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A *Salmonella typhimurium* Virulence Gene Linked to *flg*

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Isogenic pairs of strains of *Salmonella typhimurium* which differed only in whether or not they were flagellate were found to be equally virulent in C57BL/6J mice infected orally, intravenously, or intraperitoneally. Therefore, we investigated the genetic basis for our previous observation that in this mouse model, nonflagellate Δ*flgABCDE25* strains were reduced in virulence compared with isogenic wild-type flagellate strains. The recombinant plasmid pMH6, which contains several *flg*$^+$ genes and a segment of the *S. typhimurium* chromosome adjacent to the *flg* genes, was introduced into a Δ*flgABCDE25* mutant. This restored virulence in mice challenged intraperitoneally, which suggested that a virulence gene occurs adjacent to the *flg* genes. When plasmid pMH64, which lacks the chromosomal segment adjacent to the *flg* genes, was introduced into the same Δ*flgABCDE25* mutant, virulence was not restored. In contrast, the introduction of pMH71, a plasmid which retains the chromosomal segment adjacent to the *flg* genes, restored virulence. We concluded that a hitherto unknown virulence gene, which we have named *mviS*, occurs adjacent to the *flg* genes and that its absence in Δ*flgABCDE25* mutants, rather than the nonflagellate phenotype of the Δ*flgABCDE25* mutants, caused the previously reported attenuation of such mutants.

Our results in a series of previous studies led us to conclude that flagella are a virulence factor in infected C57BL/6J mice (4, 24). This conclusion was based on the relative virulence of two pairs of isogenic strains. These strains were constructed by using in vivo genetic engineering techniques (6) which allowed us to replace the wild-type *flaF*$^+$ allele of the mouse-virulent strain SL3201 with the *flaF25* allele of the nonflagellate, nonmotile strain SL488 (13). The resultant strains, St36 and St39, had the relevant genotype *flaF25* and were nonflagellate and thus nonmotile, and their virulence was much reduced (4). Their isogenic partners, St37 and St38, were flagellate, motile, and essentially as virulent as the parental strain, SL3201 (4). Our conclusion that flagella are a virulence factor was based on the assumption that mutation *flaF25* affected only one or a few of the *flaF* genes and that the affected gene(s) concerned only the formation of flagella. We later became aware of the paper of Kutsukake et al. (15), in which the strain SL488 mutation formerly called *flaF25* (13) is shown as Δ(*flaFI-flaFV*), indicating deletion of five of the cluster of flagellar genes originally designated *flaF*. This cluster is now known to comprise twelve genes, *flaFI* through *flaFX*, *flaW*, and *flaU*, recently renamed *flgA* through *flgL* (14), present in three contiguous operons (16). The division of the cluster into three operons and the chromosomal location of the cluster relative to *pyrC* is shown in Fig. 1A. The strain SL488 mutation previously called *flaF25* (13) or Δ(*flaFI-flaFV*) (15) is therefore now called Δ*flgABCDE25*, or Δ*flg-25* for brevity. We speculated that, because this deletion affected the five contiguous genes at the end of the cluster nearest *pyrC*, it could extend beyond the *flg* cluster towards *pyrC* and that deletion of a putative virulence gene located between the *flg* cluster and *pyrC*, rather than loss of flagella, might cause the reduced virulence observed in Δ*flg-25* strains (4). Since there are no known genes in the region between *pyrC* and the *flg* cluster (20), we could not distinguish between the two possibilities by a genetic analysis.

We show in this study that five nonflagellate strains, each mutated in a different *flg* gene, were equally virulent by any one of three routes of infection, as were their flagellate isogenic partners. We also present evidence for the existence of a virulence gene closely linked on the *pyrC* side to the *flg* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The growth of bacterial strains (Table 1) in liquid media and on agar plates, the preparation of inocula to challenge mice, and the use of semisolid agar (22) for testing of motility and isolation of migrating motile bacteria have been described elsewhere (4).

The three plasmids used, pMH6, pMH64, and pMH71, were obtained from M. Homma. Construction of pMH6 and pMH64 has been described elsewhere (11); that of pMH71 was as follows (M. Homma, personal communication). The same *Eco*RI chromosomal fragment of *S. typhimurium* was ligated in both possible orientations into *Eco*RI-cleaved pBR322; the two recombinant plasmids were pMH6 (11) and pMH7 (Fig. 1B). The *Cla*I-deletion derivatives of pMH6 and pMH7 constructed by *Cla*I digestion and ligation were pMH64 (11) and pMH71 (Fig. 1B), respectively.

Genetic methods. Strain P22HT *int* bacteriophage was used, and its preparation and use in transduction were as described elsewhere (4). Isogenic pairs of strains were constructed as described previously (4); a brief description of the construction of one such pair follows. P22HT *int* phage was grown on St25, and the resultant lysate was used to transduce a strain containing the *flgA1775* mutation (15). A nonmotile derivative was isolated from among the tetracycline-resistant transductants and purified, and a phage lysate was prepared. The resultant lysate was used to transduce either of two mouse-virulent strains, SL3201 or SL1898, in order to produce an isogenic pair at the *flgA*

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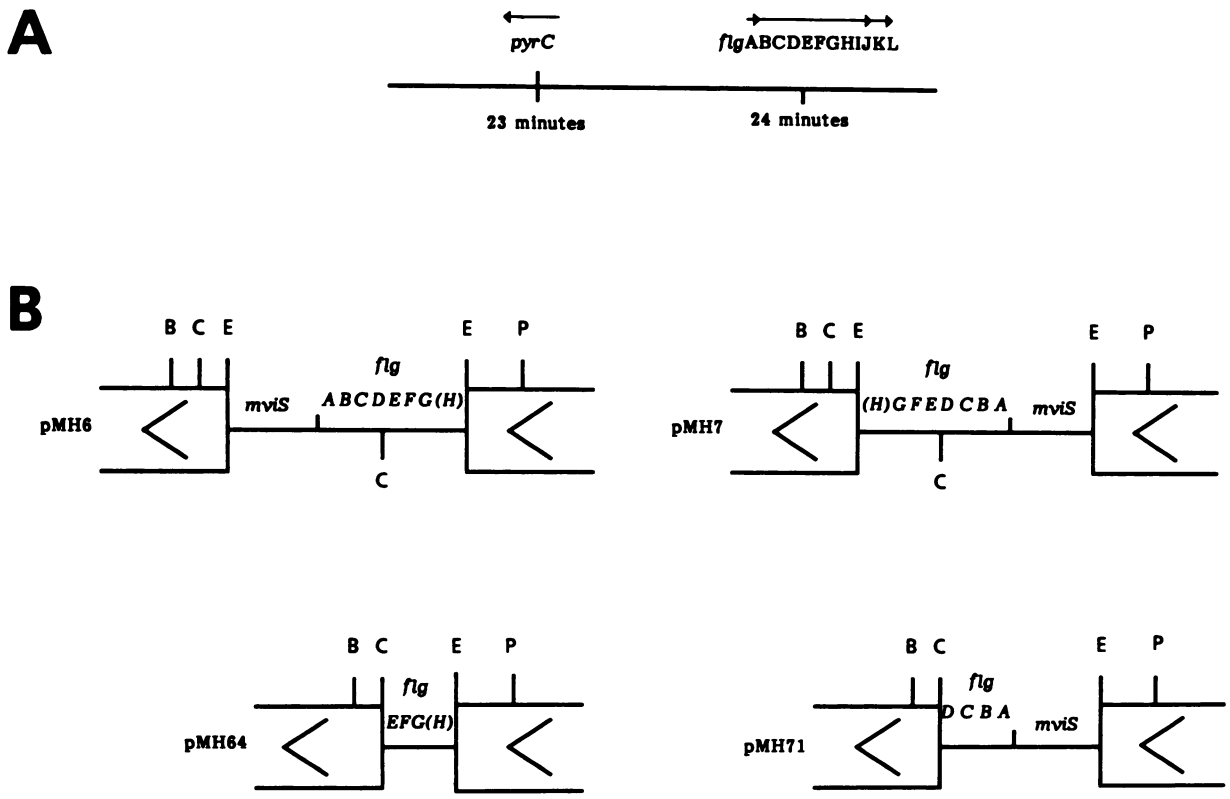

FIG. 1. (A) Relative positions and transcriptional units of the *pyrC* and *flg* genes on the chromosome of *S. typhimurium*. The *flg* genes, although not mapped precisely on the genetic map of *S. typhimurium* (20), are shown located clockwise from *pyrC*, as they occur in *E. coli* (1). Their cotransduction with *pyrC* by P22 phage, albeit at a low frequency (M. Carsiotis, unpublished data), and the approximate 1-min length of P22 transducing fragments (20) make it reasonable to assume that they occur close to 24 min. The *flg* genes are organized into three contiguous operons (16): *flgA*, *flgBCDEFGHIJ*, and *flgKL*. (B) Partial restriction maps of plasmids pMH6, pMH64, pMH7, and pMH71 (11; M. Homma, personal communication). Boxes and thin lines represent the pBR322 vector and the *S. typhimurium* chromosomal fragment, respectively. The arrowheads within the boxes indicate the directions of increase in base pair numbering in the vector. Note that in pMH6 and pMH7, the identical *Eco*RI-derived chromosomal fragment is present in opposite orientations in the same vector. The parentheses around *H* indicate that only the 5′ end is present (11). The precise location of *mviS* within the non-*flg* part of the chromosomal fragment is unknown. The entire chromosomal insert is ca. 9.5 kb, of which ca. 3.5 kb consists of non-*flg* DNA. The chromosomal inserts within the vectors have been drawn approximately to scale (11). Although no experiments in this study were done with pMH7, it is depicted here to clarify the origin of the *Cla*I deletion derivative pMH71, which was used in this study. Restriction site symbols: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; P, *Pst*I.

locus. This procedure was repeated with strains which contained the *flgB1727*, *flgC1723*, *flgD1713*, or *flgE1704* mutation (15, 23). Each of the *flg* mutations is monocistronic and nonpolar (15, 16). The terms isogenic pair, isogenic strains, and isogenic partner(s), are used to designate two or more strains that differ essentially only in the state of a single *flg* gene.

The introduction of plasmids pMH6, pMH64, and pMH71 into St39 required a two-step procedure to avoid restriction in St39. Plasmid DNA, received from M. Homma, was used (17) to evoke ampicillin-resistant (Amp^r) transformants from strain LB5000, which is *S. typhimurium* restriction negative but modification proficient for all three systems of this species (3). Phage P22HT *int* grown on one such transformant was used to transduce the now-modified plasmid into St39 by selection for Amp^r. In the construction beginning with pMH6, all of several Amp^r transductants selected appeared to be motile, as shown by spreading growth on semisolid-agar plates (22). However, their rate of spread was much slower than that of wild-type *S. typhimurium*, and examination of broth cultures by microscopy showed only a small proportion of the bacteria to be motile. We attribute the failure of pMH6, which contains functional *flgA* through *flgG*, to complement the chromosomal Δ*flg-25* mutation and thus restore motility to the absence of the gene products of *flgH*, *flgI*, and *flgJ* in the Amp^r transductants. The products are absent in Δ*flg-25* mutants, since the promoter for those genes occurs between *flgA* and *flgB* (16). The products cannot be provided by introduction of pMH6, which contains only part of *flgH* and neither *flgI* nor *flgJ* (11). Each of four poorly motile independent Amp^r transductants was stabbed into semisolid agar, and a descendant was isolated from the leading edge of growth. Each of these descendants proved to be a fully motile but ampicillin-sensitive (Amp^s) variant (see Discussion); they were designated St120A, St120B, St120C, and St120D. In the constructions that began with pMH64 and pMH71, the four Amp^r transductants of St39 (strains St129 through St132) were nonmotile, as expected, since the wild-type *flgB-flgJ* operon cannot be produced by recombination between the segment in pMH64 or in pMH71 and the nondeleted part of the operon in the chromosome of the Δ*flg-25* host.

Phenotypic testing of isogenicity. To check the isogenicity of the isogenic pairs (strains St78 through St81 and St98 through St105), we tested the following properties (data not shown): sensitivity of all derivatives to the P22 phage, amino

TABLE 1. *S. typhimurium* strains and plasmids

Strain or plasmid	Description and relevant genotype[a]	Source (reference)
Strains		
SL488	Nonflagellate derivative of LT2, previously designated *flaF25* (13) and Δ(*flaFI-flaFV*) (15) and henceforth designated Δ*flgABCDE25* (14)	Salmonella Genetic Stock Center (13, 15)
St25	Motile transductant of SL488; *flg*$^+$ *zcd-907*::Tn*10*	4
SL3201	Mouse virulent; (ColE1-30) *leu-1051 cysI1173 hisC527*(Am) FIRN biotype	B. A. D. Stocker (10)
SL1898	Mouse-virulent strain TML cured of P22; prototroph; Col$^+$	B. A. D. Stocker (9)
St39	SL3201 but Δ*flgABCDE25 zcd-907*::Tn*10*	4
St80	SL1898 but Δ*flgE1704 zcd-907*::Tn*10*	This study
St81	SL1898 but *flgE*$^+$ *zcd-907*::Tn*10*	This study
St98	SL1898 but *flgA1775 zcd-907*::Tn*10*	This study
St99	SL1898 but Δ*flgB1727 zcd-907*::Tn*10*	This study
St100	SL1898 but Δ*flgC1723 zcd-907*::Tn*10*	This study
St101	SL1898 but Δ*flgD1713 zcd-907*::Tn*10*	This study
St102	SL1898 but *flgA*$^+$ *zcd-907*::Tn*10*	This study
St103	SL1898 but *flgB*$^+$ *zcd-907*::Tn*10*	This study
St104	SL1898 but *flgC*$^+$ *zcd-907*::Tn*10*	This study
St105	SL1898 but *flgD*$^+$ *zcd-907*::Tn*10*	This study
St120A	Motile recombinant between St39 and pMH6; Amp^s	This study
St120B	Motile recombinant between St39 and pMH6; Amp^s	This study
St120C	Motile recombinant between St39 and pMH6; Amp^s	This study
St120D	Motile recombinant between St39 and pMH6; Amp^s	This study
St129	St39(pMH64) Amp^r Mot$^-$	This study
St130	St39(pMH64) Amp^r Mot$^-$	This study
St131	St39(pMH71) Amp^r Mot$^-$	This study
St132	St39(pMH71) Amp^r Mot$^-$	This study
Plasmids		
pMH6	Genes *flgABCDEFG(H)* and the ca. 3.5 kb of *S. typhimurium* chromosomal DNA adjacent to *flgA* inserted in the *Eco*RI site of pBR322	M. Homma (11)
pMH64	pMH6 deleted of *flgABCD* and ca. 3.5 kb of adjacent chromosomal DNA	M. Homma (11)
pMH71	pMH7 deleted of *flgEFG(H)*	M. Homma

[a] The chromosomal location of the silent Tn*10* insertion is designated *zcd-907*::Tn*10* since it is closely linked to *flg* (4). The *zcd* designation is according to the convention of Hong and Ames (12, 20); the allele designation 907 is from the block of numbers 901 to 1000 used by the laboratory of B. A. D. Stocker. The *flgH* gene is placed in parentheses to indicate that only the 5′ end is present (11). Symbols: *leu*, Leucine; *cys*, cysteine; *his*, histidine; *flg*, flagellar (14); Col, Colicin; Mot, motility.

acid auxotrophic requirements in derivatives of SL3201, prototrophy in derivatives of SL1898, and colicin production in all derivatives.

Animal testing. As described previously (4, 24), C57BL/6J mice were challenged orally, intravenously, or intraperitoneally (i.p.). The size of the challenge dose was determined by viable count, and mortality (number dead per number injected) was scored after 30 days. The median 50% lethal dose was determined by the method of Reed and Muench (19).

RESULTS

Virulence of nonflagellate strains which contain monocistronic *flg* mutations. The relative virulences of the *flgA*, *flgB*, *flgC*, *flgD*, and *flgE* derivatives of SL1898 and each of their isogenic *flg*$^+$ partners were determined in i.p.-infected C57BL/6J mice. The results (Table 2) indicated unequivocally that the nonflagellate, nonmotile mutants were as virulent as their isogenic flagellate, motile counterparts. Essentially identical results were obtained when a second set of the five isogenic pairs, constructed in the SL3201 parental strain, were tested in i.p.-infected C57BL/6J mice (data not shown). In addition, the 50% lethal doses of strains St100 (*flgC*) and St104 (*flgC*$^+$) were identical when C57BL/6J mice were challenged orally (2×10^4 CFU) or intravenously (<10 CFU).

Restoration of virulence by cloned chromosomal DNA. One possible explanation for the virulence of the nonflagellate monocistronic *flg*-bearing strains (Table 1) and the previously reported attenuation (4) of Δ*flg-25*-bearing strains is as follows. The Δ*flg-25* mutation may extend beyond *flgA* towards *pyrC* and thus include the deletion of a putative chromosomal virulence gene, henceforth designated *mviS*, located between *pyrC* and *flgA* (Fig. 1A). We used pMH6 (11) to test this hypothesis, since it contains a portion (ca. 3.5 kilobases [kb]) of the wild-type *S. typhimurium* chromosome between *pyrC* and *flgA*, immediately counterclockwise to *flgA*. If *mviS* is present in that chromosomal portion of pMH6, introduction of the plasmid into St39, an attenuated,

TABLE 2. Virulence of isogenic flagellate and nonflagellate strains of *S. typhimurium* in C57BL/6J mice

Strain	Genotype	Motility	Mouse mortality (no. dead/no. infected)[a]
SL1898	*flg*$^+$	+	5/5
St98	*flgA*	−	5/5
St102	*flgA*$^+$	+	5/5
St99	*flgB*	−	5/5
St103	*flgB*$^+$	+	4/4
St100	*flgC*	−	6/6
St104	*flgC*$^+$	+	5/5
St101	*flgD*	−	6/6
St105	*flgD*$^+$	+	3/5
St80	*flgE*	−	3/3
St81	*flgE*$^+$	+	3/3

[a] C57BL/6J mice were challenged i.p. with ca. 100 CFU.

TABLE 3. Restoration of virulence in infected C57BL/6J mice by chromosomal DNA adjacent to *S. typhimurium flg* gene

Strain[a]	Description and/or genotype	Motility	Mouse mortality (no. dead/no. infected)[b]
St39	Δ*flgABCDE25*	−	0/7
St120A	Amp^s *flg*$^+$ recombinant between St39 and pMH6	+	6/7
St120B	Amp^s *flg*$^+$ recombinant between St39 and pMH6	+	7/7
St120C	Amp^s *flg*$^+$ recombinant between St39 and pMH6	+	6/6
St120D	Amp^s *flg*$^+$ recombinant between St39 and pMH6	+	7/7

[a] St120A through -D are homologous, independently isolated recombinants.
[b] The i.p. challenge dose was ca. 100 CFU.

nonflagellate Δ*flg-25*-bearing strain, should restore virulence. Plasmid pMH6 was introduced into St39, and four independent derivatives (strains St120A, St120B, St120C, and St120D) were isolated and tested for virulence. All four derivatives were highly virulent in infected C57BL/6J mice (Table 3). These results are consistent with the existence of *mviS* in that portion of the chromosome between *pyrC* and the *flg* cluster which is also present in pMH6.

Localization of *mviS* in pMH71. We inferred from the partial restriction map of pMH6 and pMH7 (Fig. 1B) that the resultant *Cla*I deletion derivatives, respectively pMH64 and pMH71 (Fig. 1B), lack and contain *mviS*, respectively. Plasmids pMH64 and pMH71 were transferred via a restriction-minus intermediate (3) into attenuated strain St39. Two independent isolates were obtained from each transduction, strains St129 and St130 from pMH64 and strains St131 and St132 from pMH71; all four of the strains were nonmotile. When these strains were tested for virulence, the pMH64-bearing strains were still attenuated, whereas the pMH71-bearing strains were highly virulent (Table 4).

DISCUSSION

In each of five isogenic pairs of strains, the two members, one flagellate and motile and the other nonflagellate and nonmotile, were equally virulent when tested by i.p. infection of C57BL/6J mice (Table 2). Furthermore, the 50% lethal dose of each member of one such isogenic pair, strains St100 (*flgC*) and St104 (*flgC*$^+$), was the same for C57BL/6J mice challenged orally or intravenously. The five nonflagellate, nonmotile members of these five isogenic pairs of strains were mutated individually in all five of the *flg* genes deleted in Δ*flg-25* strains. Consequently, we concluded that attenuation of virulence in Δ*flg-25* strains is not due to their nonflagellate, nonmotile phenotype. Rather, we hypothesized (M. Carsiotis, B. A. D. Stocker, I. A. Holder, D. Weinstein, and A. D. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B-169, p. 53) that an adjacent virulence gene, here called *mviS*, had also been deleted by the Δ*flg-25* mutation. The putative *mviS* gene would occur between *pyrC* and the *flg* genes, a chromosomal region in which no other genes have been reported (20). The availability of plasmid pMH6 (11) allowed us to test this hypothesis, since this plasmid contains ca. 3.5 kb of chromosomal DNA adjacent on the *pyrC* side to the *flg* genes. After plasmid pMH6 was transduced into St39, each of four independent Amp^r transductants consisted of a mixture of motile and nonmotile derivatives. The motile derivatives, derived by secondary selection, were found to be Amp^s. Presumably, they arose by homologous recombination between pMH6 and the host chromosome, with a concomitant loss of pMH6, as evidenced by loss of the Amp^r phenotype of the plasmid. A relevant genetic implication of the recombination between the Δ*flg-25* chromosome and the chromosomal fragment in pMH6 is that the fragment overlaps each end of the Δ*flg-25* mutation. Hence, the resultant fully motile recombinants should now contain the entire chromosomal region between *pyrC* and *flg*. Therefore, if the putative *mviS* gene occurs within that region, the recombinants should be virulent. Derivatives St120A, St120B, St120C, and St120D were virulent (Table 3), a result consistent with the existence of a putative *mviS* gene linked closely to the *flg* genes. Furthermore, this result suggested that the putative *mviS* gene is present in the ca. 3.5-kb portion of chromosomal DNA in pMH6 adjacent to *flgA*. A second possibility, that restoration of virulence was due to the restoration of flagella and motility, seems untenable in light of our results with the five isogenic pairs of strains (Table 2). To determine if *mviS* is in the ca. 3.5-kb portion of chromosomal DNA in pMH6 adjacent to *flgA*, we used plasmids pMH64 and pMH71. Plasmid pMH64 is known (11) to lack this entire ca. 3.5-kb portion of chromosomal DNA (Fig. 1B), and therefore its introduction into a Δ*flg-25* mutant should not restore virulence, because *mviS* is absent. Indeed, when two such pMH64-bearing strains, St129 and St130, were tested in C57BL/6J mice, they remained attenuated (Table 4). We then introduced plasmid pMH71, which contains this entire ca. 3.5-kb portion of chromosomal DNA (Fig. 1B), into a Δ*flg-25* mutant. The two independent derivatives isolated, St131 and St132, were found to be virulent in C57BL/6J mice (Table 4). We therefore concluded that *mviS* is present in the ca. 3.5-kb chromosomal portion of pMH71 adjacent to *flgA*. Our results do not show whether *mviS* is a single gene or whether there are other virulence genes linked to *mviS*. It should be noted that these two virulent isolates, strains St131 and St132, are additional examples of nonflagellate, nonmotile strains that are virulent.

TABLE 4. Virulence of *S. typhimurium mviS* in pMH71 versus pMH64[a]

Strain	Description and/or genotype[a]	Mouse mortality (no. dead/no. infected)
St39	Δ*flgABCDE25*	0/7
St129	St39(pMH64)	0/7
St130	St39(pMH64)	0/7
St131	St39(pMH71)	6/6
St132	St39(pMH71)	7/7

[a] All strains tested were nonmotile. C57BL/6J mice were challenged i.p. with ca. 100 CFU.

A description of the variety of mutations which attenuate the mouse virulence of *S. typhimurium* has recently appeared (21). In addition, there are reports on the attenuation of virulence in *cya* and *crp* mutants (5), in purine auxotrophs (18), in *aroA* mutants (10), in a series of Tn*10* insertion mutants (8), and in partially characterized *mviA* (2, 20) and *mviC* (20) mutants. The genetic locations of *mviA* and *mviC* on the *S. typhimurium* chromosome (20) preclude their being identical to *mviS*. In a recent report (7), *phoP* mutants were

found to be attenuated in i.p.-infected BALB/c mice. It is unlikely that *mviS* and *phoP* are the same gene, since *mviS* and *phoP* map at 24 and 25 min, respectively, on the *S. typhimurium* chromosome (20). Furthermore, the *phoP* activities of a Δ*flg-25* strain and its isogenic *flg*$^+$ partner were measured and shown to be identical (E. A. Groisman, personal communication).

In both this and our previous study (4), we did not detect any phenotypic differences in several physiological properties between the members of any *flg*$^+$–Δ*flg-25* isogenic pair. Particularly noteworthy were the quantitative identities of both the lipopolysaccharide and outer membrane protein profiles of the isogenic pair examined (4). Thus, the nature of the product of *mviS* remains to be determined. Furthermore, in light of the complex nature of the Δ*flg-25* mutation, whether our results in previous studies (24) with C57BL/6J mice orally infected with a Δ*flg-25* mutant were due to the *mviS* mutation or deletion of *flgABCDE* is now uncertain. For the same reason, the more rapid in vitro clearance of a Δ*flg-25* mutant than of a *flg*$^+$ strain from murine macrophages (24) can no longer be ascribed to the nonflagellate character of the mutant. We are currently attempting to clone *mviS* to assess its role in virulence more precisely and to determine if it occurs in other *Salmonella* spp. and in *Escherichia coli*.

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