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Mutation of *flgM* Attenuates Virulence of *Salmonella typhimurium*, and Mutation of *fliA* Represses the Attenuated Phenotype

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***Salmonella typhimurium* ST39 exhibits reduced virulence in mice and decreased survival in mouse macrophages compared with the parent strain SL3201. Strain ST39 is nonmotile, carries an indeterminate deletion in and near the *flgB* operon, and is defective in the *mviS* (mouse virulence Salmonella) locus. In flagellum-defective strains, the *flgM* gene product of *S. typhimurium* negatively regulates flagellar genes by inhibiting the activity of FliA, the flagellin-specific sigma factor. In this study, *flgM* of wild-type *S. typhimurium* LT2 was found to complement the *mviS* defect in ST39 for virulence in mice and for enhanced survival in macrophages. Transduction of *flgM*::Tn*10*dCm into the parent strain SL3201 resulted in attenuation of mouse virulence and decreased survival in macrophages. However, a *flgM-fliA* double mutant was fully virulent in mice and survived in macrophages at wild-type levels. Thus, the absolute level of FliA activity appears to affect the virulence of *S. typhimurium* SL3201 in mice. DNA hybridization studies showed that *flgM*-related sequences were present in species other than *Salmonella typhimurium* and that sequences related to that of *fliA* were common among members of the family *Enterobacteriaceae*. Our results demonstrate that *flgM* and *fliA*, two genes previously shown to regulate flagellar operons, are also involved in the regulation of expression of virulence of *S. typhimurium* and that this system may not be unique to the genus *Salmonella*.**

A variety of mutations in *Salmonella typhimurium* that attenuate virulence in a mouse model of typhoid fever have been found (15). Virulent strains of *S. typhimurium* are lethal to mice and are capable of survival in peritoneal macrophages (14, 26). A nonmotile mutant strain of *S. typhimurium* (designated ST39), which carries an indeterminate deletion in and near the *flgB* operon (Δ*flg25*) and a defect in the *mviS* locus, fails to persist in murine resident peritoneal Ity^s macrophages and has reduced virulence in mice (44). Results of previous studies from this laboratory revealed that the plasmid pMH71, which contains ~6 kb of *S. typhimurium* DNA including *flgA*, part of the *flgB* operon (*flgBCDE*), and about 3.5 kb of adjacent sequences (6), complements the Δ*flg25* mutation in ST39 for virulence in mice without complementing for motility and that flagella are not required for virulence (6). These findings suggested that a gene(s) designated *mviS*, present on pMH71, is required for *S. typhimurium* to be fully pathogenic in the murine typhoid model.

Gillen and Hughes (17) recently published the DNA sequence of the *S. typhimurium flgM* gene, which encodes a negative regulator of flagellin synthesis and is in the same general region as *mviS*. Repression of expression of flagellin genes by *flgM* is seen only in strains that contain mutations in the basal body, switch, or hook flagellar genes (16). Conversely, flagellum synthesis is positively regulated by the flagellin-specific sigma factor encoded by *fliA* (24, 35). Ohnishi et al. (36) have described a novel regulatory mechanism of flagellum synthesis in *S. typhimurium* whereby FlgM acts as an anti-sigma factor to inhibit the activity of FliA by directly binding FliA.

In this study, we defined the *mviS* locus and showed that it is identical to the recently described *flgM* gene. A defect in *flgM* resulted in attenuation of *S. typhimurium* in the mouse model of typhoid fever. The attenuated phenotype of a *flgM* mutant was reversed by a mutation in *fliA*, which encodes the flagellin-specific sigma factor (35). DNA homology studies to determine the prevalence of the *flgM-fliA* system revealed that sequences at least moderately homologous to that of *flgM* of *S. typhimurium* were limited to *Salmonella* species and *Shigella* species, whereas *fliA*-related sequences were common among members of the family *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Salmonella* strains used in this study are described in Table 1. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was the cloning host. Plasmids constructed for this study are shown in Fig. 1. The cloning vectors used were pBR328 or derivatives of pBR328 since we found ColE1 replicons to be fairly stable in *Salmonella* strains. We constructed derivatives of pBR328 (pBRSK and pBRKS) which contain the promoter of β-galactosidase (P_{lac}), the T7 promoter (P_{T7}), and the multiple cloning sites from the Bluescript SK or KS vector (Stratagene). Briefly, the vectors were constructed by replacement of the 1,114-bp *Nru*I fragment of pBR328 with the 445-bp *Pvu*II fragment of pBluescript SK$^-$ or KS$^-$.

Media, enzymes, biochemicals, and radionuclides. Strains were routinely grown in L broth or brain heart infusion broth.

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TABLE 1. Bacterial strains used in this study

Salmonella strain	Relevant characteristics[a]	Source and/or reference
SL3201	Mouse virulent; FIRN biotype; Mot$^+$	B. A. D. Stocker (20)
ST39	SL3201 *flg25zcd-907*::Tn*10*; attenuated; Mot$^-$	7
JR501	r$^-$m$^+$	43
TH1886	*flgM*::Tn*10*dCm	K. Hughes
SL7395	SL3201 *flgM*::Tn*10*dCm; Mot$^+$	This study
TH1479	*fliA5059*::Tn*10*dTc; Mot$^-$	K. Hughes
SL3201::Tn*fliA*	*fliA5059*::Tn*10*dTc; Mot$^-$	This study
SL7395::Tn*fliA*	*fliA5059*::Tn*10*dTc; Mot$^-$	This study

[a] Mot, motility; FIRN, fimbriae, inositol, and rhamnose negative.

When necessary, the following antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to the medium at the indicated final concentrations (in micrograms per milliliter): ampicillin, 100; tetracycline, 10; and chloramphenicol, 15. Motility agar (10 g of tryptone, 5 g of NaCl, 5 g of agar [all per liter]) was used to test the motility phenotypes of the strains. Restriction endonucleases and calf intestinal alkaline phosphatase were purchased from Bethesda Research Laboratories; Boehringer Mannheim, Indianapolis, Ind.; or New England Biolabs, Beverly, Mass. T4 DNA ligase and Sequenase were obtained from the U.S. Biochemicals Corporation, Cleveland, Ohio. The GeneAmp PCR kit was purchased from Perkin-Elmer Cetus, Norwalk, Conn. Enzymes were used according to the manufacturers' instructions. Radionuclides were purchased from New England Nuclear Research Products, Boston, Mass.

DNA manipulations. All cloning procedures and plasmid manipulations were carried out with *E. coli* DH5α, essentially according to procedures described by Maniatis et al. (28). Plasmids to be tested were moved into ST39 by transformation (8) or electroporation (38). To bypass the restriction barrier between *E. coli* and *S. typhimurium* ST39, plasmids were passed through the r$^-$m$^+$ *S. typhimurium* strain JR501. Small-scale preparation of total DNA from 1.5-ml cultures of strains was done as described elsewhere (4). PCR was used to clone the coding sequences of *flgM* and ~150 bp of upstream DNA from pCKS150 (with the GeneAmp kit). Briefly, primers were designed to incorporate unique restriction sites into the ends of the amplified fragment which spanned from ~150 bp upstream of the start codon to a few base pairs downstream of the stop codon.

Nucleotide sequence analysis. The Sequenase kit from U.S. Biochemicals was used for nucleotide sequencing. M13mp18 and mp19 vectors were used for cloning and sequencing as described by Messing and coworkers (33). Overlapping fragments were obtained with the restriction endonucleases *Pvu*II, *Eco*RV, *Mlu*I, *Stu*I, *Fsp*I, and *Hin*dIII; when necessary, sites were made compatible by creating blunt ends (22, 28). The Genetics Computer Group program (11) was used for analysis of the DNA sequence.

DNA hybridization studies. Colonies were grown on nitrocellulose filters and processed as described previously (40). The filters were probed with nick-translated (29) *flgM*- or *fliA*-specific sequences. The *flgM* sequence corresponded to bases 129 to 455 in Fig. 2A. The *fliA* probe consisted of bases 489 to 1251 of the published sequence (35). Both probes contained the entire coding sequence of the genes, with only a small amount of upstream DNA. Total DNA was probed by in situ gel hybridization (23) with the following modifications. Gels were not prehybridized, and 20 mM sodium PP_i was included in the hybridization solution as a blocking agent. Hybridizations were done at 65°C, and washes were as follows for the filters: at high stringency, the two final washes were done at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–0.5% sodium dodecyl sulfate (SDS) for 1.5 and 0.5 h; at low stringency, all washes were at room temperature, with the final wash in 1× SSC–0.5% SDS for 0.5 h. Washing conditions for the gels at 65°C were as follows: at high stringency, one wash was done in 2× SSC for 1 h, followed by a second wash in 0.1× SSC for 0.5 h; at low stringency, two washes were done in 2× SSC for 1 and 0.5 h each.

Expression of open reading frames (ORFs). The cloning vectors pBRSK and pBRKS (described above) were constructed so as to contain a T7 promoter (P_{T7}) in the orientation opposite that of P_{lac}. These constructs allowed the direct expression of protein products from the plasmids used in the virulence complementation studies. The vectors were designed to place DNA fragments in each orientation with respect to P_{lac}, and this design also allowed each fragment to be placed under P_{T7} control. The recombinant plasmids were transformed into DH5α that contained the plasmid pGP1-2 (41). Proteins were selectively expressed from P_{T7} by thermal induction of T7 RNA polymerase from pGP1-2 and inhibition of the host RNA polymerase by rifampin (41). Proteins expressed in this system were pulse-labeled with [^{35}S]methionine, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and visualized by autoradiography.

Transductions. Bacteriophage P22HT *int* was used according to standard methods (10). Chloramphenicol-resistant colonies of SL3201 were selected following transduction of *flgM*::Tn*10*dCm from TH1886. Tetracycline-resistant colonies of SL3201 and SL7395 were selected following transduction of *fliA5059*::Tn*10*dTc from TH1479.

Assay for mouse virulence. Strains to be tested for virulence were grown at 37°C for 18 h in brain heart infusion broth, and titers were determined in sterile 0.9% NaCl. Dilutions were stored at 4°C overnight, and then the appropriate number of CFU were injected intraperitoneally into Ity^s C57BL/6J mice (6- to 8-week-old females; Jackson Laboratories, Bar Harbor, Maine). Approximately 700 CFU (equivalent to about 35 times the 50% lethal dose of SL3201 after intraperitoneal injection) was administered to each mouse per experiment. Mice were maintained on water that contained 5 g of ampicillin per liter when the test strain contained an Ap^r plasmid; the presence of the antibiotic was found to increase the stability of the plasmids in vivo. The number of viable bacteria present in the spleens of survivors was determined at about 3 weeks postinfection. In groups infected with virulent strains, a moribund animal was occasionally sacrificed for determination of CFU per spleen and confirmation that the infecting strain maintained the correct plasmid. Spleens were homogenized in sterile saline by using a stomacher and then diluted in sterile saline. To determine whether the plasmid had been lost from ST39 in vivo, homogenate dilutions were spread onto Luria

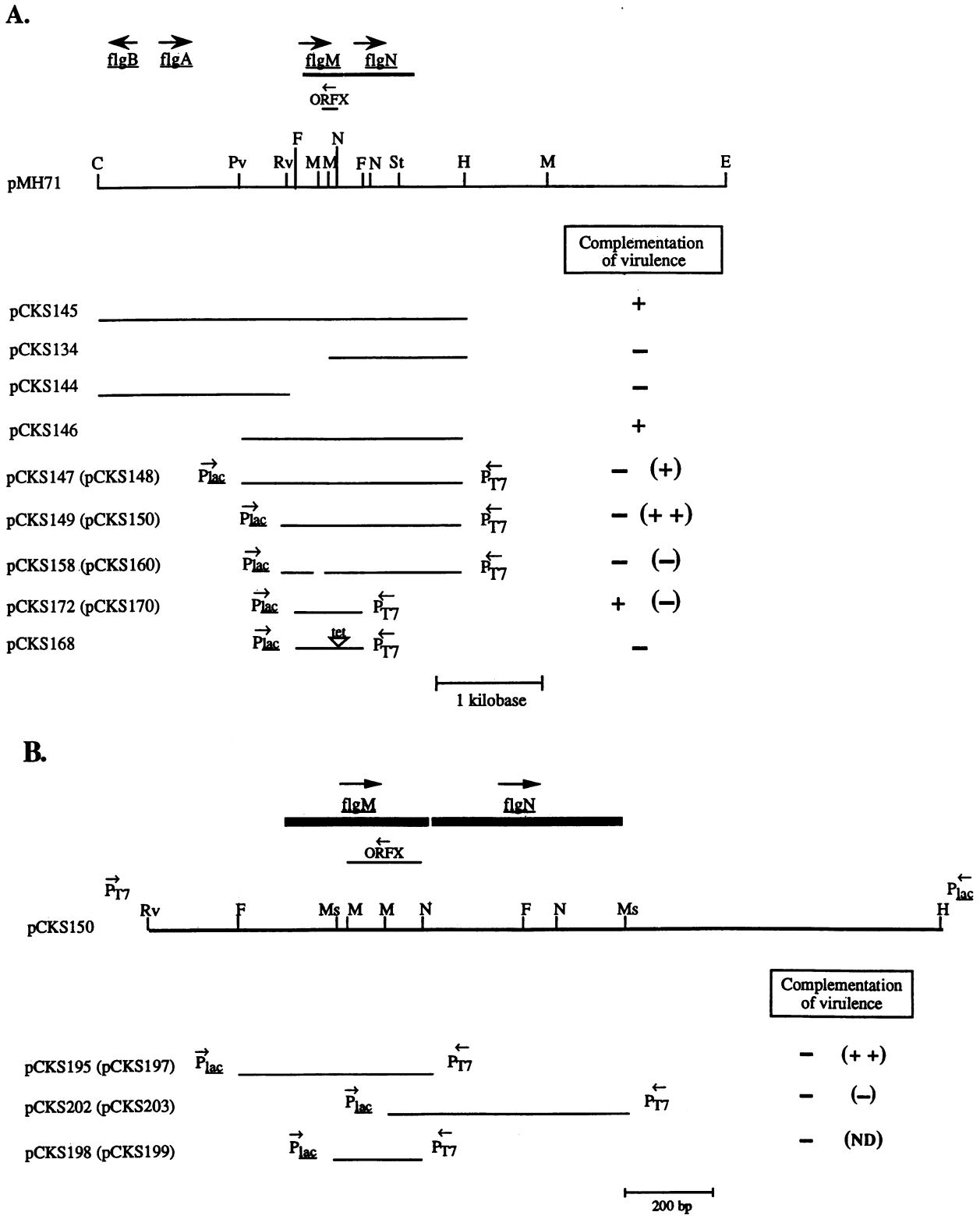

FIG. 1. Restriction map of the *mviS* region and subclones. Solid lines indicate *S. typhimurium* DNA present on plasmids listed to the left. The approximate locations of *flgM*, *flgN*, ORFX, *flgA*, and the *flgB* operon are shown above the map of pMH71 (A) or pCKS150 (B). Arrows indicate the direction of transcription. The position of, and direction of transcription from, P_{lac} and P_{T7} are shown where relevant. Parentheses indicate plasmids with inserts in the orientation opposite of that shown. Complementation of virulence in the mouse model is shown on the right: +, partial; −, none; ++, full; ND, not determined. Abbreviations for restriction sites: C, *Cla*I; E, *Eco*RI; F, *Fsp*I; H, *Hin*dIII; M, *Mlu*I; Ms, *Msp*I; N, *Nru*I; Pv, *Pvu*II; Rv, *Eco*RV; St, *Stu*I.

broth plates that contained ampicillin or no antibiotic. ST39 strains that contained plasmids to be analyzed for complementation of virulence were tested in at least three experiments. Strains containing transposon insertions in *flgM* and/or *fliA* were tested in two or more experiments. Ity^s BALB/cπ mice (Organon Teknika, Rockville, Md.) were a kind gift from Beverly Mock and Michael Potter. Animal experiments were carried out according to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* (32).

In vitro assay for survival in macrophages. The in vitro assay was done essentially as described by Lissner et al. (26) and modified by Weinstein et al. (44) with the following exceptions: ~8×10^5 macrophages were seeded per well, a multiplicity of infection of ~2.5 to 5 opsonized bacteria per adherent cell was used, and macrophages were lysed with 0.01% bovine serum albumin. Three or four infected wells were used for enumeration of CFU, and two infected wells were used for enumeration of macrophages. All possible combinations of wells for bacterial counts and wells for macrophage counts were then used for the determination of CFU/macrophage ratios.

Resistance to protamine. Approximately 10^4 log-phase bacteria were incubated with equal volumes of dilutions of protamine sulfate (ranging from 0 to 800 μg/ml) at 37°C for 1 h and plated for survival as described by Groisman et al. (18).

Nucleotide sequence accession number. The DNA sequence presented here has been assigned GenBank number U03631.

RESULTS

Complementation of virulence. Initial virulence studies (data not shown) with derivatives of pMH7, the plasmid from which pMH71 was constructed (6), suggested that the region necessary for virulence (the *mviS* locus) lies within the 3.5-kb *Cla*I-*Hin*dIII fragment (Fig. 1A). To more precisely identify the *mviS* locus, several subclones of pMH71 were constructed (Fig. 1A and B). Each plasmid was tested in the Δ*flg25* background of the attenuated *S. typhimurium* strain ST39 for complementation of virulence in mice. The control strains used were ST39/pBRSK (avirulent) and ST39/pMH71 (virulent). Full complementation was defined as lethality in most mice infected. Partial complementation was defined as lethality in a few of the mice infected, increased numbers of organisms in the spleen, and/or enlarged spleens. Initial experiments identified the virulence-complementing clone pCKS150, which contains the 1.7-kb *Eco*RV-*Hin*dIII fragment from pMH71 (results summarized in Fig. 1A).

Concurrently with these virulence studies, we determined the DNA sequence of the entire 2-kb *Pvu*II-*Hin*dIII fragment which spans the virulence-complementing region. Several potential ORFs were identified, including the *flgM* gene (relevant sequences are shown in Fig. 2A); the DNA sequence of *flgM* was published recently by Gillen and Hughes (17). We identified another ORF, designated *flgN*, which begins just 5 bp downstream of the termination codon for *flgM* (Fig. 2A). The DNA sequence of these genes suggested that *flgM* and *flgN* compose an operon. Gillen and Hughes (17) reported that FlgM shared significant homology with an ORF downstream of the *Yersinia enterocolitica invA* gene (Fig. 2B; 60% identity and 93% similarity over 60 amino acids). Similarly, analysis of the FlgN sequence revealed 45% identity and 80% similarity over a 114-amino-acid overlap with an ORF downstream of the *Yersinia pseudotuberculosis inv* gene, for which only part of the DNA sequence has been published (Fig. 2C).

We used our sequence information to construct several additional subclones and tested these plasmids in ST39 for complementation of virulence. Complementation was ablated by the introduction of a 100-bp *Mlu*I deletion into the 1.7-kb *Eco*RV-*Hin*dIII fragment of pCKS150 (compare pCKS160 to pCKS150 in Fig. 1A). This deletion was internal to *flgM* and resulted in a frameshift mutation at codon 28 with subsequent termination after 8 additional codons; however, this early termination may also have been polar on the adjacent *flgN* gene. Partial complementation was seen with pCKS172 (655-bp *Fsp*I fragment), which contains 160 bp upstream of *flgM*, all of *flgM*, and 60 of 140 codons of the adjacent downstream gene *flgN*, in the same transcriptional direction as P_{lac}. Complementation was abolished by the insertion of a tetracycline resistance cassette into the *flgM* coding region (pCKS168). Although the complementation data suggested that *flgM* is the only gene required to complement the virulence defect, involvement of *flgN* could not be discounted because this ORF is completely or partially present on all complementing clones. There is also a small ORF within the coding region of *flgM* (ORFX), albeit on the opposite strand, that is deleted in pCKS160 but present in pCKS172.

To determine definitively which of the three genes (*flgM*, *flgN*, or ORFX) was necessary for complementation of virulence, additional plasmids that contained *flgM* (plus ORFX), *flgN*, or ORFX were constructed. The inserts of these plasmids are shown in Fig. 1B in relationship to the virulence-complementing plasmid pCKS150. Infection of mice with ST39 that contained plasmids encoding only *flgN* or the potential ORFX (pCKS202, pCKS203, or pCKS198) did not result in the deaths of any mice, nor did the infections result in significant splenomegaly (results summarized in Fig. 1B). The inserts in plasmids pCKS195 and pCKS197 were generated by PCR and carried the entire *flgM* coding sequence and ~150 bp of upstream DNA but none of the *flgN* gene. Plasmid pCKS197 fully complemented the virulence defect in strain ST39 (Fig. 1B). The results of three combined experiments with the *flgM* clones pCKS195 and pCKS197 are presented in Table 2. The positive (pMH71) and negative (pBRSK) controls gave expected results. One of the *flgM* plasmids, pCKS197, gave results that were virtually identical to those seen with the positive control plasmid, pMH71. Surprisingly, of the four plasmids found to complement ST39 for virulence, three contained *flgM* in the orientation opposite that of P_{lac} (pCKS148, pCKS150, and pCKS197), while one contained *flgM* in the same orientation as P_{lac} (pCKS172).

Production of protein products with a T7 expression system. The proteins expressed from the T7 promoter (P_{T7}) present on the plasmids are shown in Fig. 3. As expected, no specific products were seen in the sample from the strain that contained only the plasmid encoding the T7 RNA polymerase, pGP1-2 (data not shown). Also, the vectors directed the expression of only one protein of about 29 kDa, which was probably β-lactamase (Fig. 3, lanes 9 and 10). Plasmids that contained the coding sequences of *flgM* under the control of P_{T7} showed a protein of about 8 kDa (lanes 2, 3, and 6), a molecular mass that is similar to those in published reports (17, 36). Plasmids that contained *flgN* under the control of P_{T7} showed a protein of about 14 kDa (lanes 2 and 8). Both FlgM and FlgN were expressed from pCKS150 (lane 2). The plasmid pCKS149 directed the production of a protein slightly larger than FlgN. This protein produced by pCKS149 is probably due to expression of an ORF on the strand opposite that of *flgM* and *flgN*. The constructs that contained the putative ORFX under the control of P_{T7} did not direct the production of a protein product (data not shown). These results revealed that all plasmids capable of conferring a virulent phenotype upon ST39 also contained the *flgM* gene. In particular, two pairs of

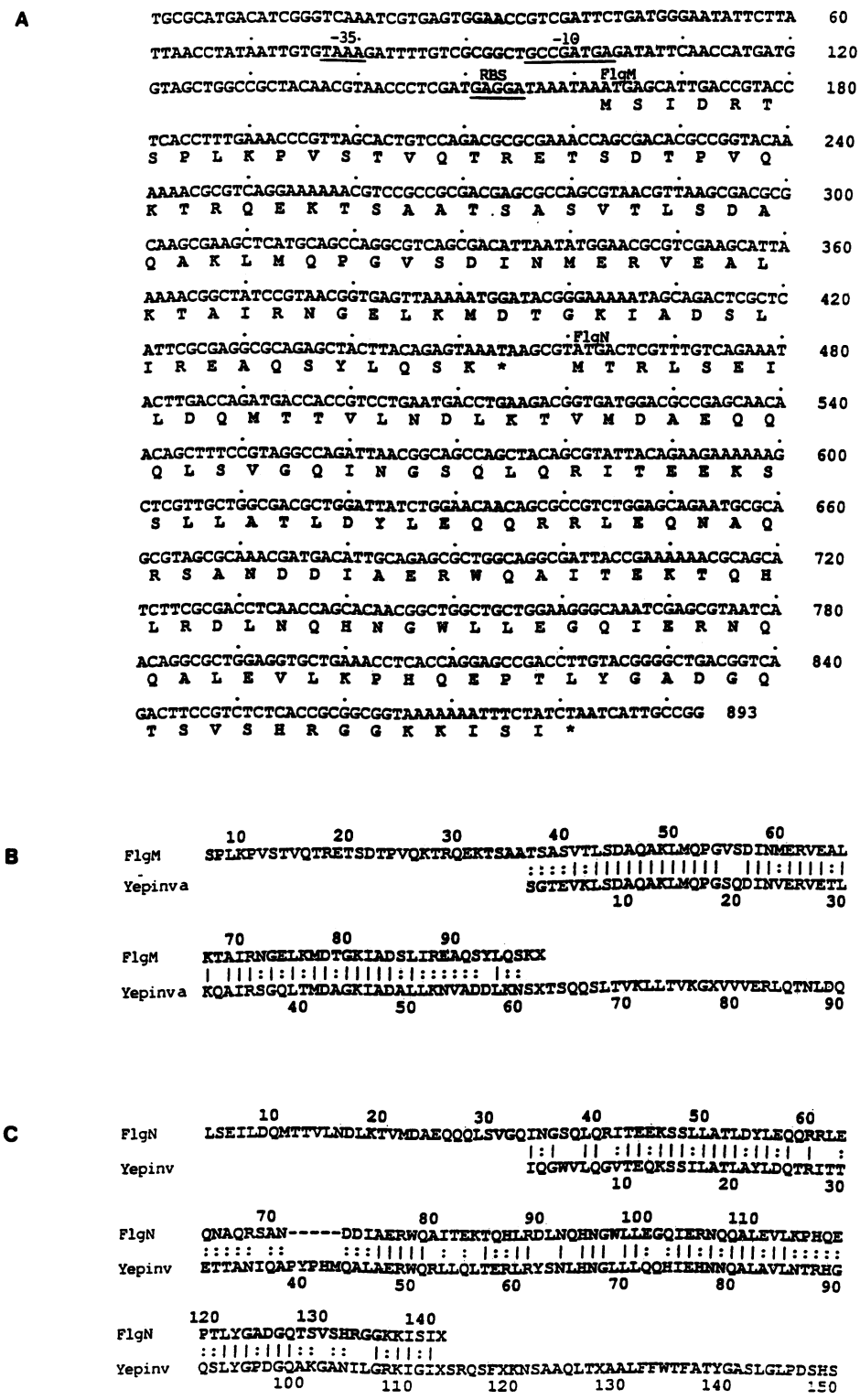

FIG. 2. (A) DNA sequence of the *flgM* and *flgN* genes. Putative transcriptional and translational elements are underlined. The deduced amino acid sequences of FlgM and FlgN are shown below the DNA sequence. (B and C) Deduced amino acid homologies of FlgM or FlgN with partial ORFs downstream of *Y. enterocolitica invA* designated Yepinva (B), or *Y. pseudotuberculosis inv*, designated Yepinv (C). Vertical lines indicate identity, and colons indicate similarity.

TABLE 2. Complementation of virulence by subclones of pMH71

Plasmid in ST39	No. of dead mice/ no. infected[a]	Mean no. of days to death	Relative spleen size[b]	CFU/ spleen[c]
pCKS195	0/15	NA[d]	+	2.5 ± 0.5
pCKS197	11/15	10	+++	7.2 ± 0.8
pMH71	12/14	9	+++	7.6 ± 0.7
pBRSK	0/15	NA	+	3.1 ± 0.3

[a] Mice were infected intraperitoneally with about 700 CFU. Moribund mice sacrificed for CFU enumeration in the spleen were included as dead.

[b] Surviving mice were sacrificed 3 weeks postinfection and the average spleen size was scored according to the following scale: +, <0.25 g; ++, 0.25 to 0.35 g; +++, 0.4 to 0.7 g.

[c] CFU were determined for each spleen. A value of 10^8 bacteria per spleen was assigned to dead mice for statistical purposes. The geometric mean ($\log_{10}$) ± the 95% confidence interval is shown for each group. The limit of detection was 100 CFU per spleen.

[d] NA, not applicable.

plasmids, pCKS170 and -172 and pCKS195 and -197, directed the expression of only one protein, FlgM, while the plasmid pair pCKS202 and pCKS203, which encode and are capable of expressing only *flgN*, failed to confer the virulent phenotype. These results provide further evidence that *flgM* alone is sufficient to confer the virulent phenotype on strain ST39.

Survival in mouse macrophages. Resident peritoneal macrophages from C57BL/6J mice were infected in vitro with ST39 containing one of the following plasmids: pCKS150, pCKS172, pMH71 (positive control), and pBR328 (negative control). Initial studies showed that the strains that contained pCKS150, pCKS172, or pMH71 displayed enhanced survival in resident peritoneal macrophages (data not shown). The strains ST39/pCKS150 and ST39/pCKS172 did not undergo as much net growth as ST39/pMH71, but these strains clearly survived better than ST39/pBR328. The in vitro macrophage assay was also done with ST39 containing one of the following plasmids: pCKS195, pCKS197, pMH71, and pBRSK. Infection of C57BL/6J macrophages with ST39 containing pCKS195, pCKS197, or pMH71 resulted in significantly higher bacterial counts per macrophage compared with ST39 containing the vector control, pBRSK (Fig. 4A). The largest difference was seen between 0 and 4 h after infection (between T_0 and T_4); the CFU of strain ST39/pBRSK per macrophage decreased approximately 70-fold, while those of the other strains dropped 10- to 18-fold. This difference in total decrease was maintained through 24 h postinfection (T_{24}), when the ratio of CFU of

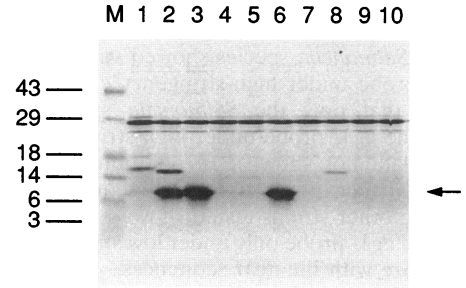

FIG. 3. Expression of protein products by T7 RNA polymerase. Samples were electrophoresed through a 15% polyacrylamide–SDS gel. Proteins produced from plasmids are shown. Lanes: 1, pCKS149; 2, pCKS150; 3, pCKS170; 4, pCKS172; 5, pCKS195; 6, pCKS197; 7, pCKS202; 8, pCKS203; 9, pBRSK; 10, pBRKS. M, molecular mass markers; masses are listed to the left in kilodaltons. The arrow points to the *flgM* gene product.

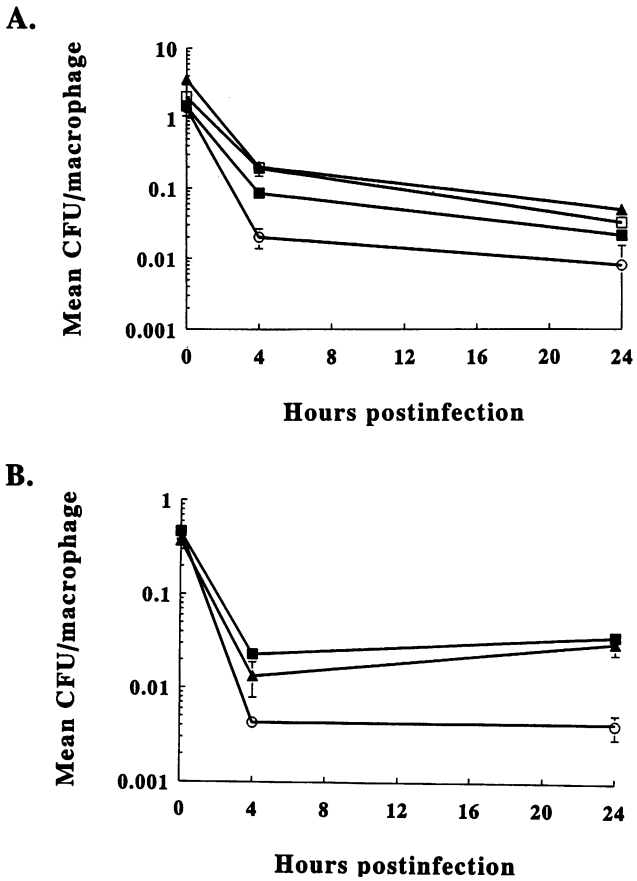

FIG. 4. Resident peritoneal macrophages were infected in vitro with the attenuated Δ*flg25* strain ST39, which contained one of the following plasmids: pCKS195 (□), pCKS197 (■), pMH71 (▲), and pBRSK (○). The numbers of viable bacteria and macrophages per well were determined at 0, 4, and 24 h postinfection. The ratios of bacteria per C57BL/6J macrophage (A) or BALB/cπ macrophage (B) ± the 95% confidence interval for each time point are shown. For panel A, the ratios were as follows for strains ST39/pCKS195, ST39/pCKS197, ST39/pMH71, and ST39/pBKSK, respectively: T_0, 2.0 ± 0.95, 1.5 ± 0.28, 3.6 ± 0.78, and 1.4 ± 0.33; T_4, 0.2 ± 0.034, 0.085 ± 0.0069, 0.20 ± 0.055, and 0.020 ± 0.0063; and T_{24}, and 0.032 ± 0.011, 0.021 ± 0.0036, 0.049 ± 0.0040, and 0.008 ± 0.0072. For panel B, the ratios at various time points were as follows for strains ST39/pCKS197, ST39/pMH71, and ST39/pBKSK, respectively: T_0, 0.46 ± 0.090, 0.37 ± 0.035, and 0.47 ± 0.083; T_4, 0.023 ± 0.0033, 0.013 ± 0.0056, and 0.0043 ± 0.00065; and T_{24}, 0.037 ± 0.0044, 0.031 ± 0.0078, and 0.00412 ± 0.0011.

ST39/pBRSK per macrophage had decreased 175-fold while those of the other strains had decreased about 70-fold. The macrophage assay was also done with macrophages from another Ity^s mouse strain, BALB/cπ. The general patterns of survival were similar to those seen with C57BL/6J macrophages except that the strains ST39/pCKS197 and ST39/pMH71 showed a slight increase between T_4 and T_{24} (Fig. 4B). The net decreases in CFU per macrophage were 114-fold for ST39/pBRSK and about 13-fold for ST39/pCKS197 and ST39/pMH71. The magnitude of the differences between the complemented strain and the *flgM* mutant as demonstrated on the log scale is consistent with the differences seen by others using this macrophage assay to demonstrate the poor survival of *Salmonella* mutants in macrophages.

Virulence of strains containing transposon insertions in

TABLE 3. Virulence of *flgM* and/or *fliA* mutants of *S. typhimurium*[a]

Strain	No. of dead mice/ no. infected[b]	Mean no. of days to death	Relative spleen size[c]	CFU/spleen[d]
SL3201 (*flgM*$^+$)	12/15	9	+	7.2 ± 1.0
SL7395 (*flgM*::Tn*10*dCm)	1/15	13	+	3.1 ± 0.8
SL7395/pCKS197	13/15	8	+++	7.4 ± 0.9
SL7395/pBRSK	0/5	NA[e]	+	3.0 ± 0.7
SL3201*fliA*	15/15	7	NA	8.1 ± 0.1
SL7395*fliA*	15/15	7	NA	8.0 ± 0.1

[a] Data are the combination of two or more experiments, except in the case of SL7395/pBRSK.
[b] Mice were infected intraperitoneally with about 700 CFU. Moribund mice sacrificed for CFU enumeration in the spleen were included as dead.
[c] Surviving mice were sacrificed 3 weeks postinfection and the average spleen size was scored according to the following scale: +, <0.25 g; ++, 0.25 to 0.35 g; +++, 0.4 to 0.7 g.
[d] CFU were determined for each spleen. A value of 10^8 bacteria per spleen was assigned to dead mice for statistical purposes. The geometric mean ($\log_{10}$) ± the 95% confidence interval is shown for each group. The limit of detection was 100 CFU per spleen.
[e] NA, not applicable.

***flgM* and/or *fliA*.** To confirm that the *flgM* gene was necessary for virulence, we created a defined mutation in *flgM* by transduction of *flgM*::Tn*10*dCm into the parental background of SL3201. Because of the reported interaction between FlgM and FliA (36), we also generated two strains that contained a mutation in *fliA* (*fliA5059*::Tn*10*dTc). This set of strains was tested for virulence in the mouse model. As expected, the *flgM* strain SL7395 was attenuated and the *flgM* plasmid pCKS197 complemented SL7395 for virulence in mice (Table 3). The *fliA* mutation alone had no effect on virulence (SL3201*fliA*). However, the presence of the *fliA* mutation in the *flgM* strain SL7395 reversed the attenuated phenotype (Table 3).

Macrophage survival of *flgM* or *fliA* transposon mutants. The following strains were tested for survival in Ity^s BALB/cπ macrophages: SL3201 (*flgM*$^+$ *fliA*$^+$), SL3201*fliA* (*flgM*$^+$ *fliA*), SL7395 (*flgM fliA*$^+$), and SL7395*fliA* (*flgM fliA*). All four strains displayed similar growth patterns in rich medium (data not shown). Significantly lower levels of viable strain SL7395 bacteria were isolated at T_4 in comparison with SL3201, but not at T_{24} (Fig. 5). The double mutant SL7395*fliA* persisted at a level equal to that of SL3201, which was consistent with the in vivo results presented above. All four strains showed a decrease of 11- to 26-fold between T_0 and T_4, a slight increase of 2- to 5-fold from T_4 to T_{24}, and a net decrease of 3- to 7-fold by T_{24}. A similar pattern of survival was seen with macrophages from C57BL/6J mice (data not shown).

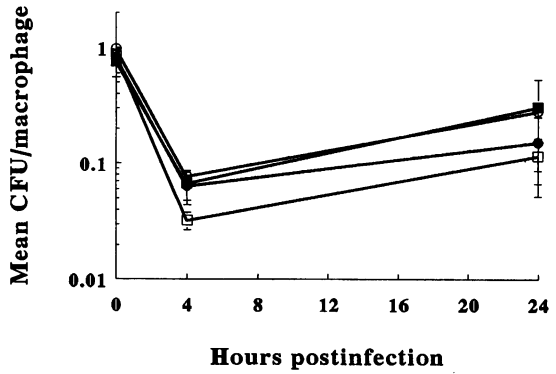

FIG. 5. Resident peritoneal BALB/cπ macrophages were infected in vitro with strains SL3201 (○), SL3201*fliA* (●), SL7395 (□), and SL7395*fliA* (■). The number of viable bacteria and macrophages per well were determined at 0, 4, and 24 h postinfection. The ratios of bacteria per macrophage ± the 95% confidence interval were as follows for strains SL3201, SL3201*fliA*, SL7395, and SL7395*fliA*, respectively: T_0, 0.97 ± 0.14, 0.82 ± 0.098, 0.85 ± 0.12, and 0.76 ± 0.21; T_4, 0.078 ± 0.0078, 0.064 ± 0.020, 0.032 ± 0.0056, and 0.067 ± 0.020; and T_{24}, 0.28 ± 0.035, 0.15 ± 0.10, 0.12 ± 0.050, and 0.31 ± 0.22.

Resistance to protamine. As an in vitro correlate to the assay of survival in macrophages, strains SL3201, SL3201*fliA*, SL7395, SL7395*fliA*, and DH5α were tested for resistance to protamine, a small, cationic antimicrobial peptide. These experiments were undertaken because resistance of *Salmonella* strains to antimicrobial peptides is correlated with survival of the organisms in macrophages and virulence in mice (13). In this study, the four *Salmonella* strains showed a similar resistance to protamine sulfate (partial resistance to 100 μg of protamine sulfate per ml) while DH5α was exquisitely sensitive to the compound. Thus, the reason for attenuation of the *flgM* mutants in vitro and in vivo is not that they display increased sensitivity to antimicrobial peptides.

Search for *flgM* and *fliA* analogs in other species. To determine whether an analog of *flgM* or *fliA* has been conserved at the level of DNA homology, other species of *Salmonella* were probed for the genes. We were particularly interested in *Salmonella* strains that are not highly virulent in the mouse model, i.e., *Salmonella typhi* (21, 34), *Salmonella gallinarum* (9), and *Salmonella pullorum* (9). In addition, representatives of other members of the family *Enterobacteriaceae* were analyzed, and these were as follows: *Shigella* species, *Vibrio* species, and enterohemorrhagic *E. coli*. *Pseudomonas aeruginosa* was also tested, as was the gram-positive organism *Staphylococcus aureus*. *P. aeruginosa* was chosen because the organism is known to contain a *fliA* gene (39). All species tested showed moderate hybridization with the *fliA* probe under low-stringency conditions, except *S. aureus*. However, only the *Salmonella* species showed strong hybridization with the *fliA* probe under high-stringency conditions. Colony blots showed that only the *Salmonella* species hybridized strongly with the *flgM* probe under high-stringency conditions (colony blot data not shown). In addition, *Shigella flexneri* showed weak hybridization with the *flgM* sequences under high stringency. The other strains tested showed very weak hybridization with the *flgM* probe only under low stringency. *S. aureus* did not hybridize with the *flgM* sequences.

Total DNA from the *Salmonella* species, several *S. flexneri* isolates, *Shigella dysenteriae* 3818T, and *E. coli* DH5α was examined by in situ gel hybridization with the *flgM* probe. All of the salmonellae contained an *Eco*RI fragment of about 9.5 kb that hybridized with the *flgM* probe (Fig. 6A). The *S. flexneri* strains showed a band of about 6.5 kb that hybridized with the *flgM* probe, while a band of about 7.5 kb from *S. dysenteriae*

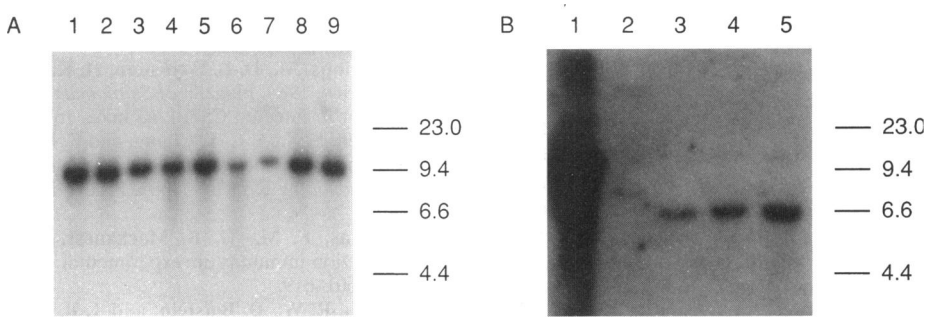

FIG. 6. In situ gel hybridization of *Salmonella* and *Shigella* isolates. Total DNAs were digested with *Eco*RI and hybridized with a probe internal to the *flgM* coding sequences. Washing was at high stringency for panel A and at low stringency for panel B. (A) *Salmonella* species. Lane 1, *S. typhimurium* SL3201; lane 2, *S. typhimurium* TML; lane 3, *S. typhi* TY-2; lane 4, *Salmonella dublin*; lane 5, *Salmonella darby*; lane 6, *S. gallinarum*; lane 7, *Salmonella choleraesuis* 2822; lane 8, *S. choleraesuis* 2863; lane 9, *S. pullorum* 2922. (B) Lane 1, *S. dublin*; lane 2, *S. dysenteriae* 3818T; lane 3, *S. flexneri* 2457; lane 4, *S. flexneri* 4243; lane 5, *S. flexneri* SA101.

hybridized with the probe (Fig. 6B). *E. coli* DH5α also contained a band of approximately 6.5 kb that hybridized with the *flgM* probe (data not shown).

DISCUSSION

FlgM has been shown to be a repressor of flagellin synthesis in strains that are defective in the basal body, hook, or switch genes (16). Ohnishi et al. (36) recently described the activity of FlgM as an anti-sigma factor responsible for inhibition of the flagellum-specific sigma factor FliA (σ^{28}) in strains that contain a defect in the flagellum synthesis pathway. Our studies show that strains that contain the Δ*flg25* mutation are attenuated because of the lack of a functional *flgM* gene. Furthermore, our results indicate that FlgM and FliA normally interact even in Fla$^+$ backgrounds, because the Fla$^+$ (motile) *flgM* mutant SL7395 was attenuated. The *flgM* gene was also found to be required by strain ST39 for enhanced survival in mouse peritoneal macrophages. These results were confirmed by the introduction of a *flgM* mutation into the wild-type parent strain SL3201, which resulted in attenuation.

Although our results indicate that the *flgM* gene is essential for complementation, we found that the orientation of the cloned *flgM*$^+$ fragments in the vectors was an important determinant of complementation. Only one of the *flgM*$^+$ plasmids from each pair of plasmids constructed was found to complement, and the orientations of these complementing plasmids were not consistent. The complementing plasmids pCKS150 and pCKS197 contained *flgM* in the orientation opposite that of P_{lac}, while pCKS172 carried *flgM* in the same orientation as P_{lac}. Several possible explanations may account for this inconsistency: (i) the virulent phenotype may be dependent on normal, wild-type regulation of *flgM* and transcription from P_{lac} may interfere with *flgM* expression; (ii) normal regulation of *flgM* may be sensitive to the context within the vector; and (iii) the different amounts of flanking DNA present in the various *flgM*$^+$ clones may have contained *cis*-acting elements required for normal regulation of *flgM* or for stability of the *flgM* transcript.

Because of the interaction between FlgM and FliA, we tested *fliA* mutants for virulence in mice. Although the *fliA* mutation alone had no effect on virulence, mutation of *fliA* was found to reverse the attenuated phenotype of a *flgM* mutant. These results suggest that the effect of *flgM* on virulence is indirect and that the sigma factor FliA may regulate or modulate the expression of genes involved in virulence. Although the mechanism through which FliA exerts its effects is unknown, we propose three models that describe how FlgM and FliA may function to influence expression of genes associated with virulence. First, FliA may positively regulate a gene(s) that interferes with virulence when the gene is overexpressed because of the increased FliA activity thought to be present in the absence of FlgM. Thus, the attenuated phenotype of a *flgM* mutant may be a consequence of overexpression of a FliA-regulated gene whose expression is normally modulated in a *flgM*$^+$ background. Second, FliA may positively regulate a repressor of a virulence gene. Increased FliA activity in a *flgM* mutant strain would result in elevated repressor levels and thus reduced expression of the virulence gene. Third, FliA could act as both an activator of flagellar genes and a repressor of a gene(s) associated with virulence. An example of this type of regulation is the putative sigma factor encoded by *katF* of *E. coli*. KatF appears to positively regulate expression of $bolAp_1$, a gene involved in controlling *E. coli* septum formation, while negatively regulating an operon encoding the peptide antibiotic microcin B17 (5).

In all three models, FlgM would be required to modulate the activity of FliA to balance the expression levels of the target gene(s). Miller and Mekalanos (31) found that balanced levels of the regulator PhoP are important for virulence. Constitutive expression of *phoP* results in attenuation of *Salmonella* strains and decreased survival in mouse macrophages. It is not clear whether the attenuation of these mutants is due to the overexpression of *phoP*-activated genes (*pag* genes) or the reduced expression of other genes (*prg* genes) (31). Consistent with the models we have proposed, overexpression of *fliA* by a plasmid resulted in attenuation of the wild-type strain (zero of five mice died; data not shown). However, these results were confounded by the observation that SL3201 containing the *fliA*$^+$ plasmid displayed a slower growth rate in vitro compared with the vector control, and a slower growth rate alone could cause *Salmonella* strains to be attenuated in vivo.

The data presented here reveal a clear link between regulation of flagellar synthesis and virulence and suggest that the flagellar and virulence systems may share regulatory features. Fields et al. (14) found that a high percentage (8 of 83) of macrophage-sensitive transposon mutants of *S. typhimurium* were nonmotile. However, subsequent studies by us and others showed that flagella per se are not required for virulence (6, 27). Consistent with this finding was our observation that the nonmotile *fliA* mutant of SL3201 (as well as ST39 complemented by a *flgM*$^+$ plasmid) is virulent. Thus, the virulence-associated target gene(s) for FlgM and FliA regulation appears to affect virulence in a manner independent of flagellum

synthesis. There is at least one other example of the involvement of an alternate sigma factor in virulence: the *katF* gene encodes a sigma factor that has been implicated in the virulence of *S. typhimurium* (12).

The presence of flagellar genes that are controlled by a σ^{28}-like promoter is not limited to *Salmonella* strains (19). *E. coli* (3), *P. aeruginosa* (42), *Caulobacter crescentus* (37), *Bacillus subtilis* (25), and *Vibrio parahaemolyticus* (30) also contain flagellar genes with σ^{28}-like promoters. Similarly, several different genera of bacteria have virulence-associated genes that may also be controlled by a σ^{28}-like sigma factor. The *lcrD* gene of *Yersinia pestis* contains a σ^{28}-like promoter (2), as does the *lcrD* homolog of *Shigella flexneri mxiA* (2). The *flaA* gene of *Bordetella bronchiseptica* also contains a σ^{28}-like promoter (1). Since the BvgAS system coordinately regulates virulence genes of *Bordetella* and has recently been shown to regulate the flagellar gene *flaA* in *B. bronchiseptica*, Akerley and Miller have also proposed a relationship between motility and virulence in *B. bronchiseptica* (1). They hypothesize that BvgAS acts to regulate *flaA* through an analog of the sigma factor FliA (σ^{28}). The above-mentioned studies, combined with our findings, suggest that dissimilar organisms which contain a *fliA* analog may also contain a *flgM* analog even if the bacteria are nonmotile. In fact, we found sequences in nonmotile *Shigella* strains that were homologous to *flgM*. Moreover, we and others (17) found a high degree of similarity between FlgM and a partially sequenced ORF in *Y. enterocolitica*. We also found similarity between FlgN and a partial ORF in *Y. pseudotuberculosis*. Since *flgN* is downstream of *flgM*, these two findings suggested that a FlgM analog may be present in *Yersinia* species, as well as *fliA*-specific promoters (2). The FlgM-FliA regulatory system may act to control diverse functions composed of flagellar and virulence-associated genes so that each class is expressed at the appropriate time. We are currently searching for *fliA*-regulated genes that are associated with virulence.

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