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Identification of New Flagellar Genes of *Salmonella enterica* Serovar Typhimurium

Jonathan Frye,[2] Joyce E. Karlinsey,[1] Heather R. Felise,[1] Bruz Marzolf,[3] Naeem Dowidar,[3] Michael McClelland,[2] and Kelly T. Hughes[1]*

Department of Microbiology, Box 357242, University of Washington, Seattle, Washington 98195[1]; Sidney Kimmel Cancer Center, 10835 Altman Row, San Diego, California 92121[2]; and The Institute for Systems Biology, 1441 N 34th Street, Seattle, Washington 98103[3]

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RNA levels of flagellar genes in eight different genetic backgrounds were compared to that of the wild type by DNA microarray analysis. Cluster analysis identified new, potential flagellar genes, three putative methyl-accepting chemotaxis proteins, STM3138 (McpA), STM3152 (McpB), and STM3216(McpC), and a CheV homolog, STM2314, in *Salmonella*, that are not found in *Escherichia coli*. Isolation and characterization of Mu*d-lac* insertions in *cheV*, *mcpB*, *mcpC*, and the previously uncharacterized *aer* locus of *S. enterica* serovar Typhimurium revealed them to be controlled by σ^{28}-dependent flagellar class 3 promoters. In addition, the *srfABC* operon previously isolated as an SsrB-regulated operon clustered with the flagellar class 2 operon and was determined to be under FlhDC control. The previously unclassified *fliB* gene, encoding flagellin methylase, clustered as a class 2 gene, which was verified using reporter fusions, and the *fliB* transcriptional start site was identified by primer extension analysis. RNA levels of all flagellar genes were elevated in *flgM* or *fliT* null strains. RNA levels of class 3 flagellar genes were elevated in a *fliS* null strain, while deletion of the *fliY*, *fliZ*, or *flk* gene did not affect flagellar RNA levels relative to those of the wild type. The *cafA* (RNase G) and *yhjH* genes clustered with flagellar class 3 transcribed genes. Null alleles in *cheV*, *mcpA*, *mcpB*, *mcpC*, and *srfB* did not affect motility, while deletion of *yhjH* did result in reduced motility compared to that of the wild type.

What is a flagellar gene? Loss of motility when a gene is disrupted might seem a good definition of a flagellar gene. However, the disruption of many genes not considered flagellar genes results in a motility-defective phenotype. These include genes such as *hns*, *crp*, *cya*, *dnaJ* and *dnaK*, *pss*, and *psd* (6). These are global regulators that affect gene expression in a number of regulons, including the flagellar regulon. There are a number of genes that are directly responsible for the structure and assembly of the bacterial flagellum and its normal function in chemotaxis. These originally defined the flagellar genes, and their characterization has led to the discovery that the flagellar regulon is a hierarchy of coordinately transcribed genes (30). Structural genes are assigned to three assembly classes of early, middle, and late depending on when their products are needed for assembly and function (7). The promoters responsible for flagellar gene expression are also assigned to three classes, class 1, class 2, and class 3, according to their temporal expression after induction of the flagellar regulon (25). A number of new flagellar genes have been identified, not by their effect on motility but by the fact that they are transcribed from flagellar promoters in operons with known flagellar genes. Some of these, such as *flhE*, *fliY*, *fliZ*, and *fliT*, have little or no effect on motility (37), but in the case of *fliZ* and *fliT* they have positive and negative effects on transcription of other flagellar genes (29).

* Corresponding author. Mailing address: Department of Microbiology, Box 357242, University of Washington, Seattle, WA 98195. Phone: (801) 581-6517. Fax: (801) 581-4668. E-mail: Hughes@biology.utah.edu.

The flagellar regulon of *Salmonella enterica* serovar Typhimurium includes over 60 genes. Our current understanding of this process is diagrammed in Fig. 1 (reviewed in references 6 and 34). At the top of the transcriptional 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. As mentioned above, a number of global regulatory signals influence transcription of the *flhDC* promoter, which has been shown to contain at least six different transcriptional start sites (52). The FlhD and FlhC proteins form a heteromultimeric complex ($FlhD_2C_2$) that acts as a transcriptional activator to promote σ^{70}-dependent transcription from the class 2 flagellar promoters (32, 33). The FlhDC complex also acts to autorepress transcription of the class 1 promoter (26).

The class 2 promoters direct transcription of the flagellar middle assembly genes needed for the structure and assembly of the hook-basal body (HBB) structure. Also transcribed from class 2 promoters are three late assembly genes, *flgK*, *flgL*, and *fliD*, whose products are assembled immediately after HBB completion (27). In addition to structural genes, a number of regulatory genes are also transcribed from class 2 promoters, including the *fliA* and *flgM* genes (17, 23). The *fliA* gene encodes the flagellar-specific transcription factor, σ^{28}, which directs transcription from class 3 promoters (38). It is held inactive prior to HBB completion by the anti-σ^{28} factor, FlgM (25, 39). Upon completion of the HBB, there is a switch in secretion specificity of the flagellar type III secretion apparatus from HBB-type (middle gene assembly) substrates to late secretion substrates that include FlgK, FlgL, FliD, FlgM, and flagellin (either FliC or FljB) (14, 19). These late secretion

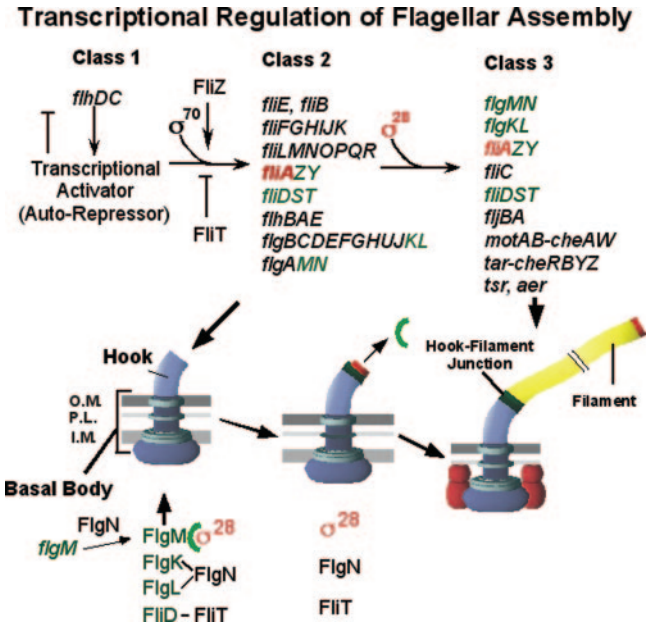


FIG. 1. Flagellar regulon expression coupled to flagellum assembly. The flagellar genes are organized into a transcriptional hierarchy that is coupled to assembly of the flagellar organelle as described in the text. The σ^{28} structural gene, *fliA*, is labeled in red. The genes labeled in red and green are transcribed from both class 2 (FlhDC- and σ^{70}-dependent) and class 3 (σ^{28}-dependent) promoters. The structures are imbedded in the inner, cytoplasmic membrane (I.M.), the peptidoglycan layer (P.L.), and the outer membrane and lipopolysaccharide (O.M.).

substrates all have corresponding type III secretion chaperones (TTSCs), which facilitate substrate secretion either directly or indirectly by preventing proteolytic degradation of substrates in the cytoplasm prior to secretion (4, 12). The TTSCs also have roles as regulators of flagellar gene expression. The TTSC for FlgK and FlgL is FlgN (13). Loss of FlgN results in reduced intracellular FlgM levels due to a decrease in *flgM* gene translation (24). The TTSC for FliD is FliT (13). Loss of FliT results in an increase in class 2 transcription (29). The TTSC for FlgM is σ^{28} (K. Hughes and P. Aldridge, unpublished results). Loss of σ^{28} results in no class 3 transcription (38). The TTSC for flagellin is FliS (3). This study demonstrates that the loss of FliS results in an increase in class 3 transcription.

A set of genes that includes the late secretion substrates and their secretion chaperones and important regulatory proteins are transcribed from both class 2 and class 3 promoters. These are the *flgM*, *flgN*, *flgK*, *flgL*, *fliA*, *fliD*, *fliS*, and *fliT* genes (15, 23, 27). The *flgM* and *flgN* genes are in the class 2 *flgAMN* and class 3 *flgMN* transcripts. The *flgK* and *flgL* genes are in the class 2 *flgBCDEFGHIJKL* and class 3 *flgKL* operons. The *fliAZY* operon is transcribed from a class 2 promoter, and the shorter *fliAZ* operon is from a separate class 3 promoter (23). The role that transcription from multiple promoters plays in the coupling of flagellar gene expression to flagellar assembly has yet to be determined.

In the work presented here, we compared levels of RNA from strains of *S. enterica* serovar Typhimurium LT2 defective in different flagellar regulatory genes to the levels of RNA expressed in the wild-type strain. This was done by DNA microarrays prepared from PCR-amplified open reading frames (ORFs) derived from the annotated sequence of the *S. enterica* serovar Typhimurium LT2 genome. Using a clustering analysis of eight mutant backgrounds, the flagellar genes were grouped to the known transcriptional classes (class 2 or class 3) to assign previously uncharacterized flagellar genes and the genes expressed from both class 2 and class 3 promoters. In addition, novel *Salmonella* flagellar genes were identified.

TABLE 1. List of bacterial strains

Strain	Genotype	Source or reference[a]
LT2	Wild type	J. Roth
MH111	*fliC*::Tn*10* Δ*fljB*	M. Homma
MJW708	*srfB*::Mu*dJ* (14028s background)	51
TH453	*fliC*::Tn*10*	
TH1059	*fli-5448*(IS*200*IV)::Tn*10d*Cm	
TH2788	*fliY5221*::Tn*10d*Tc (−86 from ATG of *fliY*)	
TH3070	*flgK5289*::Mu*dK*	1
TH3234	*fliA5394*::Mu*dJ* (Lac$^-$)	
TH3236	*flgK5396*::Mu*dJ*	1
TH3358	Δ*flgM5301*	
TH3730	P_{flhDC}*5451*::Tn*10d*Tc[*del-25*]	25
TH4707	Δ*fliT*::Km	53
TH4722	*flgK5726*::Mu*dK*	1
TH4911	Δ*flgN5626*::FKF	
TH4912	Δ*flk-5627*::FKF (Δ*flk* bp1-999 coding)	
TH5071	Δ*flgM5628*::FKF	
TH5360	Δ*fliA5647*::FCF	
TH5711	Δ*fliZ5737*::FKF	
TH5720	*flhC2035*	16
TH5721	*flhD2040*	16
TH5736	*fla-2039*(Δ*tar-flhD*)	16
TH5737	Δ*fliS5720*::FKF	
TH6554	Δ*flgM5628*::FKF Δ*fliA5647*::FCF	
TH6555	*fla-2039*(Δ*tar-flhD*) Δ*flgM5628*::FKF	
TH6572	*fla-2039*(Δ*tar-flhD*) Δ*flgM5628*::FKF Δ*fliA5647*::FCF	
TH6719	*fliB5968*::Mu*dJ*	
TH6720	*fliB5740*::Mu*dK*	
TH7409	Δ*fliA5805*::*tetRA fliB5968*::Mu*dJ*	
TH7410	Δ*fliA5805*::*tetRA fliB5470*::Mu*dK*	
TH7411	*fla-2039*(Δ*tar-flhD*) *fliB5968*::Mu*dJ*	
TH7412	*fla-2039*(Δ*tar-flhD*) *fliB5470*::Mu*dK*	
TH7458	*fliA5999 fliB5968*::Mu*dJ*	
TH7544	*fliA5999 fliB5470*::Mu*dK*	
TH7718	*fla-2039*(Δ*tar-flhD*) *flgK5396*::Mu*dJ*	
TH7719	*fla-2039*(Δ*tar-flhD*) *flgK5726*::Mu*dK*	
TH7720	*fla-2039*(Δ*tar-flhD*) *flgK5289*::Mu*dK*	
TH7721	*fliA5886 flgK5396*::Mu*dJ*	
TH7722	*fliA5886 flgK5726*::Mu*dK*	
TH7723	*fliA5886 flgK5289*::Mu*dK*	
TH7942	*srfB*::Mu*dJ*	
TH7943	*srfB*::Mu*dJ fla-2039*(Δ*tar-flhD*)	
TH7944	*srfB*::Mu*dJ fliA5999*	
TH9670	CRR4109[*metC1975*::Tn*10*(Tc^S)] P_{flhDC}*5451*::Tn*10d*Tc[*del-25*]	
TT10427	pNK972/LT2	
TT10604	Δ*proAB47*/F′ Pro$^+$ Lac$^+$ *zzf-1836*::Tn*10d*Cm	11

[a] Unless indicated otherwise, all strains were constructed during the course of this work. The FKF (FRT-Km-FRT) and FCF (FRT-Cm-FRT) cassettes have the Flp recognition target sites flanking either a kanamycin (Km) or chloramphenicol (Cm) resistance gene followed by a ribosome-binding site (8). The resistance gene can be removed by expression of FLP recombinase to leave a single FRT site followed by a ribosomal binding site (8).

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this study and their origins are listed in Table 1. Plasmid pRP1 was constructed as follows. Strain TH3234 carrying a Mu*dJ* insertion in the *fliA* gene (*fliA*::Mu*dJ*) was converted to a Mu*d*-P22 insertion (*fliA*::Mu*d*P) (54). The *fliA*::Mu*d*P strain was induced with mitomycin C, and DNA was isolated from the induced phage lysate (the DNA packaged from the induced *fliA*::Mu*d*P is enriched for chromosomal DNA flanking the 5′ end of the *fliA* locus [54]; the DNA was digested with EcoRI [New England Biolabs]). A 3.5-kbp fragment that includes the $fliB^+$ and $fliC^+$ genes and portions of the adjacent IS*200* and *fliD* sequences was ligated (with T4 DNA ligase [New England Biolabs]) into the pBluescript II KS^- vector (Stratagene) digested with EcoRI to create pRP1.

Media and standard genetic manipulations. Media, growth conditions, transductional methods, and motility assays were as described previously (15, 16). The generalized transducing phage of *S. enterica* serovar Typhimurium P22 *HT105/1 int-201* was used in all transductional crosses (9). Transductions involving tetracycline-sensitive (Tc^s) selection was done as follows: 0.1 ml of a 10-fold dilution from a standard phage prep was mixed with 0.1 ml of an overnight culture (yielding a final mixture of 10^8 to 10^9 phage and 2×10^8 cells). As a control for spontaneous tetracycline sensitive mutants, 0.1 ml of 0.85% saline solution was mixed with 0.1 ml of the same overnight culture. Both solutions were allowed to stand at room temperature for about 30 min and then diluted 10- and 100-fold prior to plating on tetracycline-sensitive selection plates and incubated at 42°C (35). It takes 2 days for Tc^s colonies to appear. If the transduction works well, there should be at least 100 times the number of Tc^s colonies on the experimental plates as there is on the no-phage control plates.

Isolation of targeted flagellar gene deletions. *S. enterica* serovar Typhimurium chromosomal gene deletion and replacement with drug resistance cassette FRT-Kan-FRT (FKF) or FRT-Chlor-FRT (FCF) were performed by the lambda Red phage mediated recombination system (8). The oligonucleotide sequences below in uppercase are the sites of recombination in the chromosome. The lowercase sequences are sites of P1 or P2 for PCR amplification of pKD4 (FKF) or pKD3 (FCF) (8). *TH4911* Δ*flgN5626*::FKF (deletion of entire coding region) 1frtflgN, 5′-ATTCGCCAGGCGCAGAGCTACTTACAGAGTAAATAAGCGTgtgtaggctggagctgctcc-3′; 2frtflgN, 5′-CAGGCCGGAAAGGCGCAACGTCGCCATCCGGCAATGATTAcatatgaatatcctccttag-3′; TH4912 Δ*flk-5627*::FKF (deletion of entire coding region) 1FRTFLK, 5′-GCATATATTGCTCAGATTTATGGTTAAAGGATAATTAATTgtgtaggctggagctgcttc-3′; 2FRTFLK, 5′-GGTTGGCATCCTGGTGACGATATTATCGTTCCGGCGCGGTcatatgaatatcctcctta g-3′; TH5071 Δ*flgM5628*::FKF (deletion from codon 5 to 97 and the intervening region between *flgM* and *flgN*) FlgMP1, 5′-AACGTAACCCTCGATGAGGATAAATAAATGAGCATTGACCGgtgtaggctgcagctgcttc-3′; FlgNATGP2, 5′-ACGGTGGTCATCTGGTCAAGTATTTCTGACAAACGAGTCATcatatgaatatcctcctta-3′; TH5360 Δ*fliA5467*::FCF (deletion from codon 9 to 218) fliAP1, 5′-GTGTAATGGATAAACACTCGCTGTGGCAGCGTTATGTACCgtgtaggctggagctgcttc-3′; fliAP2, 5′-AATCGTTTGATGGCCTGACTATGCAACTGGCTGACCCGCGcatatgaatatcctccttag-3′; TH5711 Δ*fliZ5737*::FKF (deletion of entire coding region) fliZF1, 5′-CGAAAAGTGCCGCACAACGTATAGACTACCAGGAGTTCTCgtgtaggctggagctgcttc-3′; fliZF2, 5′-GTTTCACCAACACGACCTCTGCTACATCTTATGCTTTTTAAcatatgaatatcctccttag-3′; TH5737 Δ*fliS5720*::FKF (deletion from codon 2 to 135) 5′fliS-FRT, 5′-AATTTACAGCTATGAACAAGTCCTGATAACAGAGGTCACCATgtgtaggctggagctgcttcg-3′; 3′fliS-FRT, 5′-ACGCTGCCAACGGTTGATAAACTCCACGGTTGAGGTCATTAcatatgaatatcctccttag-3′.

Isolation of a Tn*10d*Cm insertion in the IS*200* element near the *fliB* gene. To target Mu*d* insertions to specific regions within the flagellar *fli* region, a number of Tn*10d*Cm insertions were isolated that were linked to a *fliC*::Tn*10* allele, which was transduced into strain LT2 (to create TH453) from strain MH111 (Table 1). P22 was grown on strain TT10604, which caries a Tn*10d*Cm insertion in the F plasmid, and used to transduce strain TT10427 to chloramphenicol resistance. TT10427 carries plasmid pNK972 that constitutively expresses Tn*10* transposase (50). Because there was no F plasmid in strain TT10427, it was not possible to inherit the Tn*10d*Cm transposon by homologous recombination. The chloramphenicol-resistant (Cm^r) transductants were inherited by transposition into the recipient chromosome. Greater than 50,000 Cm^r transductants were pooled together, and a P22 lysate was prepared on the pooled cells carrying Tn*10d*Cm insertions located at positions throughout the chromosome. To isolate Tn*10d*Cm insertions in the *fliC* region, the pooled lysate was used to transduce TH453 (*fliC*::Tn*10*) to Cm^r on L-Cm plates, and the colonies were screened for loss of the *fliC*::Tn*10* allele by replica printing onto L-Cm-Tc plates and screening for those that became Tc^s upon transduction to Cm^r. This allows for the isolation of Tn*10d*Cm insertions near *fliC*, because the recipient does not carry Tn*10* transposase, so the Tn*10d*Cm elements from the pooled donor lysate are inherited by homologous recombination. Those close to *fliC* can inherit Tn*10d*Cm-encoded Cm^r, and if they also coinherit the $fliC^+$ allele by cotransduction, they become Tc^s. A total of 19 Cm^r Tc^s transductants were kept for further analysis. Of these 19, 10 were found to be nonmotile, indicating that they had inserted into one of the flagellar genes in the *fli* region, 1 was nonmotile in a *fljB* null strain only, indicating that it was inserted in the *fliC* gene, and 8 were motile. Flair complementation analysis was performed on the 10 nonmotile *fli*::Tn*10d*Cm insertions. Of the 10, 5 were located on the *fliD* gene, 1 was located in *fliF* and 4 were located in *fliQ*. Linkage analysis of the 8 motile insertions revealed that four were linked to the *fliR* locus (located counterclockwise of *fliR* on the standard *S. enterica* serovar Typhimurium linkage map), two were located between the *fliT* and *fliE* genes, one was located between the *fliC* and *fliD* genes, and one was between the *fliC* and *fliA* genes. DNA sequence analysis was performed on the insertion between the *fliC* and *fliA* genes, named *fli-5448*::Tn*10d*Cm. The *fli-5448*::Tn*10d*Cm transposon had inserted counterclockwise after base pair 25 of the presumed ATG start codon of the transposase gene of the IS*200* element (IS*200*IV) located between the *fliA* and *fliB* loci.

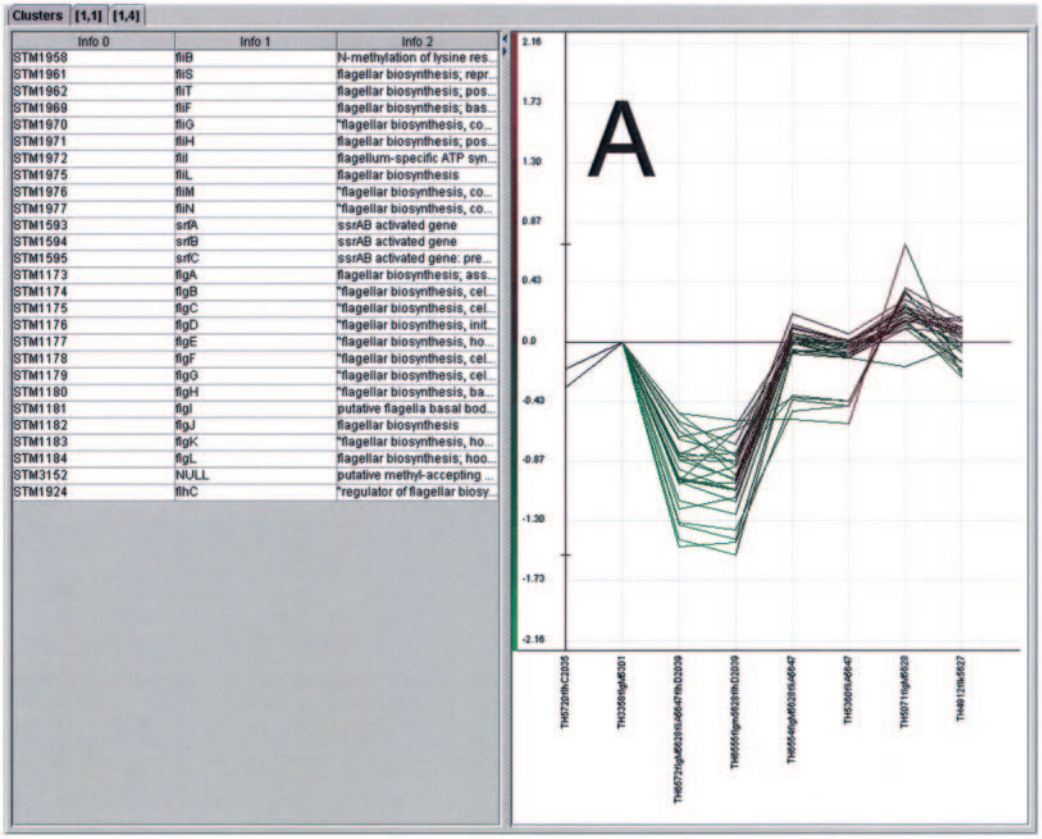
Isolation of the *fliB5968*::Mu*dJ* and *fliB5740*::Mu*dK* insertions. Strain TH3730 carries a Tn*10d*Tc[*del-25*] (T-POP) insertion located between the class 1 *flhDC* promoter and the *flhDC* structural genes. This insertion places the *flhDC* operon and thus the entire flagellar regulon under control of the inducible *tetA* promoter (25). The Mu*dJ* transposon was introduced into strain TH1059 (*fli-5448*::Tn*10d*Cm) carrying the Tn*10d*Cm insertion in the IS*200*IV element next to the *fliB* gene by the method of transitory *cis* complementation (20). Greater than 50,000 Mu*dJ*-encoding kanamycin-resistant (Km^r) transductants were pooled, and P22 transducing phage was grown on the pooled cells. The lysate was used to transduce strain TH3730 to Cm^r on L-Cm plates, and these plates were then replica printed to L-Cm-Km plates to identify Cm^r Km^r recipients that had also inherited a Mu*dJ* insertion along with the *fli-5448*::Tn*10d*Cm allele, presumably by cotransduction. The Cm^r Km^r transductants were screened for motility and expression of the *lac* genes in the presence of Tc (*flhDC*-inducing condition). Putative *fliB*::Mu*dJ* isolates were defined as being greater than 85% linked to the *fli-5448*::Tn*10d*Cm allele by P22 transduction, exhibited a Tc-inducible Lac^+ phenotype in the presence of the P_{flhDC}::T-POP insertion, and were motile (loss of *fliB* does not affect motility). Subsequent PCR and DNA sequence analysis identified the *fliB5968*::Mu*dJ* transposon to be inserted after base 38 of the *fliB* coding region (*fliB* is transcribed counterclockwise on the standard *S. enterica* serovar Typhimurium linkage map).

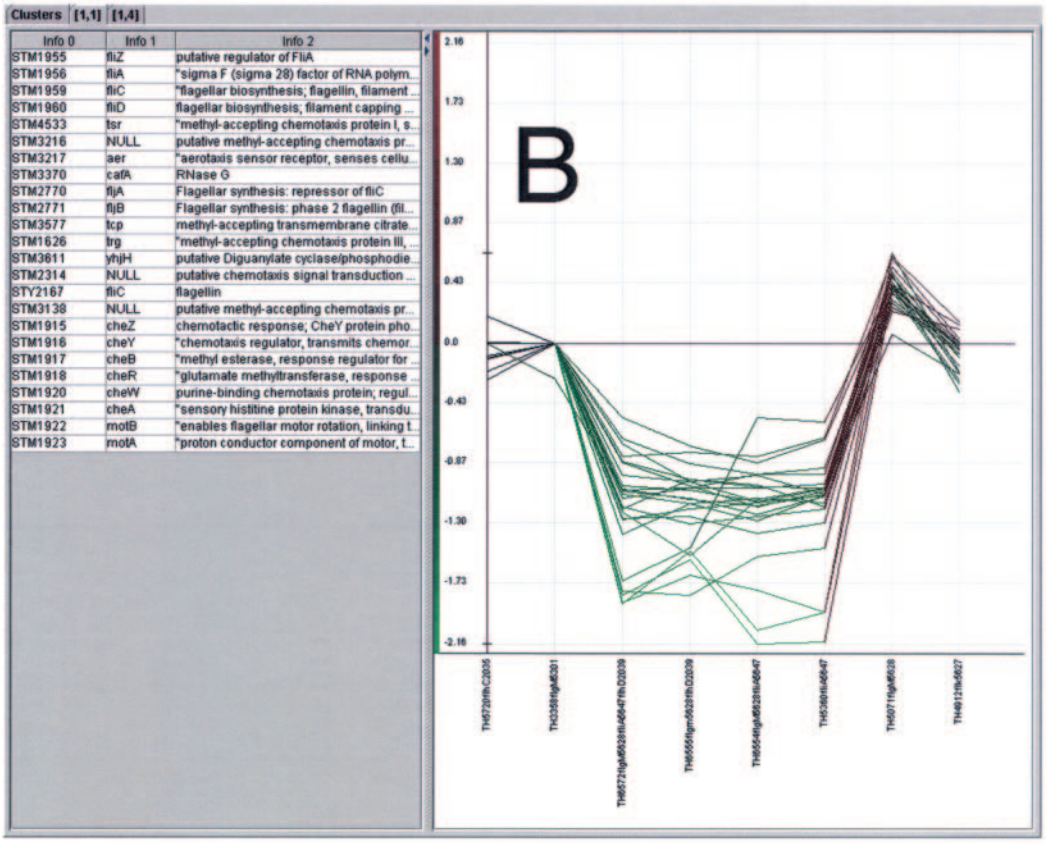
The *fliB5740*::Mu*dK* insertion was isolated in a hunt for Mu*dK* insertions in the *fliC* gene (5). Mu*dK* insertions greater that 85% linked to the *fli-5448*::Tn*10d*Cm allele that were motile were analyzed by PCR and DNA sequence analysis. One, *fliB5740*::Mu*dK*, was found to be inserted 813 nucleotides into the *fliB* coding region, resulting in a translational fusion of the first 271 amino acids of FliB to LacZ, and it was used for further studies.

Isolation of Mu*dJ* insertions in the *cheV*, *mcpB*, *mcpC*, and *aer* loci. To isolate insertions of Mu*dJ* in *cheV*, we obtained a linked Δ*pmrD*::Cm allele from Eduardo Groisman. About 50,000 random Mu*dJ* insertion mutants in a Δ*pmrD*::Cm-containing strain were pooled, and P22-transducing phage was used to transduce this pool into strain TH3730, which has the *flhDC* operon under control of the *tetA* promoter. Four Mu*dJ* insertions that showed Tc-dependent Lac expression were tested by PCR using a primer that hybridized just outside the 3′ end of *cheV* and a primer that hybridizes to the left end of Mu, which reads out of the Mu*dJ* element. All four putative *cheV*::Mu*dJ* insertions gave PCR products of sizes expected for insertions in *cheV*.

To isolate insertions of Mu*dJ* in the *mcpC* and *aer* genes, we utilized a Tn*10d*Cm insertion already present in the strain collection, which by DNA sequence analysis revealed it to be at the very 3′ end of the *mcpB* gene (data not shown). About 50,000 random Mu*dJ* insertions mutants in the *mcpC*::Tn*10d*Cm-containing strain were pooled, and P22 transducing phage was used to transduce this pool into strain TH3730, which has the *flhDC* operon under control of the *tetA* promoter. Six Mu*dJ* insertions that showed Tc-dependent Lac expression were separated from the *mcpC*::Tn*10d*Cm insertion by P22 transduction. They were then tested by PCR using either a primer that hybridized just outside the 3′ end of *mcpC* or a primer that hybridized just outside the 3′ end of *aer* as well as a primer that hybridizes to the left end of Mu, which reads out of the Mu*dJ* element. Two Mu*dJ* insertions were located on the *mcpC* gene, and four were located on the *aer* gene.

The Mu*dJ* pools isolated above were used with strain TH9670 that was defective in the *mcpB*-linked *metC* gene and has the *flhDC* operon under control of the *tetA* promoter. Three Mu*dJ* insertions that showed Tc-dependent Lac expression were tested by PCR using a primer that hybridized just outside the 3′ end of *mcpB* and a primer that hybridizes to the left end of Mu, which reads out of the





Mu*dJ* element. All three putative *mcpB*::Mu*dJ* insertions gave PCR products of sizes expected for insertions in *mcpB*.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Maloy (35). Cells were grown to an optical density at 600 nm of 0.6 to 0.8. Each data point was the sum of three independently grown bacterial cultures each assayed twice, and the values were recorded as β-galactosidase units (nanomoles per minute per unit of optical density at 650 nm per milliliter).

Primer extension. RNA was isolated as described previously (47). RNase inhibitor (Boehringer Mannheim) and DNase (Bethesda Research Laboratories) were added to the RNA in RNase-free water (sterile irrigation water; Baxter Healthcare Corp.). ^{32}P labeling of primer FliBPE (5′-GGGTGACAAAGGCAG GTTCAGTGACGGTGA-3′) was done with T4 polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear) (2). Primer extensions were carried out (2) with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and buffer supplied by the manufacturer at 42°C for 30 min.

Microarray design and manufacture. The *S. enterica* serovar Typhimurium LT2 microarray was constructed from specific PCR products for each gene. These were amplified from LT2 genomic DNA, and whenever possible these encompassed the whole ORF. The initial array set was used in all studies, except for two of the comparisons of the Δ*flhDC* and LT2 strains. For these, the microarray contained 4,442 whole ORF PCR products, representing 96.6% of the *Salmonella enterica* serovar Typhimurium LT2 genome and the pSLT plasmid. For the latter two experiments, the overall *S. enterica* serovar Typhimurium genome coverage for the array was 99.4% (4,466 genes). The arrays also contained PCR products representing the genes found on the LT2 virulence plasmid pSLT. The DNAs were spotted onto Ultra-GAPS glass slides (Corning Inc., Corning, N.Y.) in 50% dimethyl sulfoxide and were hybridized by the method of Brown et al. (http://cmgm.stanford.edu/brown/protocols/4_Ecoli_RNA.txt). Details of the construction of the *Salmonella* array were described previously (41).

RNA labeling and hybridization. cDNA probes were labeled with Cy3- and Cy5-dye-linked dUTP by direct incorporation during reverse transcription from total RNA to cDNA, following the method described by Pat Brown (http://cmgm.stanford.edu/pbrown/protocols/4_Ecoli_RNA.txt), with the following modifications. Fifty micrograms of total RNA and 2.4 μg of random hexamers were resuspended in 30 μl of water, and subsequently the amounts and volumes of all components were doubled compared to those of the Brown protocol. Two microliters of RNAsin (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was added to the reverse transcription, and the reaction was incubated at 42°C for 2 h. After the first hour of incubation, an additional 2 μl of Superscript II reverse transcriptase was added. Probes were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and eluted in 1 mM Tris-HCl, pH 8. 0.

Hybridization and data acquisition. Probes were hybridized to the *Salmonella* array overnight in 25% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate at 42°C using a hybridization chamber (Corning) submerged in a water bath. Protocols suggested by the manufacturer for hybridizations in formamide buffer (http://www.corning.com/Lifesciences/technical_information/techDocs/gaps_ii_manual_protocol_5_02_cls_gaps_005.pdf) were applied for prehybridization, hybridization, and posthybridization processing. The cDNA probes from wild-type and mutant strains were hybridized to three arrays, and then dyes were switched and samples were hybridized to three additional arrays. Scans were performed on a ScanArray 5000 Laser scanner (Packard BioChip Technologies, Billerica, MA) using ScanArray 2.1 software.

Data analysis. Signal intensities were quantified using the QuantArray 3.0 software package (Packard BioChip Technologies, Billerica, MA). Spots were analyzed by adaptive quantitation, and local background was subsequently subtracted from the recorded spot intensities. Ratios of the contribution of each spot to total signal in each channel were calculated (data normalization). Negative values (i.e., local background intensities higher than spot signal) were considered no data. The expression ratio of each gene was then calculated as the median of the six ratios from the six hybridizations. RNA measurements were analyzed by calculating ratios and standard deviations between the mutant versus wild-type strains. Genes with signals less than two standard deviations above background in both conditions were considered not detected and were removed prior to any analysis.

RESULTS

Effect of flagellar regulatory mutations on flagellar gene expression. Two DNA microarray experiments were performed to identify novel flagellar genes of *Salmonella enterica* serovar Typhimurium. In one experiment, genomic RNA samples isolated from different flagellar mutant backgrounds were compared to RNA samples isolated from the wild-type strain LT2 (see Materials and Methods). In this experiment, each comparison was done between one sample from each of eight different strains and LT2. A cluster analysis program was then used to identify new genes that clustered with known flagellar genes. In a second experiment, four independent comparisons of RNA levels from a strain deleted for the flagellar master operon, *flhDC*, were compared to RNA levels from wild-type strain LT2.

Cluster analysis. The expression levels of whole cellular RNAs from the eight different flagellar mutant strains relative to LT2 were subject to cluster analysis using the J-Express program (MolMine AS, Bergen, Norway). These strains included TH3358 (Δ*flgM5301*), TH4912 (Δ*flk-5627*), TH5071 (Δ*flgM5628*), TH5360 (Δ*fliA5647*), TH5720 (*flhC2035*), TH6554 (Δ*flgM5628* Δ*fliA5647*), TH6555 (Δ*flgM5628* Δ*flhDC2039*), and TH6572 (Δ*flgM5628* Δ*fliA5647* Δ*flhDC2039*). Two clusters were found to include predominantly class 2 or class 3 flagellar genes and are presented in Fig. 2.

In the class 2 flagellar cluster (Fig. 2A), we observed many of the genes required for hook-basal body (HBB) formation. The late secretion substrate genes, *flgK* and *flgL*, and late secretion chaperone genes, *fliS* and *fliT*, which are transcribed from both class 2 and class 3 flagellar promoters, also clustered with the class 2 genes. In addition, the *flhC* gene clustered with class 2 genes, which probably reflects the fact that the *flhDC* operon is under autogenous control. The *fliB* gene, whose promoter has not been characterized, also clustered with the class 2 genes, which suggested that *fliB* was independently transcribed from the upstream class 3 *fliC* gene. In results presented below, we show that this is the case.

In addition to known flagellar genes, three genes from a presumed virulence gene operon, *srfABC*, and an uncharacterized gene, STM3152, annotated as a methyl-accepting chemotaxis protein clustered with the class 2 genes. We show below that the *srfABC* operon is under FlhDC control but not σ^{28} control, indicating that this operon is a flagellar class 2 operon. STM3152 was found to be a σ^{28}-dependent class 3 gene, al-

FIG. 2. Cluster analysis of flagellar genes using J-Express. A. Cluster [1,1] included structural genes of the hook-basal body (*fliF-N* and *flgA-J*), the flagellin methylase (*fliB*), secretion chaperones *fliS* and *fliT*, a putative methyl-accepting chemotaxis gene, STM3152, and the *srfABC* operon. These are all expressed from class 2 promoters, except for the class 1 *flhC* gene and class 3 STM3152 (*mcpB*) gene. The *fliST* genes are also expressed from a class 3 promoter. The *flhC* gene was artifactual, since it was deleted in three of the strains included in the cluster analysis. B. Cluster [1,4] included genes of the chemosensory system (*tsr*, STM3216, *aer*, *tcp*, *trg*, STM2314, STM3138, *cheZ*, *cheY*, *cheB*, *cheR*, *cheW*, *cheA*, the flagellar motor force generator genes [*motAB*], the flagellin genes [*fliC* and *fljB*], the flagellin cap gene [*fliD*], and regulatory genes *fliZ*, *fliA* [σ^{28}], and *fljA*). The RNase G structural gene, *cafA*, and the *yhjH* gene were also part of this cluster. All genes are transcribed from flagellar class 3 promoters, except for *cafA*. The clustering of *cafA* with flagellar genes was most likely an array error (see the text). The *fliA* and *fliZ* genes are also expressed from class 2 promoters.

TABLE 2. FlhDC-dependent *Salmonella* genes[a]

Rank and gene name[b]	Ratio[c]	Rank and gene name[b]	Ratio[c]
1. *fliC*	0.031	48. *fliN*	0.23
2. *fljB*	0.034	49. *fliI*	0.26
3. *flgK*	0.043	50. *fliJ*	0.28
4. *cheA*	0.044	51. *flhB*	0.31
5. *flgG*	0.060	52. *fliP*	0.36
6. *fliA*	0.050	53. *modC*	0.41
7. *flgD*	0.058	54. *modA*	0.36
8. *fliD*	0.060	55. *ycgR*	0.38
9. *cheW*	0.073	56. *flgN*	0.49
10. *cheM*	0.10	57. STM3152	0.32
11. *trg*	0.085	58. *fliO*	0.43
12. *flgE*	0.072	59. *spaO*	0.50
13. *flgL*	0.074	60. *prgJ*	0.47
14. *motA*	0.092	61. *modB*	0.46
15. *flgF*	0.079	62. STM3155	0.61
16. *flgB*	0.10	63. *prgH*	0.38
17. *cheZ*	0.095	64. *fliE*	0.58
18. *aer*	0.089	65. *sopB*	0.34
19. *cheY*	0.095	66. *fimA*	0.55
20. *fliH*	0.097	67. *flhA*	0.53
21. *cheR*	0.12	68. *invJ*	0.49
22. *cheB*	0.12	69. *invI*	0.50
23. *motB*	0.12	70. *sipB*	0.42
24. *fliZ*	0.13	71. *invG*	0.42
25. *tsr*	0.14	72. *sipC*	0.42
26. *fliM*	0.13	73. STM1133	0.31
27. *fliF*	0.14	74. *invC*	0.55
28. *flgH*	0.14	75. *hilD*	0.53
29. *fliL*	0.14	76. STM1328	0.57
30. *fliB*	0.14	77. *hilA*	0.45
31. *fliG*	0.16	78. *sicA*	0.54
32. *fliS*	0.15	79. *prgK*	0.44
33. *flgM*	0.15	80. *prgI*	0.55
34. *3138*	0.15	81. *sprB*	0.50
35. *2314*	0.15	82. *2787*	0.40
36. *flgC*	0.16	83. *sipD*	0.51
37. *flgI*	0.17	84. *flhE*	0.55
38. *flgJ*	0.16	85. *hilC*	0.46
39. STM3216	0.16	86. STM4080	0.55
40. *fljA*	0.19	87. *yjcH*	0.53
41. *srfA*	0.21	88. *invA*	0.49
42. *tcp*	0.19	89. *sipA*	0.57
43. *srfB*	0.20	90. *yjcG*	0.53
44. *yhjH*	0.20	*fliY*[d]	0.57
45. *srfC*	0.20	*fliR*	0.81
46. *flgA*	0.22	*fliQ*	0.98
47. *fliT*	0.23		

[a] The top 90 genes were ranked from 4 independent array data sets.
[b] The ranking in this table reflects their average ranking from all four data sets. Hence, some genes ranked higher than others, even though their overall FlhDC dependence was lower.
[c] The number represents the average ration of RNA in the *flhDC* deletion background to the wild-type LT2 strain.
[d] The effect of FlhDC on the *fliY*, *fliQ* and *fliR* genes, which did not make the top-90 cut, is also shown.

though it did not cluster with the class 3 genes. A large number of other flagellar genes were not present in the class 2 cluster. While the entire *flgB-L* operon and the *flgA* gene were in the class 2 cluster, the *flhBAE* operon and *flhD*, *fliE*, *fliJ*, and *fliOPQR* genes were not. It is possible that the *flhBAE* and *fliE* operons have different expression patterns in the different flagellar mutant strains compared to the other class 2 genes, but other genes cotranscribed with *flhD*, *fliE*, *fliJ* and *fliOPQR* did cluster as class 2 genes. It is likely that the low level of expression of these genes did not produce the significant changes in expression needed in the various mutant backgrounds to cluster with the other class 2 genes. Genes at the 3′ end of a transcript could be at low RNA levels due to mRNA degradation. Also, in *E. coli* it was shown that σ^{28} regulates expression of some class 2 operons (such as *fliL-R*) but not others (such as *flhBAE*) (33). This could account for lower levels of the *flhBAE* operon genes. The *fliK* gene was not present in the array slides used for these studies.

In the class 3 flagellar cluster (Fig. 2B) we observed primarily late assembly genes, the motor force generator genes *motAB*, and the genes of the chemotaxis system. Unlike *flgKL* and *fliST*, the other late secretion substrate gene, *fliD* clustered with the class 3 genes (as did the *fliAZ* operon), which are expressed from both class 2 and class 3 promoters. In addition

TABLE 3. β-Galactosidase assays using the Mu*dJ lac* operon fusion vector as transcriptional reporter[a]

Strain background	*srfB*	STM2314 (*cheV*)	STM3152 (*mcpB*)	STM3216 (*mcpC*)	*aer*	*flgK*	*fliB*
Wild type	13 ± 2	120 ± 36	46 ± 15	75 ± 39	79 ± 36	71 ± 1.4	47 ± 1.6
Δ*flhDC*	<1 ± 0.1	7.7 ± 4.6	1.7 ± 0.7	2.9 ± 1.3	2.1 ± 0.6	<1 ± 0.1	<1 ± 0.1
fliA	11 ± 2.1	2.2 ± 1.4	2.2 ± 1.4	3.2 ± 1.8	1.5 ± 0.5	17 ± 1.4	27 ± 1.0
Δ*hin-5717*::FCF		35 ± 9.9	35 ± 9.9	92 ± 30	100 ± 33		
Δ*hin-5718*::FCF		11 ± 14	11 ± 14	62 ± 38	120 ± 9		
Δ*hin-5717*::FCF Δ*flhD2039*		<1 ± 1	<1 ± 1	3.8 ± 1.5	1.9 ± 0.8		
Δ*hin-5717*::FCF *fliA*		1.9 ± 0.4	1.9 ± 0.4	3.4 ± 1.3	1.9 ± 0.4		

[a] The expression of *srfB*::Mu*dJ*, *flgK*::Mu*dJ* and *fliB*::Mu*dJ* fusions were assayed in strains defective in either *fliA* (σ^{28}) or FlhDC compared to expression in the isogenic wild-type strain. The *fliA* allele used in these assays was *fliA5999*(R91C, L207P) and carries two point mutations that make this nonpolar mutant form of σ^{28} defective in binding both the −35 (L207P) and −10 (R91C) regions of flagellar promoters. The expression of *cheV*::Mu*dJ*, *mcpB*::Mu*dJ*, *mcpC*::Mu*dJ*, and *aer*::Mu*dJ* fusions was assayed in strains defective in either *fliA* (σ^{28}) or FlhDC compared to expression in the isogenic wild-type strain and in strains locked in either the $fliC^{ON}$ $fljB^{OFF}$ (Δ*hin-5717*::FCF) or $fliC^{OFF}$ $fliB^{ON}$ (Δ*hin-5718*::FCF) state of flagellar phase variation to determine if these genes were affected by flagellar phase variation.

to known flagellar genes, the *cafA* gene, encoding RNase G, clustered as a class 3 gene, but data below will show this to be an error of the particular set of array chips used early in this study. In addition to known flagellar genes, a putative dicyclic GMP phosphodiesterase gene, *yhjH*, identified previously to have a class 3 flagellar promoter clustered with the flagellar class 3 genes as did three uncharacterized genes, STM2314, STM3138, and STM3152, annotated to be *cheV* (a *cheW-cheY* hybrid gene known from *Bacillus subtilis*) and two methyl-accepting chemotaxis proteins, respectively. We did not observe the *flgMN* operon in either cluster, and the *tar* (*cheM*) gene was not in the array slides.

FlhDC-dependent flagellar genes. In a second set of experiments, we looked at genes dependent on the *flhDC* master operon for expression. Using one set of microarray chips, two independent RNA isolates from a Δ*flhDC* strain were compared for reduced expression from two independent RNA isolates from strain LT2 (see Materials and Methods). This was repeated with an independently constructed set of microarray chips, and the top 90 FlhDC-dependent genes from all four comparisons are shown in Table 2. As expected, flagellar gene expression was reduced compared to that of pathogenesis genes. The flagellar regulatory system was shown to affect the regulation of many virulence-associated genes (10, 46, 48). The list of FlhDC-dependent genes includes those identified by the cluster analysis as novel flagellar genes, *yhjH* and STM2314 (*cheV*), as well as putative MCP genes STM3138 (*mcpA*), STM3152 (*mcpB*), and STM3216 (*mcpC*). We have renamed the STM3138, STM3152, and STM3216 loci *mcpA*, *mcpB*, and *mcpC* due to their homology to known methyl-accepting chemotaxis protein genes. The *srfABC* operon that clustered with flagellar class 2 genes ranked highest of all the virulence-associated genes on the FlhDC-dependent list, which was consistent with its clustering with flagellar genes. The flagellar genes that did not cluster (*flgMN*, *flhBAE*, *fliE*, *fliJ*, *fliOPQR*, and *fliY*) were less affected by the loss of *flhDC* than the genes that did. The *fliY* gene is expressed by a σ^{70}-dependent promoter in addition to flagellar promoters (23), which would explain the reduced sensitivity to the loss of *flhDC*. Surprisingly, two flagellar class 2 genes were, in fact, not significantly affected by the absence of the *flhDC* operon. The *fliR* gene averaged only 10% lower RNA levels in the absence of the *flhDC* operon, while the *fliQ* gene was unaffected. This likely reflects low levels of *fliQR* mRNA in the presence of *flhDC*. Finally, the *cafA* gene, which clustered with flagellar class 3 genes, showed FlhDC dependence using the first set of array slides (Δ*flhDC*/LT2 ratio, 0.17) but not in the second independently constructed set of array slides (Δ*flhDC*/LT2 ratio, 1.3), suggesting that the initial clustering with flagellar genes was due to some error in array slide construction. Also, *cafA* appears to be part of a large operon that includes the *mreBCD* genes, which were not FlhDC dependent on either set of array slides.

Isolation and characterization of Mu*d-lac* operon fusions to new flagellar genes. To verify the microarray results for the identification of novel flagellar genes, we took advantage of the fact that a Mu*d-lac* reporter fusion to the *srfB* gene had been isolated and generously provided to us by Fred Heffron. We also took advantage of the close linkage of STM3152 to the *metC* gene and the fact that we already had an insertion of Tn*10d*Cm at the very 3′ end of the STM3216 gene to isolate Mu*d-lac* insertions in STM3152, STM3216, and the *aer* locus (see Materials and Methods) as well as the close linkage of the *cheV* homolog gene, STM2314, to a Δ*pmrD*::Cm insertion mutation generously provided to us by Eduardo Groisman.

Insertions of the *lac* transcriptional reporter vector Mu*dJ* in STM2314, STM3152, STM3216, and *aer* loci were isolated (see Materials and Methods). These, along with the *srfB*::Mu*dJ* insertion kindly provided by Fred Heffron, were assayed for effects of deletions in *flhDC* and *fliA* (σ^{28}) on expression of these fusions to determine if these genes were transcribed from flagellar class 2 or class 3 promoters.

Expression of the *srfB*::Mu*dJ* insertion in strains carrying null alleles of *flhDC* or *fliA* confirmed that the *srfABC* operon was a flagellar class 2 operon. Transcription was 30-fold reduced in the absence of FlhDC but was not significantly affected by the *fliA* null allele (Table 3).

Mu*dJ* insertions in the previously uncharacterized putative *Salmonella cheV* (STM2314) and *mcp* genes, STM2314 (*mcpA*), STM3152 (*mcpB*), STM3216 (*mcpC*), and *aer*, were isolated and assayed in strains carrying null alleles of *flhDC* or *fliA* and in strains locked in either the $fliC^{ON}$ or $fljB^{ON}$ orientations of the flagellar phase variation system to determine if any were under control of flagellar phase variation (Table 3). The Mu*dJ* insertion in STM2314 was σ^{28} dependent and not affected by flagellar phase variation. As with the *cheV*::Mu*dJ* insertion, Mu*dJ* insertions in the STM3152 (*mcpB*), STM3216 (*mcpC*), and *aer* loci were σ^{28}-dependent class 3 flagellar genes and were not affected by flagellar phase variation. The *cheV*::Mu*dJ*, *mcpB*::Mu*dJ*,

mcpC::Mu*d*J, and *aer*::Mu*d*J fusions were not affect by flagellar phase variation. Only *mcpB*::Mu*d*J showed a somewhat reduced level in the *fliC*^{OFF} *fljB*^{ON} (D*hin-5718*::FCF) orientation, but the level observed was still within the error range of the β-galactosidase assays. While we did not obtain a Mu*d*J insertion in STM3138, we presumed that its clustering with the flagellar class 3 genes, its dependence of FlhDC for expression (RNA levels were 67-fold lower in Δ*flhDC* compared to LT2 [Table 2]), its amino acid homology to MCP proteins, and the presence of a σ^{28} consensus promoter sequence upstream (the consensus −10 sequence is 80 bases upstream of the ATG start codon) was sufficient evidence to include it with the new *Salmonella* flagellar class 3 genes.

We included the characterization of recently isolated *flgK*::Mu*d*J in these studies (1). The *flgKL* operon is transcribed from both class 2 and class 3 promoters (27), and it clustered with the class 2 flagellar genes (Fig. 2). We tested the expression of the *flgK-lac* transcriptional fusion in wild-type and Δ*flhDC* and *fliA* mutant strains (Table 3). The loss of σ^{28}-dependent class 3 transcription resulted in a fourfold reduction in *flgK-lac* transcription, and transcription was abolished in the Δ*flhDC* strain background. This is consistent with the *flgKL* operon being transcribed from both class 2 and class 3 promoters.

Finally, we also isolated and characterized *fliB*::Mu*d-lac* reporter constructs. The *fliB* gene was shown over 40 years ago to encode a protein that methylates the lysine residues of flagellin (45). Prior to this array analysis, we presumed that the *fliB* gene was transcribed in an operon with the *fliC* gene flagellin. However, it was not determined if the *fliC* and *fliB* genes were cotranscribed in an operon, and it had not been reported where the *fliB* gene resided within the flagellar regulatory hierarchy. Given its role as a flagellin methylase, it was expected to be transcribed from a class 3 promoter. However, the DNA array data showed that *fliB* transcription was primarily dependent on FlhDC and not on σ^{28}, indicating that it was transcribed in a class 2 transcript (Fig. 2A). Transcriptional and translational *lac* reporter fusions to the *fliB* reporter were constructed using the Mu*d-lac* transposons Mu*d*J and Mu*d*K (see Materials and Methods) (18). The effect of FlhDC and σ^{28} on the *fliB*::Mu*d-lac* fusion constructs was determined and is presented in Table 3. Loss of σ^{28} resulted in a twofold reduction in *fliB* transcription using the *fliB*::Mu*d*J (*lac* operon) transcriptional fusion vector, consistent with other class 2 genes (28). Loss of FlhDC resulted in complete loss of β-galactosidase activity for the *fliB*::Mu*d*J fusion. These results support the DNA array data and indicate that transcription of the *fliB* gene was from a class 2 promoter. Low levels of β-galactosidase were obtained with the *fliB*::Mu*d*K construct, suggesting that there may be additional regulation of *fliB* gene expression at the posttranscriptional level.

Primer extension assay of the *fliB* gene. The classification of *fliB* as a class 2 transcript predicted the presence of a promoter between the 3′ end of the class 3 *fliC* transcript and the beginning of the *fliB* open reading frame. Flagellar class 3 promoters have −10 (GCCGATAA) and −35 (TAAAGTTT) regions that are well conserved (21), but the class 2 promoters lack a conserved −35 region, and even the −10 sequences are not well conserved, although 4 out of 10 class 2 promoters have an identical −10 sequence (GATAAT) (22). The closest sequence

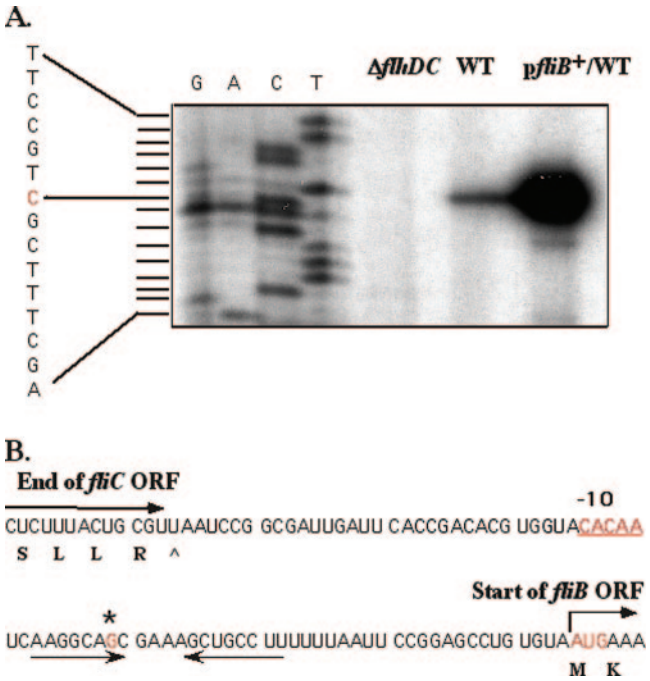


FIG. 3. A. Transcriptional start site mapping for the *S. enterica* serovar Typhimurium *fliB* gene. Lanes G, A, C, and T are the sequencing ladder. Primer extensions were measured in isogenic wild-type (WT), Δ*flhDC*, and p*fliB*$^{+}$ wild-type (p*fliB*$^{+}$/WT) strains. The base shown in red is the transcriptional start site. B. Sequence upstream of the *fliB* start codon is shown, and the transcriptional start site is marked by an asterisk. The putative flagellar class 2 promoter −10 sequence is shown in red. The inverted sequence marked by arrows overlapping the *fliB* transcriptional start site followed by a run of U's is the putative Rho-independent transcriptional terminator for the *fliC* transcript.

upstream of the *fliB* start codon that corresponded to this sequence was CACAAT, 44 bases upstream of the *fliB* coding region. We performed primer extension assays on RNA samples isolated from isogenic wild-type (LT2), Δ*flhDC* (TH2231), and p*fliB*$^{+}$/WT (pRP1/LT2) strains to locate a transcriptional start site for the *fliB* gene. The results of the primer extension analysis are shown in Fig. 3. The observed FlhDC-dependent start site maps to a guanine position (marked by an asterisk) that was located 9 bases downstream from the putative class 2 CACAAT (−10) sequence mentioned above. In addition, a polar Tn*10* insertion in *fliC* had no effect on *fliB-lac* expression (data not shown). These results confirm that *fliB* was transcribed independently from the *fliC* promoter from its own class 2 flagellar promoter.

Motility phenotypes of strains defective in the *srfB*, *yhjH*, *cafA*, and *fliB* loci. In the original array, the original slides had *cafA* clustered with the flagellar genes. It was also identified by another group using the original *Salmonella* array slides as a gene coregulated with the flagellar genes (48). Using the most recent set of *Salmonella* array slides, *cafA* did not show FlhDC-dependent regulation. The location of the *cafA* gene in an operon of other nonflagellar genes and lack of any potential flagellar promoter sequences supports that *cafA* is not a flagellar gene. We deleted the *cafA* and *yhjH* loci, and the D*cafA* and D*yhjH* mutant strains were tested for motility along with

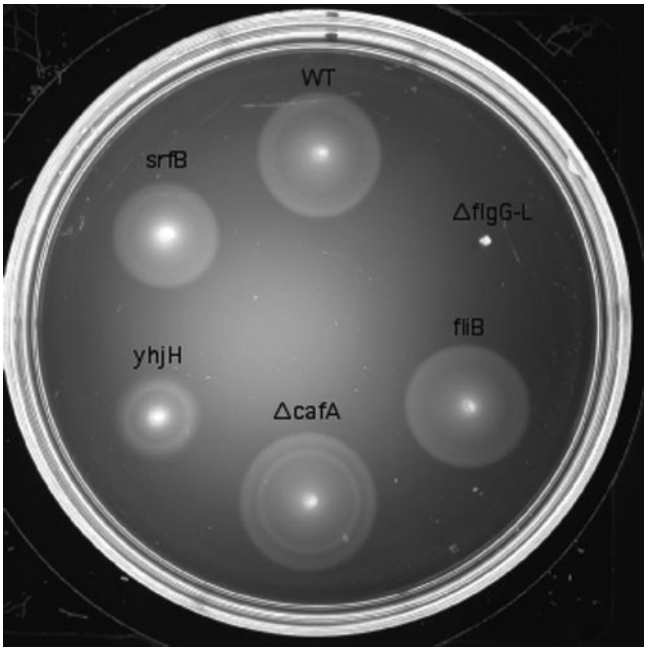


FIG. 4. Motility assays of novel flagellar genes. Null alleles in *fliB* (flagellin methylase), *cafA* (RNase G), *yhjH* (unknown), and *srfB* (unknown) were tested and compared to the wild-type strain LT2 (positive control) and a strain deleted for hook-basal body structural genes (Δ*flgG-L*).

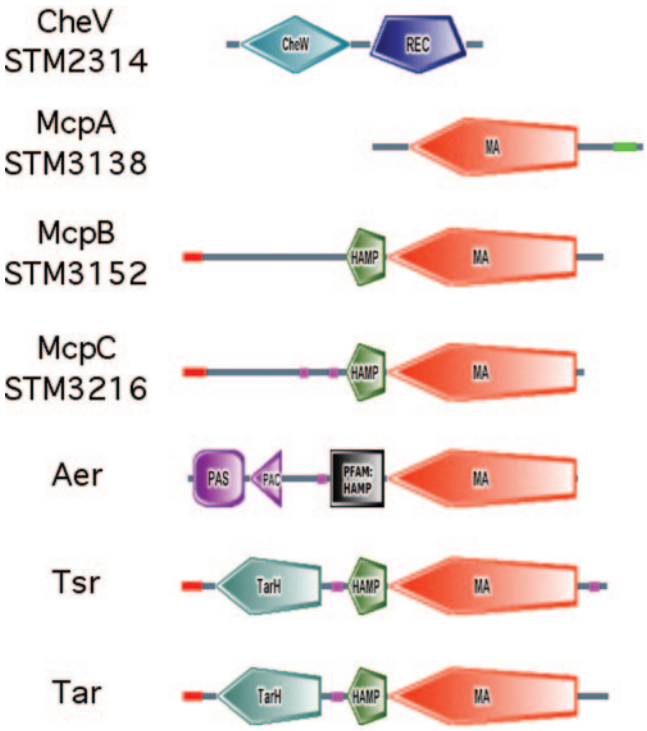


FIG. 5. Analysis of functional domains in new *Salmonella* flagellar genes *cheV*, *mcpA*, *mcpB*, *mcpC*, and *aer*. The functional domains were determined using the SMART domain-based sequence annotation resource (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) (31). The Tar and Tsr MCP chemoreceptors were included for comparison.

strains containing Mu*d-lac* null alleles of *srfB* or *fliB* (Fig. 4). The D*cafA* strain was unaffected in motility. However, a polar, null allele in *srfB* was also unaffected in motility, even though it was verified to be dependent on an FlhDC-dependent class 2 promoter for its expression. Loss of *fliB* was also not affected for motility. This is consistent with the idea that flagellin methylation by FliB is required for *Salmonella* virulence, not for flagellin function (K. Hughes and R. Curtiss III, unpublished data). The deletion of *yhjH* caused a motility defect, as had been reported previously (43). The *yhjH* gene product was annotated as a dicyclic GMP phosphodiesterase. The effect of *yhjH* on motility has yet to be determined.

DISCUSSION

In this study, we screened the *Salmonella* genome for novel genes that were coregulated with known flagellar genes using microarrays. The *Salmonella* flagellar genes clustered primarily according to whether they were expressed from class 2 or class 3 promoters. One additional operon, the *srfABC* operon, was also included in the flagellar class 2 gene cluster. The *srfC* locus encodes a homolog to an ADP ribosyltransferase that is secreted by the virulence-associated Hrp type III protein secretion system of *Pseudomonas syringae* pv. tomato DC3000 (40). The *srfABC* operon includes 3 of over 20 genes controlled by SsrAB, a two-component system located within *Salmonella* pathogenecity island 2 (49). Thus, the *srfABC* operon is thought to be associated with *Salmonella* virulence. The operon showed the strongest regulation of all virulence genes by FlhDC. A *srfB*::Mu*dJ* insertion exhibited normal motility on a soft agar plate, indicating that the *srf* operon is not required for flagellar construction.

We were surprised that several flagellar structural genes, the *flhBAE* genes, and the *fliE*, *fliJ*, and *fliOPQR* genes did not cluster with class 2 genes, even though they are known flagellar class 2 genes. It is noteworthy that all flagellar genes that showed the least dependence on FlhDC were at the 3′ end of operons (Table 2). This leads us to suspect that the mRNA of these operons was degraded from the 3′ end so the levels of 3′ gene mRNA was low. We are intrigued by the possibility that microarrays, if set up to be quantitative, can be used to study mRNA degradation pathways. This would be an important use for DNA microarrays in analyzing operon expression.

We were also surprised a little by the overlap between the FlhDC-dependent genes of *Salmonella* and those of *E. coli*. In *E. coli*, six nonflagellar operons were identified that require FlhDC for expression not picked up in this study of *Salmonella* FlhDC-dependent genes. These were genes required for cytoskelatin genes, *mreBCD*, and genes required for anaerobic respiration, *glpABC*, *napFAGHBC*, *nrfABCDEFG*, *dmsABC*, and *hydNhypF* (42). It is not clear why these were not picked up as FlhDC-dependent genes in *Salmonella* and may reflect the difference in lifestyles of the two organisms.

There are four new class 3 chemotaxis genes present in *Salmonella* that are not present in *E. coli*. These genes, formerly named STM2314, STM3138, STM3152, and STM3216,

are here renamed *cheV*, *mcpA*, *mcpB*, and *mcpC*, respectively. These genes clustered with the known class 3 flagellar genes. The structural motifs of these new genes are shown in Fig. 5 along with Aer and the well-characterized Tar and Tsr MCP receptors. The CheV protein is a fusion of CheW to a CheY receiver domain. McpA, McpB, McpC, and Aer all have a methyl-accepting signaling domain (MA domain [chemotaxis sensory transducer]) common to all MCP proteins. Aer of *Salmonella* is essentially the same as Aer from *E. coli* (data not shown). McpB and McpC are similar to Tar and Tsr, with a putative periplasmic ligand-binding domain flanked by transmembrane domains followed by a HAMP domain (histidine kinases, adenylyl cyclases, methyl binding proteins, and phosphatases) and the MA domain. McpA is unusual. McpA appears to have just a cytoplasmic MA domain followed by a coiled-coil region (green). Perhaps McpA interacts with the C-terminal domains of other Mcp receptors to influence their signaling. Expression analysis of Mu*d-lac* insertions in *cheV*, *mcpB*, and *mcpC* confirmed that they are under the control of σ^{28}-dependent promoters. The *mcpC* gene is in an apparent operon downstream of the *Salmonella mcp* gene required for aerotaxis, the *aer* gene. We also obtained Mu*d-lac* insertions in the previously uncharacterized *Salmonella aer* gene and verified that it was also expressed from a σ^{28}-dependent promoter.

The three novel MCPs not present in *E. coli* cluster to the same region of the *Salmonella* chromosome in the vicinity of the *metC* locus. This is one of the largest regions of uncharacterized genes in the *S. enterica* serovar Typhimurium genome (44), with a high density of *S. enterica* serovar Typhimurium-specific (STM) genes (36). The *mcpA* (STM3138) and *mcpB* (STM3152) loci are located only 12.6 kbp from each other, and *mcpC* (STM3216) is another 64.8 kbp clockwise of *mcpB* on the *S. enterica* serovar Typhimurium chromosome (36). Curiously, *mcpC* (STM3216) is in an operon with the aerotaxis MCP gene *aer*, which is present in both *S. enterica* serovar Typhimurium and *E. coli*. The location of *mcpA* immediately adjacent to 22 STM genes is suggestive that it may be part of a uncharacterized STM pathogenecity island, and future studies on a possible role of *cheV* and *mcpA*, *mcpB*, and *mcpC* in *S. enterica* serovar Typhimurium pathogenesis or survival as a commensal organism in *S. enterica* serovar Typhimurium hosts are warranted.

The *fliB* gene was identified as a class 2 gene, and primer extension analysis revealed the presence of an FlhDC-dependent promoter located between *fliC* and *fliB*. There is apparent overlap between the 3′-untranslated region of the *fliC* transcript and the promoter region of *fliB* (Fig. 3). The *fliB* transcriptional start site is located within the apparent Rho-independent termination signal for *fliC*, suggesting that transcription of *fliC* might interfere with that of *fliB*. However, a polar Tn*10* insertion in *fliC* did not affect expression of an *fliB-lac* reporter construct (data not shown). Expression of *fliB* from a class 2 promoter suggests that the FliB methylase must be temporally expressed before the *fliC* or *fljB* filament gene product on which it acts is produced. The exact role in flagellin methylation on *Salmonella* virulence has yet to be determined, but methylation of exposed lysine residues would remove the reactive positive charge of the lysine side group that could aid in avoiding the host immune response.

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