which was followed by screening for loss of the Tn10dTc allele (Tc^s). By chance, one Tn10dCm insertion (STM1081) was found to be 90% linked to this Tn10. PCR amplification of a strain carrying both closely linked insertions with a primer that would recognize the ends of Tn10dTc and Tn10dCm gave a product which, after DNA sequence analysis, revealed the Tn10dTc insertion to be in the *ycwW* locus and the Tn10dCm insertion to be in the STM1081 locus. The other Tn10dTc insertion was subject to semirandom PCR (42), and the sequenced product revealed it to reside in the *hpaB* gene. The STM1081::Tn10dCm insertion was 78% linked to *serT* and 35% linked to *hpaB* by P22 transduction.

Site-directed mutagenesis. The UCA codons in *flgM* and *flgN* were changed to AGC codons by site-directed mutagenesis using the bacteriophage lambda recombination system (λ -Red) of Datsenko and Wanner (15). Insertion of the *tetA* and *tetR* genes (*tetRA*) from transposon Tn10 were previously placed at the 5' (TH7953) and 3' (TH8191) ends of the *flgM* gene (V. Rosu and K. T. Hughes, unpublished). Large primers (Integrated DNA Technologies) that contained the TCA-to-AGC substitutions in *flgM* and *flgN* were purchased. For FlgM, primer FlgM-AGC7mut (5'-AGCTGGCCGCTACAACGTAACCCCTCGATGAGGATAAAATAATGAGCATTGACCGTACCAGCCCTTTGAAACCCGTTAGCACTGTCAGACGCGCGAAACCA-3') was made double stranded by hybridization with primer FlgM-AGC7Rev (5'-TGGTTTCGCGCGTCTGGA-3'), which was followed by fill-in reaction with ThermalAce (Invitrogen) according to the manufacturer's instructions and purification by ethanol precipitation. For FlgN, primer FlgN-AGC5mut (5'-CTCGCTCATTGCGAGGCGCAGAGCTACTTACAGAGTAATAAGCGTATGACTCGTTTGTAGCGAAATACTTGACCAGATGACCACCGTCTCTGAATGACCTGA-3') was made double stranded by hybridization with primer FlgN-AGC5mutRev (5'-TTCAGGTCATTCAGGACG-3'), which was followed by fill-in reaction with ThermalAce (Invitrogen) according to the manufacturer's instructions and purification by ethanol precipitation. For each recombination reaction, 1 μ g of filled-in FlgM and FlgN DNA products were electroporated into electrocompetent TH7953 and TH8191, respectively, that were grown in L medium containing ampicillin (Ap) and arabinose (added 1 h prior to harvesting). Selection was made on Tc^s plates (37). Mutational changes were verified by DNA sequence analysis.

β -Galactosidase assays. β -Galactosidase assays were performed as described by Maloy (37). Cells were grown at 37°C until mid-log phase. Assays were performed in triplicate, and the values are reported as activity units in nmol/min/optical density at 650 nm/ml.

TABLE 1. List of bacterial strains

Salmonella serovar Typhimurium strain	Genotype	Source ^a
E12A2	<i>pipB</i> ::Tc- <i>lux</i>	B. Finlay
LT2	Wild type	J. Roth
TH2430	Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH2893	Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fliA5059</i> ::Tn10dTc <i>fljBenx</i> <i>vh2</i>	
TH3933	<i>motA5461</i> ::MudJ	
TH3227	<i>ataA</i> ::[P22 <i>sieA44</i> Kn-9 P _{<i>fliC</i>} (-435→+6)- <i>cat</i> 9 ⁺]	
TH3228	<i>ataA</i> ::[P22 <i>sieA44</i> Kn-9 P _{<i>fliC</i>} (-435→+6)- <i>cat</i> 9 ⁺] <i>flgM5208</i> ::MudB	
TH3238	<i>flu-5398</i> (<i>serT</i>) <i>ataA</i> ::[P22 <i>sieA44</i> Kn-9 P _{<i>fliC</i>} (-435→+6)- <i>cat</i> 9 ⁺] <i>flgM5208</i> ::MudB	
TH6652	<i>hpaB</i> ::Tn10dTc	
TH6654	STM1081::Tn10dCm	
TH7012	<i>hpaB</i> ::Tn10dTc <i>fla-5398</i> (<i>serT</i>)	
TH7368	<i>yccW</i> ::Tn10dTc	
TH7953	pKD46/ <i>flgM6085</i> :: <i>tetRA</i> (start)	V. Rosu
TH8191	pKD46/ <i>flgM6101</i> :: <i>tetRA</i> (stop)	V. Rosu
TH9390	<i>flgM6421</i> (S7, UCA:ACG)	
TH9392	<i>flgN6423</i> (S5, UCA:ACG)	
TH9515	<i>flgM6421</i> (S7, UCA:ACG) <i>flgN6430</i> (S5, UCA:ACG)	
TH9524	<i>flgM6421</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9525	<i>flgM6423</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9526	<i>flgM6421</i> <i>flgN6430</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9623	<i>hpaB</i> ::Tn10dTc <i>fla-5398</i> (<i>serT</i>) <i>flgM6421</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9624	<i>hpaB</i> ::Tn10dTc <i>fla-5398</i> (<i>serT</i>) <i>flgN6423</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9625	<i>hpaB</i> ::Tn10dTc <i>fla-5398</i> (<i>serT</i>) <i>flgM6421</i> <i>flgN6430</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9626	<i>hpaB</i> ::Tn10dTc <i>flgM6421</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9627	<i>hpaB</i> ::Tn10dTc <i>flgN6423</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9628	<i>hpaB</i> ::Tn10dTc <i>flgM6421</i> <i>flgN6430</i> Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9784	<i>hpaB</i> ::Tn10dTc Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9785	<i>hpaB</i> ::Tn10dTc <i>fla-5398</i> (<i>serT</i>) Δ <i>flgG</i> -L2157 <i>fliC5050</i> ::MudJ <i>fljBenx</i> <i>vh2</i>	
TH9786	<i>motA5461</i> ::MudJ <i>flgM6421</i>	
TH9787	<i>motA5461</i> ::MudJ <i>flgN6423</i>	
TH9788	<i>motA5461</i> ::MudJ <i>flgM6421</i> <i>flgN6430</i>	
TT10423	Δ <i>proAB47</i> /F' <i>pro</i> ⁺ <i>lac</i> ⁺ <i>zzf-1831</i> ::Tn10dTc	J. Roth
TT10604	pNK972 (Ap ^r Tn10 transposase)/LT2	J. Roth
TT10427	Δ <i>proAB47</i> /F' <i>pro</i> ⁺ <i>lac</i> ⁺ <i>zzf-1836</i> ::Tn10dCm	J. Roth

^a Unless indicated otherwise, all strains were constructed during the course of this work.

RESULTS

Isolation of mutants defective in FlgM-LacZ inhibition of *fliC* promoter transcription. A P22 phage that constitutively expresses kanamycin resistance (Km^r) and carries the chloramphenicol acetyltransferase (*cat*) gene expressed from the *fliC* promoter (P_{*fliC*}-*cat*) was constructed. When lysogenized into the wild-type *Salmonella* serovar Typhimurium strain LT2 by selection for Km^r , the cells become Cm^r , due to σ^{28} -dependent transcription of P_{*fliC*}-*cat*. We have previously described the isolation of *lacZ* translational fusions to the *flgM* gene using a Mud-*lac* translational fusion vector that result in the expression of FlgM-LacZ fusion proteins (23). One allele, *flgM5208*::MudB, results in a fusion of the first 86 amino acids of the 97-amino-acid FlgM protein to LacZ and retains the anti- σ^{28} activity of FlgM (25). When the *flgM5208*::MudB insertion is introduced into wild-type strain LT2 or in a strain that retains a wild-type copy of the *flgM* gene, the cells become nonmotile, presumably because the FlgM-LacZ fusion cannot be secreted. Thus, the dominant anti- σ^{28} activity of the FlgM-LacZ fusion is not coupled to HBB completion, and inhibition of σ^{28} by FlgM-LacZ is constitutive, resulting in loss of motility. Another possibility was that the FlgM-LacZ fusion blocks flagellar secretion by itself, getting stuck in the flagellar secre-

tion machinery during the secretion process. This was shown not to be the case (see below).

Motile revertants of strains carrying the FlgM-LacZ fusion that remained LacZ⁺ were isolated (25). Two of these were mutants with single-amino-acid-substitutions in FlgM, I58L and L66S. These were later shown to be specifically defective in interactions with σ^{28} (9, 10, 16). Other motile revertants in strain LT2 expressing the FlgM-LacZ fusion were mutants with single amino acid substitutions in σ^{28} that were defective in interactions with FlgM (9, 10, 16). The fact that mutants specific to FlgM- σ^{28} interactions resulted in motility in the presence of the FlgM-LacZ fusion and that intact FlgM with these substitutions is secreted normally argues that the FlgM-LacZ fusion does not block the flagellar secretion apparatus.

We decided to exhaustively search for mutants defective in FlgM- σ^{28} interactions in an attempt to uncover any essential genes that might be involved in this flagellar regulatory process. Strain TH3228 was constructed for this purpose. It harbors a bacteriophage P22 lysogen with a P_{*fliC*}-*cat* construct and the dominant FlgM-LacZ fusion (*flgM5208*::MudB). Strain TH3228 is Cm^s due to FlgM-LacZ inhibition of σ^{28} -dependent transcription of P_{*fliC*}-*cat*. If either the I58L or the L66S substitution is introduced into the FlgM-LacZ fusion construct, the

cells express $P_{flic-cat}$ and are Cm^r . One hundred fifty independent cultures of TH3228 were plated on LB medium containing chloramphenicol (12.5 $\mu\text{g/ml}$) and incubated at 30°C. After overnight incubation, Cm^r colonies appeared, and the plates were replica printed to two LB- Cm plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 80 $\mu\text{g/ml}$) and incubated separately at 30°C and 42°C. From each of the initial 20 independent cultures, three Cm^r colonies were chosen; one Lac^+ (X-Gal; blue), one $Lac^{+/-}$ (X-Gal; light blue), and one Lac^- (X-Gal; white) colony were isolated and checked for linkage to the *flgM* and *fliA* loci (see Materials and Methods). The 40 light-blue and white Cm^r mutants were all linked to the *flgM* locus and are presumed defective in the expression of the FlgM-LacZ fusion. Of the 20 independent Lac^+ (X-Gal; blue) Cm^r mutants isolated, 8 were linked to the σ^{28} structural gene (*fliA*), 10 were linked to *flgM*, and 2 carried mutations in both the *flgM* and *fliA* loci. For the remaining 130 independent cultures tested, only Lac^+ colonies were screened for linkage; 62 were linked to *flgM*, 46 were linked to *fliA*, and 22 were linked to both *flgM* and *fliA*. From the 150 independent cultures screened, only 12 yielded mutants that grew on Cm at 30°C but not at 42°C. One Cm^r mutant mapped to *flgM*, and one mapped to *fliA*. The remaining 10 were not linked to *flgM* or *fliA*. Five mapped to the *flg* operon downstream of *flgM*, presumably in HBB structural genes or the *flg*-linked RNase E gene (*rne*), but were not further characterized. We tested if any were temperature sensitive on LB medium in the absence of chloramphenicol as potential mutants with changes in essential genes, and only one, the *fla-5398* mutant, was found to be unable to grow on LB medium at 42°C and was characterized further.

Mapping the *fla-5398* temperature-sensitive lethal mutation. A $Tn10dTc$ transposon insertion that was 50% linked by P22 transduction to the *fla-5398* ts-lethal phenotype was isolated (see Materials and Methods). DNA sequence analysis revealed the $Tn10dTc$ insertion to be in the *hpaB* gene (see Materials and Methods). We checked the linkage of the *fla-5398* ts-lethal phenotype to other insertions in the vicinity of *hpaB*. A *putA::MudCm* allele was 1% linked to *fla-5398* by P22 cotransduction; a *pipB::Tc* insertion was 89% linked, suggesting that the *fla-5398* allele was close to the *pipB* locus. In order to facilitate mapping, an insertion of $Tn10dCm$ that was 78% linked to the *fla-5398* allele and 35% linked to the *hpaB::Tn10dTc* insertion by P22 cotransduction was isolated. DNA sequence analysis revealed this insertion to be in the STM1081 gene. Three-factor cross mapping (not shown) between the $Tn10dCm$ insertion in STM1081, the *hpaB::Tn10dTc* insertion, and the *fla-5398* allele revealed the *fla-5398* allele to be located between STM1081 and *hpaB*.

The *fla-5398* allele is a mutation in a serine tRNA gene, *serT*. The precise location of the *fla-5398* allele was determined by estimation of the distance of *fla-5398* from the *hpaB::Tn10dTc* and STM1081:: $Tn10dCm$ insertions using the modified formula of Wu (46, 55). Examination of the annotated sequence of the *Salmonella* serovar Typhimurium LT2 genome did not reveal an obvious gene that when mutated might account for the phenotype of inhibition of FlgM function at 30°C and lethality at 42°C. It was not until we looked at the actual DNA sequence of the region that we found that a potential essential gene, *serT*, coding for tRNA^{Ser}_{cmo5UGA}, was located where the

TABLE 2. Effect of mutant G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} on FlgM anti- σ^{28} activity

Strain	Relative genotype or phenotype	β -Galactosidase activity ^a
TH9784	HBB ⁻ <i>serT</i> ⁺	2.0 \pm 0.23
TH9626	HBB ⁻ <i>serT</i> ⁺ FlgM(ACG)	1.7 \pm 0.26
TH9627	HBB ⁻ <i>serT</i> ⁺ FlgN(ACG)	4.1 \pm 0.01
TH9628	HBB ⁻ <i>serT</i> ⁺ FlgM(ACG) FlgN(ACG)	1.8 \pm 0.12
TH9785	HBB ⁻ <i>serT</i>	23.6 \pm 0.91
TH9623	HBB ⁻ <i>serT</i> FlgM(ACG)	1.3 \pm 0.10
TH9624	HBB ⁻ <i>serT</i> FlgN(ACG)	6.8 \pm 0.21
TH9625	HBB ⁻ <i>serT</i> FlgM(ACG) FlgN(ACG)	1.2 \pm 0.11

^a In nmol/min/optical density at 650 nm/ml.

fla-5398 allele mapped. We sequenced the *serT* locus and identified a single nucleotide change where G₁₀ is replaced by A₁₀ in the D-stem. Thus, a base substitution mutation in the serine tRNA, tRNA^{Ser}_{cmo5UGA} (G₁₀:A₁₀), resulted in a defect in the FlgM-LacZ inhibition of σ^{28} -dependent $P_{flic-cat}$ transcription at 30°C and lethality at 42°C.

Effect of the tRNA^{Ser}_{cmo5UGA}-specific UCA codon on *flgM* and *flgN* gene expression. The tRNA^{Ser}_{cmo5UGA} species (SerT) recognizes the UCA, UCG, and UCU codons. However, two of these codons, UCG and UCU, are recognized by alternative serine tRNA species, tRNA^{Ser}_{GGA} and tRNA^{Ser}_{CGA} species, respectively. No other tRNA is known to read the UCA codon. Thus, the UCA codon is specifically recognized by tRNA^{Ser}_{cmo5UGA}. In FlgM, only one serine residue at amino acid 7 (S7) is coded for by UCA, while there are eight UCA codons in *lacZ*. The presence of 9 UCA codons in the *flgM-lacZ* fusion is likely to make the translation of the fusion less efficient in a *serT* allele background (see Discussion). We moved the *fla-5398* (*serT*) allele into an *flgM*⁺ strain defective in HBB formation ($\Delta flgG-L$) that also carries the *lac* operon expressed from the σ^{28} -dependent *fliC* promoter ($P_{flic-lac}$ [*fliC5050::MudJ*]). In an HBB⁻ (*serT*⁺) strain, FlgM is not secreted, and *fliC-lac* expression is inhibited 100-fold compared to an isogenic HBB⁺ strain (21). However, if we introduce the *serT* (*fla-5398*) allele into the HBB⁻ strain, β -galactosidase activity increases 10-fold at the permissive temperature (30°C) (Table 2; compare TH9784 to TH9785). Thus, even though only one UCA codon is present in *flgM* without the *lacZ* fusion, the *serT* allele results in a defect in FlgM anti- σ^{28} activity. The fact that the reporter is *lacZ* and sensitive to the *serT* mutation (40) suggests that the effect might be even larger if we used a reporter that did not contain UCA codons (see Discussion). The *flgM* S7 UCA codon was changed to AGC by site-directed mutagenesis (see Materials and Methods). When the S7 UCA codon was changed to the alternative serine codon AGC, the *serT* (*fla-5398*) allele no longer prevented FlgM inhibition of σ^{28} -dependent *fliC-lac* transcription (Table 2; compare TH9623 to TH9785 and TH9784). The change of UCA to AGC did not affect *fliC-lac* transcription in a *serT*⁺ background (Table 2; compare TH9784 to TH9626). In the absence of the *serT* allele, the AGC codon had no effect on FlgM activity: the strains had the same motility as the wild type, and there was no effect on σ^{28} -dependent transcription (data not shown).

We also examined the effect of the *serT* (*fla-5398*) allele on

flgN gene activity. Loss of FlgN results in reduced FlgM levels in the cell, leading to a derepression of *fliC-lac* expression in HBB⁻ strains (27). The *flgN* gene also has a single UCA codon that codes for serine at amino acid 5 of FlgN (33). We changed this codon to AGC and found that it had a slight effect (two-fold) on *fliC-lac* transcription in a *serT*⁺ background (Table 2; compare TH9784 to TH9627) but resulted in restoration of inhibition of *fliC-lac* transcription, although not to the same degree as the change from UCA to AGC in *flgM* (Table 2; compare TH9624 to TH9623 and TH9785). The double changes from UCA to AGC in both *flgM* and *flgN* had the same effect as just the single change in *flgM* (Table 2; compare TH9625 to TH9785, TH9623, and TH9624). Thus, the *serT* (*fla-5398*) allele can affect both *flgM* and *flgN* gene translation.

DISCUSSION

An extensive search was performed with 150 independent cultures in order to look for novel regulatory mutants affecting FlgM inhibition of σ^{28} -dependent transcription of the *fliC* promoter. The strain in which the selection was set up had an FlgM-LacZ fusion that would inhibit σ^{28} -dependent transcription even in strains with a functional HBB structure, because the fusion of LacZ to FlgM prevents secretion through the HBB structure (25). The selection utilized a fusion of the *fliC* promoter (P_{fliC}) to the *cat* gene ($P_{fliC-cat}$), so that when P_{fliC} was transcribed, the cells became Cm^r. The selection was done at 30°C and then coupled to a screen for inability to grow at 42°C. In this way, we hoped to identify previously uncharacterized essential genes that might play a role in FlgM inhibition of σ^{28} -dependent transcription.

The selection yielded some unexpected results. All but one of the Cm^r revertants (non-ts) carry mutations that mapped to either the *flgM* or the *fliA* region. We expected mutants defective in the *clpXP* protease genes. It had been shown that loss of ClpPX protease results in a more stable σ^{28} protein that can overcome FlgM inhibition of σ^{28} -dependent transcription in HBB mutant strains (3, 53). We propose that the addition of LacZ to the C terminus of FlgM enhances the stability of FlgM, resulting in a selection that is specific for mutations that are specific to FlgM- σ^{28} interactions, such that loss of ClpXP protease does not stabilize σ^{28} enough to overcome the excess inhibition by the FlgM-LacZ fusion. The fact that 22 of the 150 cultures yielded double mutants carrying mutations that mapped to both the *flgM* and *fliA* regions suggests that the selection used (Cm^r by $P_{fliC-cat}$ in the presence of FlgM-LacZ) required a significant loss in the anti- σ^{28} activity of the FlgM-LacZ fusion. However, the mutants were not screened for the presence of mutations in *clpXP* protease, so it is possible that many of the mutants that had either an *flgM*-linked or an *fliA*-linked allele also carried a second mutation in *clpXP*.

In the screen for the identification of essential genes involved in FlgM regulation of P_{fliC} transcription, one ts-lethal mutant from the 150 independent cultures that prevented FlgM-LacZ inhibition of $P_{fliC-cat}$ transcription at the permissive temperature and failed to grow on LB medium at 42°C was isolated. This mutant had a G₁₀:A₁₀ substitution mutation in the D-stem of an essential tRNA gene, *serT* (4), which encodes tRNA^{Ser}_{cmo5UGA} (5). The *serT* gene is essential, because only tRNA^{Ser}_{cmo5UGA} will recognize the UCA codon (26, 49).

It was fortuitous that we used $P_{fliC-cat}$ and not $P_{fliC-lac}$ as our reporter with some other nonsecreted FlgM fusion. If we compare strains TH9784 (*hpaB::Tn10dTc ΔflgG-L2157 fliC5050::MudJ fljBenx vh2*) and TH9785 (TH9784 *serT*), we find that they are isogenic *serT*⁺ and *serT* strains, respectively. The β -galactosidase assays shown in Table 2 show that TH9784 has 10-fold-higher levels of activity and that the cells are darker blue on X-Gal (not shown). This all fits with the *serT* mutants having reduced FlgM levels corresponding to increased transcription of $P_{fliC-lac}$. However, if we compare the strains on MacConkey lactose indicator plates, we find that TH9784 is pink in color after overnight incubation, indicating low levels of lactose utilization, while TH9785 is completely white, indicating no lactose fermentation (not shown). How can this be if TH9785 has 10-fold-higher levels of β -galactosidase? It takes many more genes than just the β -galactosidase gene to ferment lactose to achieve the Lac⁺ phenotype. It is likely that many genes required to ferment lactose are defective in the presence of the SerT-G₁₀:A₁₀ tRNA, resulting in the Lac⁻ phenotype, and that the expression of only one gene is required for the LacZ⁺ phenotype. Because *lacZ* mRNA has 8 UCA codons, the 10-fold increase in β -galactosidase in TH9785 probably represents a lower limit of increased transcription of $P_{fliC-lac}$ due to reduced translation of *lacZ* mRNA in the presence of the SerT-G₁₀:A₁₀ tRNA.

The textbook picture of a tRNA is the secondary cloverleaf structure: an amino acid acceptor stem on top; an anticodon stem and loop on the bottom; a left arm known as the D-stem and D-loop, due to the presence of many dihydrouridine modifications in the D-loop; and a right arm known as the T ψ C stem and T ψ C loop, due to the presence of a conserved pseudouridine (ψ) residue (5). When folded into the tertiary structure, the D-stem and D-loop become fused with the anticodon stem and loop (29, 43). The base substitution of A for G would result in the loss of a G:C base pair in the D-stem that might allow a conformational change in the tRNA tertiary structure at 42°C. Such a conformational change probably does not result in a nonfunctional tRNA, because several proteins containing UCA codon products are normally expressed in an *Escherichia coli serT* mutant strain with the identical temperature-sensitive lethal G₁₀:A₁₀ allele (1).

The identical (G₁₀:A₁₀) substitution mutation in the *serT* gene had been isolated previously in *Escherichia coli* and named *divE42* because it affected cell cycle-dependent enzymes (39). When shifted to 42°C, the cells grew until they doubled in volume, but when shifted down to low temperature, they began to divide synchronously. The mutant also exhibited low β -galactosidase levels at the nonpermissive temperature (42°C) (1, 40). Synthesis of a number of cell cycle-dependent proteins was decreased significantly in the *divE42* mutant at 42°C (47). DNA sequence analysis revealed *divE42* to be a mutation in tRNA^{Ser}_{cmo5UGA}, and it was renamed *serT* (50). The *divE42* allele could be complemented in *trans* by a wild-type copy of the *serT*⁺ gene expressed from an F' plasmid, indicating that it was a recessive allele (47). Remarkably, a pBR322-derived plasmid expressing the *divE42* allele could also complement the chromosomal *divE42* allele for growth at 42°C, implying that stability or a concentration-dependent interaction required for translation of essential genes is lost at 42°C (57). Furthermore, the *E. coli divE42* allele is more labile at

44°C than at 42°C, suggesting that the mutant phenotype shows a gradient effect with temperature (1). Presumably, the translation of essential genes is reduced at elevated temperatures to the degree that the cell can no longer grow. It was fortuitous that the *Salmonella serT* mutant was defective in *flgM* translation at 30°C, since this enabled it to be picked up in our mutant screen.

In *Salmonella* serovar Typhimurium, *serT* was identified by DNA sequence analysis as flanking *Salmonella* pathogenicity island 5 (54). The *serT* gene is located basically at the same positions in both the *E. coli* and *Salmonella* serovar Typhimurium chromosomes. Further studies with the *divE42* allele from *E. coli* suggested that many proteins with serine residues expressed from UCA codons are made at normal levels in the presence of the *divE42* allele, suggesting that the context of translation may be critical to the effect of *divE42* on protein expression. The temperature-sensitive phenotype of the *divE42* mutant could be suppressed by a double mutation in *rne* (RNase E) and *pnp* (polynucleotide phosphorylase) (31), members of the mRNA degradosome (8). The *lacZ* mRNA, containing eight UCA codons, is unstable in the *divE42* mutant, but stability is restored in a strain that also carries the *rne-1* and *pnp-7* mutations (1). This suggests that a defect in translation of UCA by the G₁₀:A₁₀ substitution in tRNA^{Ser}_{cmo5UGA} could lead to mRNA instability.

Other suppressors of the temperature-sensitive growth of the *E. coli divE42* allele were isolated. Intragenic suppressors include C₂₅:U₂₅, which can now form a base pair with A₁₀, and G₆₇:A₆₇, which forms a base pair with the acceptor stem (41). One unusual mutant, resulting from a single amino acid substitution of amino acid 275 of trigger factor (TF), actually reversed the phenotype: the *tig divE42* double mutant grew at 42°C but not at 30°C, and the growth defect at 30°C could not be complemented by *divE*⁺ but could be complemented by *tig*⁺ (38). TF is a ribosome-associated chaperone that associates with the DnaK protein-folding system to catalyze proper folding of newly synthesized cytosolic proteins. TF is nonessential except in *dnaK* mutants (17, 52). TF consists of an N-terminal domain that mediates ribosome binding and by itself is sufficient to complement for chaperone activity (30). A central domain with peptidyl-prolyl *cis/trans* isomerase activity, substrate binding activity, and a C-terminal domain contributes to the chaperone activity (30). Amino acid 275 is located at the beginning of the C-terminal domain (amino acids 248 to 432) (30). In their model, Nakano et al. propose that reduced TF at 42°C compensates for the reduction of an essential cell protein (X) produced at low levels in the *divE42* mutant at 42°C. They propose that the ratio of TF to X determines lethality. In *divE42 tig*⁺ cells, the TF⁺:X ratio is too high at 42°C, and cell division is inhibited. In the *divE42 tig* double mutant at 42°C, low TF activity resulting from the *tig* mutation and low X levels resulting from the *divE42* mutation result in normal TF:X ratios, and the cells grow. At 30°C, X levels are wild type in the *divE42* mutant, but TF is low, so the TF:X ratios are too low.

The serine codon usages in *Salmonella* serovar Typhimurium per thousand are 8.4 (UCU), 10.6 (UCC), 8.0 (UCA), 9.5 (UCG), 8.6 (AGU), and 17.7 (AGC), and in *E. coli* are 8.4 (UCU), 8.6 (UCC), 7.1 (UCA), 8.9 (UCG), 8.8 (AGU), and 16.0 (AGC). While UCA usage is the lowest, UCA is far from rare. Suppression of the G₁₀:A₁₀ substitution mutation in

tRNA^{Ser}_{cmo5UGA} by mutants in the mRNA degradosome (*rne-1* and *pnp-7* mutations) suggests that stalling plays a critical role in the phenotype. The temperature-sensitive nature of the mutant is as follows: defective at 30°C (evidenced by reduction in *flgM* translation), more defective at 42°C (evidenced by inhibition of cell division and significant reduction in levels of some proteins), and even more defective at 44°C (evidenced by further reduction in affected proteins at 44°C). Self-complementation of *divE42* by the *divE42* allele present in high copy numbers is consistent with the loss of a concentration-dependent interaction with G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} that is dependent on the G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} tertiary structure.

We rule out the possibility that the UCA codon early (amino acid codon 7) in the *flgM* gene accounts for sensitivity to the *serT* mutation. It was possible that G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA}-dependent stalling of translation early in the *flgM* gene transcript resulted in enhanced *flgM* mRNA degradation. We examined the 12 *flg*-linked flagellar hook-basal body structural genes for numbers and locations of UCA codons and obtained the following results (with gene, number of amino acids, number of serine residues, and number of UCA codons listed in that order and followed by the UCA codon position[s], if any, in parentheses): *flgA*, 219, 13, 1 (49); *flgB*, 138, 8, 0; *flgC*, 134, 9, 1 (107); *flgD*, 232, 15, 0; *flgE*, 400, 34, 3 (18, 173, 180); *flgF*, 250, 15, 2 (30, 198); *flgG*, 260, 20, 2 (4, 64); *flgH*, 232, 20, 0; *flgI*, 365, 28, 3 (37, 109, 281); *flgJ*, 316, 26, 0; *flgK*, 553, 46, 4 (61, 105, 440, 490); and *flgL*, 317, 31, 1 (261). We observed 17 UCA codons/265 serine codons (0.64%) that were located throughout the genes. One gene, *flgG*, had a UCA at the position corresponding to amino acid 4, a position similar to that in the *flgM* gene. Given that the *serT* mutant shows normal motility at 30°C, it seems likely that the flagellar genes are translated at normal efficiencies.

As mentioned above, certain proteins with serine residues encoded by UCA are present at lower levels in the *divE42* mutant strain, and the number of proteins affected increases with increasing temperature. One interpretation of the observed effect of the *serT* mutant tRNA is that there is less-efficient charging of it. This should give a lower concentration of the ternary complex and thereby stalling of the ribosome and less-efficient translation. This could significantly affect mRNA instability of some transcripts but not that of others.

Another interpretation is that the context of the UCA codon in the mRNA is critical for translation by G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA}. Such a context effect could be due to an adjacent base influencing the ability to read the UCA codon or the fitting of an adjacent tRNA to the UCA codon. The binding efficiency of tRNA^{fMet} for the initiation codon, AUG, of the QB coat gene was shown to increase more than threefold when the G residue adjacent to the 3' side of the AUG was replaced by an A residue (51). Structures of tRNA species can be altered to read four codons, consistent with the possibility that even a given triplet-specific tRNA could be influenced by adjacent bases (44). There are several lines of evidence to suggest that efficient translation of UCA codons by G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} might be dependent on which tRNAs read adjacent codons. The efficiency of the *supE* amber suppressor in translating the UAG codon varies over an order of magnitude depending on the nucleotide adjacent to the 3' side of the codon (7). The frameshift suppressor tRNA SufJ will read four

codons where the first three are ACC and the fourth base can be anything, suggesting that the SufJ tRNA sterically prevents another tRNA from reading the fourth codon (6). The influence of reading context on the efficiency of nonsense suppression has been observed by a number of labs (2, 13, 14, 18–20, 45, 56). Thus, context is critical for translational efficiency, and it is likely that the fact that some UCA-containing mRNAs are translated more efficiently than others represents an example of context effects. If the G₁₀:A₁₀ substitution in tRNA^{Ser}_{cmo5UGA} affects translation in a context-dependent manner, the suppression of *divE42* by itself in high copy numbers would be consistent with an effect on the 5' side. This would mean that the ability of G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} to interact with the tRNA in the P site of the ribosome is more likely to be sensitive to G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} concentration than the ability of a tRNA to enter the A site with G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} present in the P site. It remains to be determined if the ability of G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} to read the UCA codon is affected by context. To our knowledge, the involvement of position 10 in the D-stem of tRNA in tRNA-tRNA interactions during translation has not been demonstrated. However, tRNA-tRNA interactions during translation have not been characterized to any significant extent. The finding that some proteins with UCA codons are translated normally, while others are not, argues against a simple loss-of-function type of mutant. It is possible that G₁₀:A₁₀-tRNA^{Ser}_{cmo5UGA} results in a conformational change that affects its ability to sterically fit adjacent to specific tRNA species in the ribosome during translation. The results presented here remind us that context effects can be critical for gene expression and could play an important role in gene regulation by determining the level at which a given gene is expressed in the cell and also in gene evolution.

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