The *Salmonella typhimurium* Locus *mviA* Regulates Virulence in *Ity*^{s} but Not *Ity*^{r} Mice: Functional *mviA* Results in Avirulence; Mutant (nonfunctional) *mviA* Results in Virulence

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Summary

The virulent *Salmonella typhimurium* strain WB600 carries the *mviA* allele of the gene mouse virulence A. As shown here, the virulent phenotype of WB600 is the result of a nonfunctional *mviA* gene. As compared to the functional allele *mviA*$^+$, *mviA* increases virulence in *Ity*^{s} mice, but not in *Ity*^{r} mice. A specific BglII site, *mviA4185*, between *osmZ* and *galU*, located at ~35 min on the salmonella chromosome, was within *mviA*. Insertion of an antibiotic cassette in the *mviA4185* site of *mviA*$^+$ or the homologous *mviA4093* site of *mviA* DNA resulted in virulence when either cassette was recombined into the chromosome. When *mviA* and *mviA*$^+$ were both expressed in the same strain with one carried in the chromosome and the other on a plasmid, avirulence was dominant. Replacement of the *mviA* allele of strain WB600 using P22 transductions of linked antibiotic cassettes cloned into the chromosome of virulent *S. typhimurium* strains (SR-11, TML, SL1344, C5, ATCC14028, W118-2, and WB600) showed that all but WB600 contained the avirulent *mviA*$^+$ allele. Southern hybridizations provided no evidence for a second *mviA* allele anywhere in the genome of the six non-WB600 strains.

Worldwide, there are >1.25 × 10^7 cases per year of salmonella-caused enteric fever in man (1). The ability of salmonella to cause enteric fever is highly species specific. *Salmonella typhi* is the primary salmonella species causing enteric fever in man. Enteric fever caused by other species of salmonella result in significant economic losses in cattle (*S. dublin*, and less frequently, *S. typhimurium*), swine (*S. choleraesuis*), and poultry (*S. pullorum*, *S. gallinarum*) (2). In mice, enteric fever is caused by *S. typhimurium* and *S. enteritidis* (3–6). In mice as in other animals, the natural route of acquisition is oral, usually via food or water. Unlike salmonella strains that cause gastroenteritis, the focus of enteric fever infections is the spleen and liver (3, 7). It is likely that most of the early salmonella growth in the target organs occurs within cells (8, 8*a*). Investigators disagree about which is the major cell type involved, with evidence for salmonella survival and growth in cells as diverse as hepatocytes, epithelial cells, and macrophages (9–14).

Regulation of the growth rate of salmonella in vivo appears to be an important defense mechanism. Among inbred mouse strains considerable polymorphism exists for the alleles *Ity*^{r} (resistant) and *Ity*^{s} (susceptible), which have a major effect on the resistance of mice to infections with *S. typhimurium* (15–17). Although the *Ity* locus has been shown to have an effect on the killing of salmonella taken up by phagocytes (18, 19), its major in vivo effect is on the growth of salmonella (19, 20). It has also been shown that prior infection with mouse hepatitis virus can result in a slower salmonella growth rate in *Ity*^{s} but not *Ity*^{r} mice (21, 22).

The mechanism by which the *Ity* locus affects in vivo salmonella growth is not known. Direct studies are difficult because the effect of the *Ity* locus on growth has not been observed in vitro (12). Studies of the mechanism of action of salmonella genes that affect the virulence of salmonella in *Ity*^{s} but not *Ity*^{r} mice should provide insights into the mechanism of action of the *Ity* locus. In a previous study, we showed that salmonella strain SR-11 has a gene, or genes, that affect the virulence of salmonella in an *Ity*-dependent manner (23). In this study, we have examined a virulence gene, *mviA* (mouse virulence A, which maps at ~35 min in strain WB600 *S. typhimurium*) (24). In the original studies, this gene was identified by Hfr matings and transductional crosses from the virulent WB600 background to the avirulent LT2-Z background. In our present studies, we have examined the effect of *mviA* and *mviA*$^+$ alleles in the WB600 background. The *mviA* allele confers virulence in *Ity*^{s} mice but not *Ity*^{r} mice and appears to be distinct from the genes responsible for the virulence of strain SR-11.

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Materials and Methods

Mice. The mice used in this study included *Ity*^{r} BALB/cAnPt and *Ity*^{r} C.D2-*Idh-1*^{b} (N20) (25), raised from a stock obtained from M. Potter at the National Cancer Institute (NIH, Bethesda, MD). C.D2-*Idh-1*^{b} mice are congenic with BALB/cAnPt mice for a 20-cm region of DNA containing the *Ity* locus. The C.D2-*Idh-1*^{b} mice were prepared by backcrossing the 20-cm portion of DBA/2 chromosome 1 containing the *pep3* and *Idh-1*^{b} loci 20 times into *Ity*^{s} BALB/cAnPt mice. It was found that the *Ity*^{r} locus was also transferred as a passenger gene (25). *Ity*^{s} C57BL/6J and *Ity*^{r} LAF1, (C57L/J × A/J)F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Male and female mice were 6–8 wk of age when infected.

Bacterial Strains. Bacterial strains used in this study are listed in Table 1. The virulent *S. typhimurium* background used in most of the study is that of WB600 (24), a strain derived from TT289, an LT2 strain obtained from J. Roth (University of Utah). The avirulent allele of *mviA* was derived from JL3404, an LT2-Z strain from J. Ingraham (University of California Davis). Although both WB600 and LT2-Z are LT2 derivatives, our previous studies revealed almost a 4-log difference in CFU obtained from the liver and spleen 6 d after infection of *Ity*^{r} mice with strains of the two backgrounds (19, 24).

Transduction. Transductions were done with P22 HT105/1 *int201* as previously described (24, 35). Several of the wild-type virulent strains such as SR-11, ATCC14028, and W118-2 are not lysed very well with P22, thus making preparation of transducing lysates difficult. It is possible that the transductions of these wild-type strains were facilitated by an endogenous, uncharacterized, lysogenic bacteriophage in these salmonella and not the P22 with which we attempted to make the lysates.

Electroporation. Ligation mixtures were dialyzed before being electroporated into LE392. Supercoiled plasmids were prepared by alkaline lysis (36) and electroporated directly. Electroporation was performed with a gene pulser and capacitance extender (Bio-Rad Laboratories, Richmond, CA). Cells were washed in cold 10% glycerol and pulsed at 2.5 kV with a 25-μF capacitor and 400-Ω parallel resistor. The cells were allowed to express antibiotic resistance in SOB (37) for 1 h before being plated on selective media.

Bacterial Culture Media. Unless indicated otherwise, bacteria were grown in Luria-Bertani medium and on plates made with LB plus 1.5% agar. Selective plates were made by adding kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), ampicillin (50 μg/ml), spectinomycin (50 μg/ml), or streptomycin (100 μg/ml). Additional media used to compare the in vitro growth rate of *mviA* and *mviA*$^+$ isogenic strains of salmonella were: MOPS minimal enterobacteria media with 0, 50, 200, and 400 mM NaCl (38); and Vogell Bonner minimal media adjusted to pH 4.5 and 7, and with 0.44 M sucrose added (39).

Cloning of the mviA Region. Salmonella DNA was cloned first in *Escherichia coli* LE392 and then transferred to salmonella strains by electroporation. All enzymes were used according to manufacturers' recommendations. Cloning procedures were done as described by Maniatis et al. (40). Cloning of specific fragments was accomplished by excising bands out of Tris acetate–buffered agarose gels (40) and using Geneclean (Bio 101 Inc., La Jolla, CA) to recover the DNA in a form suitable for ligation. Subclones were made by ligating the BglII fragments into the low copy number vector pGB2, which has a pSC101 origin and codes for resistance to spectinomycin and streptomycin. The cloned fragments were then ordered by Southern hybridization with overlapping clones made by cloning with other restriction enzymes. Field inversion gel electrophoresis was performed in a vertical electrophoresis box using a pulse controller (PC750; Hoefer Scientific Instruments, San Francisco, CA).

Insertion of Antibiotic Cassettes in the Chromosome. Incompatible plasmids were used to select for directed insertion of antibiotic cassettes into the chromosome (41, 42). Selectable markers were introduced into the chromosome of *S. typhimurium* by ligating the 1.3-kb kanamycin resistance cartridge from pUC4K (43) into unique sites in *S. typhimurium* DNA from the region of *mviA* cloned into pGB2. We selected for integration into the chromosome by using an incompatible plasmid pWB3097, which is the 2-kb HaeII fragment of pGB2 (27) containing the origin of replication of pSC101, ligated to the 1.25-kb HaeII fragment of pHSG422-containing chloramphenicol resistance (44). The new plasmid (pWB3097) has the origin of replication of pSC101 and chloramphenicol as its only antibiotic resistance. The two incompatible plasmids, for instance, pWB3097 (cm only) and pWB4093 km (km and streptomycin), were electroporated into *S. typhimurium* sequentially followed by selection for kanamycin and chloramphenicol. Cm^r km^r colonies were streaked for isolation, and large colonies were patched to streptomycin. Those that retained the streptomycin resistance of the pWB4093 km were suspected to have resulted from rearrangements between the plasmids and were discarded. Streptomycin-sensitive colonies were suspected to be those where the antibiotic resistance cassette of the donor plasmid (in this case pWB4093) had recombined into the chromosome before the loss of the donor plasmid. DNA from streptomycin-sensitive colonies was tested by agarose electrophoresis for loss of the larger plasmid (in this case pWB4093::km), which contained the cassette to be inserted in the chromosome. The inserted DNA was then transduced to the same background, but lacking the incompatible plasmid. This procedure was carried out to insert antibiotic cassettes in nine restriction sites of six different plasmids (Table 3, see also Fig. 2). All inserts were transduced with P22 to check recombination frequency with other inserts. Each insert was tested for linkage to the *opp3*::Tn*10* insert. As more inserts were found, we tested for linkage of km^r and cm^r to each other. This allowed us to determine, for example, that all *zde4093* inserts were 100% linked to each other and also to *zde4185* inserts.

A chloramphenicol (*cat*) cartridge was made by blunt ending the pUC4K (43) vector after digestion with PstI and ligating in a blunt-ended 1.25-kb HaeII fragment from pHSG422 (44), which carried the chloramphenicol acetyl transferase gene. This produced a chloramphenicol cassette that could be excised with EcoRI, BamHI, SalI, AccI, and HincII. Cassette inserts could be made in any chromosomal site with overhangs compatible with those produced by these enzymes. To affect recombination of the fragments with the cm inserts into the chromosome, we used the incompatible plasmid pWB3096. This plasmid was made by ligating a 1.3-kb kanamycin resistance fragment from pHSG422 and the 2-kb pGB2 HaeII fragment discussed above. The chromosomal inserts in known restriction sites and *opp::3* Tn*10* and *zde/5410*::Tn*10* were used to select for recombination in fairly short regions of the chromosome in order to map the location of *mviA* on the chromosome.

Results

Effect of mviA on Virulence in Ity^r *and Ity*^{s} *Mice.* The *mviA* gene was originally described in strain WB600 and its allele *mviA*$^+$ in strain WB101 (24). For the present studies, isogenic *mviA* WB600 and *mviA*$^+$ WB335 strains on the WB600 background were used to infect congenic *Ity*^{s}

BALB/cAnPt and *Ity*^{r} C.D2-*Idh-1*^{b} (N20) mice as well as *Ity*^{s} C57BL/6J and *Ity*^{r} LAF1 mice. As shown in Fig. 1, high levels of *mviA* salmonella were recovered from the *Ity*^{s} mouse strains 6 d post-infection: $>10^7$ salmonella were recovered from BALB/c mice and five of five C57BL/6 mice were already dead ($\geq 10^8$ CFU). About 1/10,000 as many *mviA*$^+$ than *mviA* salmonella were recovered from the two *Ity*^{s} strains. Quite different results were observed after infection of the *Ity*^{r} mice. Both of the *Ity*^{r} mouse strains showed high resistance to *mviA* and *mviA*$^+$ salmonella. Only three- to fourfold more salmonella were recovered from *Ity*^{r} mice infected with *mviA* vs. *mviA*$^+$ salmonella, and the difference was not statistically significant. These data demonstrate that the presence or absence of *mviA* has a large effect on virulence in *Ity*^{s} mice but little if any effect in *Ity*^{r} mice. Even though *mviA*$^+$ salmonella were recovered in only low numbers from *Ity*^{s} mice, there were several-fold more *mviA*$^+$ salmonella in *Ity*^{s} than *Ity*^{r} mice (Fig. 1).

Cloning of mviA and Flanking DNA. We previously used Hfr matings and P22 cotransductions to map *mviA* to the region between *trp* and *galU* (24). To clone *mviA*, we used these flanking genes as selectable markers. Because *trp* and *galU* genes are ~30 kb apart, we first used the in vivo cloning vector pULB113 (28, 45) to obtain random insertions in the WB600 chromosome via the mini-Mu contained in this plasmid. Because pULB113 is conjugable, we were then able to mate the pool of insertion mutants with the *E. coli* strain WB9945 and select for an R-prime capable of complementing the *galU* and *trp* mutations in this strain. Streptomycin was used to select against the donor. An ~150-kb plasmid (pWB3007) containing an ~100-kb insert and both markers was obtained in *E. coli*. A spontaneous deletion of ~50 kb that included *trp* resulted in the GalU$^+$ Opp$^+$ plasmid pWB3023. A partial restriction map of the insert DNA was obtained. A portion of this map is shown in Fig. 2. When the same probes were used in hybridizations of restriction digests of WB600 and pWB3023 DNA, identical maps were obtained.

Mapping of mviA. To locate *mviA* on the restriction map, antibiotic resistance cassettes inserted into the subclones described above (shown in Fig. 2) were driven to the chromosome of the *mviA*$^+$ strain WB335, and the resulting strains were used to infect mice. To accomplish the chromosomal integration of these fragments, we inserted antibiotic cassettes at the indicated (Fig. 3) restriction sites of clones pWB4005, pWB4050, pWB4089, pWB4173, pWB4093, and pWB4185. Incompatible plasmids were then introduced in order to select for insertion of the cassette into the *mviA* and *mviA*$^+$ chromosomes by homologous recombination. These antibiotic inserts were used in two and three factor crosses to determine the location of *mviA*, using virulence in mice as the test for *mviA*. Other markers used to map *mviA* were a Tn*10* closely linked to *osmZ* (*zde/5410*::Tn*10*) and a Tn*10* inserted in *opp* (*opp3*::Tn*10*) (Fig. 2). Orientation of this region on the chromosome was determined from linkage of *opp3*::Tn*10* to *Δtrp-opp24* and then from the restriction map generated from the cloned fragments. Virulence was found to be clockwise of *zde4005*, *zde4006*, and *zde4173* (Table 2). Antibiotic cassettes inserted into site *zde4093* were found to be linked to virulence in four of four recombinants, indicating that *mviA* must be very near the BglII site at position *zde4093* (Fig. 2).

Demonstration that the mviA Phenotype Is the Result of a Nonfunctional mviA Gene. To confirm that the *zde4093*::km insert was close to *mviA*, cloned fragments of *mviA* DNA with the antibiotic inserts were forced to recombine into the chromosome of *mviA* WB600 and *mviA*$^+$ WB335 salmonella. Only inserts in site *zde4093* conferred virulence when forced into the chromosome of avirulent strain WB335 (Table 3). This finding confirmed the location of *mviA* very near, or at, the pWB4093 BglII site (see Figs. 2 and 3). Using the pWB4093 *mviA* DNA as a probe, we found that JL3404 (*mviA*$^+$) and WB600 (*mviA*) each had a HindIII fragment of 7.5 kb and a ClaI fragment of 3.5 kb. This enabled us to clone the same fragment from JL3404 by ligating 3.5-kb ClaI fragments from a genomic digest of JL3404 to pGB2 and probing with the pWB4093 insert fragment to identify the proper insert. The restriction map of the JL3404 clone (pWB4184) was identical to that of pWB4093 shown in Fig. 3. We inserted the km antibiotic cassette into the BglII site of pWB4184 and then forced the cassette into the chromosome of the *mviA*$^+$ strain WB335. Two independent insertions were used to infect mice. As seen in Fig. 4, these mutants were as virulent in *Ity*^{s} mice as WB600, indicating that interruption of *mviA*$^+$ resulted in virulence. This BglII insertion site in *mviA*$^+$ DNA was designated *mviA4185*.

Effect of Plasmid Expression of mviA and mviA$^+$. If avirulence results from MviA function and virulence from a lack of MviA function, it would be expected that avirulence would be dominant in salmonella bearing both *mviA* and *mviA*$^+$, one on the chromosome and the other on a plasmid. Two sets of experiments tested this hypothesis. In one, all 10 fragments of *mviA* DNA, shown in Fig. 2, were cloned into pGB2 and then transformed into the *mviA*$^+$ strain WB335. The resulting strains were used to infect mice. Two of these clones (pWB4EH and pWB4093) extended 1.5–2 kb on either side of *mviA4093* and should have contained all of *mviA*. We observed that none of these clones was able to confer virulence on the recipient strain despite stable maintenance of the recombinant plasmid during the infections (data not shown).

In the other set of experiments, the plasmid pWB4184, carrying the fragment of *mviA*$^+$ DNA corresponding to that of *mviA* plasmid pWB4093, was stably maintained in the WB600 background strain WB4169. WB4169 is *mviA* and is isogenic with *mviA*$^+$ WB335. WB4169 was made by the insertion of a km insert in site *mviA4093* of WB335. As a control, some mice were infected with WB4169 harboring the plasmid pWB4186, which is pWB4184 containing an antibiotic cassette in *mviA4185*. Two independent strains were made with each plasmid. Salmonella containing the plasmid bearing unaltered *mviA*$^+$ DNA were avirulent (Fig. 5) in *Ity*^{s} mice, even though the recipient strain WB4169 was virulent in *Ity*^{s} mice (data not shown). The salmonella containing the identical plasmid, except with an insert in *mviA4185*, were virulent (Fig. 5). Taken together,

Table 1. *Bacterial Strains Used in this Study*

Strain*	Relevant genotype	Source and comment
Strains used for infections and for genetic constructions		
ATCC14028	Virulent	(26)
BS167	*Shigella flexneri galU*::Tn*10*	R. Curtiss (Washington University, St. Louis, MO)
C5	Virulent	(17)
JC3272	LT2 *gal300 trp*	J. Gougen (University of Massachusetts, Worcester, MA)
JL3404	LT2-Z *galE1122 mviA*$^+$	(24)
LE392	*E. coli hsdR514*	Laboratory collection
LT2-Z	*mviA*$^+$	C. Turnbough (UAB, Birmingham, AL)
NH337	*zde/5410*::Tn*10* (95% linked to *osmZ*)	N. P. Higgins (UAB, Birmingham, AL)
pGB2	Low copy number cloning vector	T. Elliott (UAB, Birmingham, AL) (27)
SA2876	pULB113 (RP4::miniMu Ap Tc Km)	K. Sanderson (University of Calgary) (28)
SL1344	Virulent	B.A.D. Stocker (University of California, Stanford) (29)
SR-11	Virulent	J. Berry (University of Texas) (30, 31)
TML	Virulent	A. O'Brien (Uniform Services, Bethesda, MD) (15, 32)
TT289	LT2 *mviA purE884*::Tn*10*	J. Roth (University of Utah) (33)
W118-2	Virulent	T. Eisenstein (Temple University) (34)
WB43	*mviA Δtrp-opp24*	WB600 *trpD*::Tn*10* (FA^r triornithine^r [24])
WB101	*gyrA mviA*$^+$	P22 (LT2-Z) × JL3404[b]
WB166-1	*Δtrp-opp24 opp3*::Tn*10 mviA*$^+$	P22 (WB167-1) × WB43
WB167-1	*opp3*::Tn*10 mviA*$^+$	WB101-Tn*10* hop select triornithine^r (24)
WB170	*opp3*::Tn*10 mviA*	P22 (WB167-1) × WB43
WB335	*mviA*$^+$	P22 (JL3404) × WB43[‡]
WB273	*zde4005*::km	SR-11 (pWB4005::km + pWB3097)
WB274	*zde4005*::*km*	TML (pWB4005::km + pWB3097)
WB276	*zde4005*::*km*	SL1344 (pWB4005::km + pWB3097)
WB278	*zde4005*::km	C5 (pWB4005::km + pWB3097)
WB280	*zde4005*::km	W118-2 (pWB4005::km + pWB3097)
WB282	*zde4005*::km	ATCC14028 (pWB4005::km + pWB3097)
WB290	*zde4005*::km	JL3404 (pWB4050::km + pWB3097)
WB296	*zde4005*::km *opp3*::Tn*10 mviA*	P22 (WB170) × WB290
WB600	*mviA*	P22 (LT2-Z) × TT289 (24)
WB3023	*galU*::Tn*10 trp*/pWB3007 Trp$^+$ Gal$^+$	WB600 pULB113 → WB9945[§]
WB3024	*galU*::Tn*10 trp*/pWB3024 Opp$^+$ Gal$^+$	Spontaneous Δ of pWB3007
WB3096	LE392 pWB3096	Origin of replication pSC101 km^r
WB3097	LE392 pWB3097	Origin of replication pSC101 cm^r
WB9944	*E. coli trp*	P1 (LE392) × JC3272[‡]
WB9945	*E coli, galU*::Tn*10 trp rpsL*	P1 (BS167) × WB9944[‡]

continued

the two complementation studies indicate that *mviA*$^+$ is dominant over *mviA*. This result was consistent with the possibility that *mviA* is a nonfunctional gene. These studies also indicate that plasmid pWB4184 carries a functional *mviA*$^+$ gene.

mviA Is Not Present in Six Other Virulent S. typhimurium Strains. In a previous study, an Hfr mating between a highly mouse-virulent strain, SR-11, and LT2-Z yielded *S. typhimurium* strain WB500, which was virulent in *Ity*^{s} but not *Ity*^{r} mice (23). Since LT2-Z was avirulent in *Ity*^{s} and *Ity*^{r} mice, the observation demonstrated that WB500 had acquired a gene(s) from SR-11 required to exploit the salmonella resistance defect of *Ity*^{s} mice. Since *mviA* is in the portion of the genome transferred during the construction of WB500 (23), it was expected that the SR-11 virulence gene was the same as *mviA*. However, subsequent Hfr conjugations into the

Table 1. *(continued)*

Strain*	Relevant genotype	Source and comment
Salmonella with inserts used to map *mviA*		
WB286	WB335 *zde4005*::km	Antibiotic insert, this study
WB288	WB600 *zde4005*::km	Antibiotic insert, this study
WB4073	WB600 *zde4006*::Am	Antibiotic insert, this study
WB4085	WB600 *zde4089*::km	Antibiotic insert, this study
WB4095-1	WB600 *zde/5410*::Tn*10* *mviA*$^+$	P22 (NH337) × WB600‡
WB4095-2	WB600 *zde/5410*::Tn*10* *mviA*	P22 (NH337) × WB600‡
WB4161	WB600 *zde4050*::km	Antibiotic insert, this study
WB4163	WB600 *zde4093*::cm	Antibiotic insert, this study
WB4165	WB600 *zde4093*::km	Antibiotic insert, this study
WB4166	WB600 *zde4093*::cm	Antibiotic insert, this study
WB4167	WB335 *zde4050*::km	Antibiotic insert, this study
WB4168	WB335 *zde4093*::km	Antibiotic insert, this study
WB4169	WB335 *zde4093*::cm	Antibiotic insert, this study
WB4170	WB335 *zde4089*::km	Antibiotic insert, this study
WB4171	WB335 *zde4089*::cm	Antibiotic insert, this study
WB4175	WB335 *zde4173*::km	Antibiotic insert, this study
WB4187	WB335 *zde4185*::km	Antibiotic insert, this study
WB4188	WB335 *zde4185*::km	Antibiotic insert, this study
WB4227	WB335 *zde4185*::cm	Antibiotic insert, this study

* Unless otherwise indicated, all strains are *S. typhimurium*.
‡ This designation indicates transduction crosses; for example, P22 (LT2-Z) × JL3404 indicates that JL3404 was infected with a P22 lysate grown on LT2-Z and selected for the phenotype of interest, in this case growth on galactose.
§ This designation indicates conjugation of the R-prime plasmid.

LT2-Z background suggested that SR-11 and also WB500 were *mviA*$^+$. To test this possibility, and to determine whether other virulent strains also carried the *mviA*$^+$ gene, P22 transductions were performed between the virulent strains and an *mviA* recipient (WB43) that carried the deletion *Δtrp-opp24*. Selection was for repair of *Δtrp-opp24*. This approach was based on the fact that by cotransduction with P22, repair of this deletion on the WB600 background was linked by ~50% to *mviA* (data not shown). Because the recipient in the present studies was *mviA*, we expected both *mviA*$^+$ and *mviA* Trp$^+$ transductants from *mviA*$^+$ donors. From *mviA* donors, the only type of transductants expected were *mviA*.

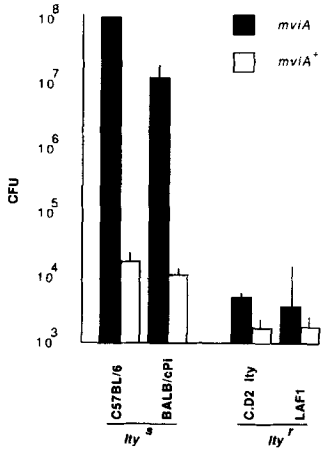

Figure 1. Effect of *mviA* and *mviA*$^+$ in *Ity*^{s} and *Ity*^{r} strains. Inbred mice were infected intravenously with 100 CFU of isogenic *mviA* (WB600) or *mviA*$^+$ (WB335) *S. typhimurium*. The bars indicate the geometric mean of the numbers of salmonella recovered in the livers and spleens (combined) of groups of 5–15 infected mice 6 d post-infection. Numbers of salmonella in dead mice were recorded as 10^8 for calculation of geometric means. All C57BL/6 mice died.

When WB600 (*mviA*) was used as a donor, all Trp$^+$ transductants were virulent. When the *mviA*$^+$ strains LT2-Z and WB335 were used as donors, two of four and four of four tested transductants were avirulent, verifying the close linkage of *mviA* with *Δtrp-opp24* (Table 4). The failure to observe virulent transductants when WB335 was used as donor was probably due to the small number of transductants tested. When the six mouse-virulent donors with unknown *mviA* were tested, the majority of the transductants obtained from each were also avirulent (Table 4). Since the above studies demonstrated that strains with the WB600 background were virulent, unless they have a functional *mviA*$^+$ gene, the results in these studies indicated that the virulent donor strains (other than WB600) had an equivalent of *mviA*$^+$ closely linked to the DNA required to repair *Δtrp-opp24*. This finding also suggested that the SR-11 gene(s) that increased the virulence of WB500 in *Ity*^{s} but not *Ity*^{r} mice (23) was not *mviA*.

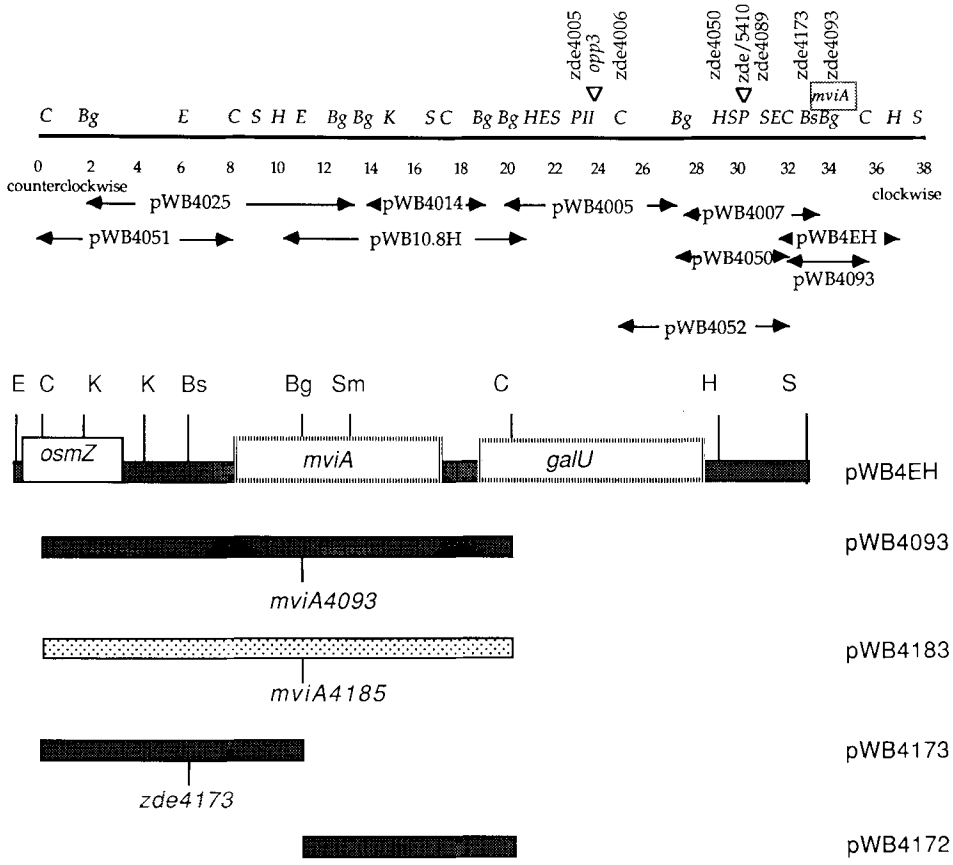

Figure 2. Restriction map of *S. typhimurium* chromosomal DNA in the region of *mviA*. This map is based on restriction maps of the overlapping cloned fragments, including the nine shown. The localization of *mviA* on the map is based on matings described in Table 2 and Fig. 3, and recombination with cassettes inserted in the chromosome described in Table 3. The *zde* numbers represent the positions of inserted antibiotic cassettes. (▽) Positions of Tn*10* insertions. Restriction enzyme sites are indicated by *Bg*, BglII; *Bs*, BstXI; *C*, ClaI; *E*, EcoRI; *H*, HindIII; *K*, KpnI; *PII*, PvuII; *P*, PstI; *S*, SalI.

Figure 3. A higher resolution map of chromosomal DNA including and immediately adjacent to *mviA*. Mapping of *mviA* on this fragment was accomplished in part by matings described in Table 2 and recombination with cassettes inserted in the chromosome described in Table 3. The numbers 4093 and 4173 etc. indicate the positions of inserted antibiotic cassettes and correspond to zde numbers from Fig. 2. The clones represented by black bars were cloned from *mviA* strain WB600, and the stippled bars represent clones from *mviA*$^+$ JL3404.

Because of this unexpected result, we more precisely mapped the position of the avirulence gene from the mouse-virulent strains. This time we used SR-11, TML, SL1344, and C5 as virulent *S. typhimurium* donors, and the *mviA* (*mviA4093*::cm) WB4163 salmonella strain as the recipient. The virulent recipient carried cm in the *mviA4093* site of the WB600 chromosome. To prepare the donor strains, the km antibiotic cassette was cloned into the PvuII site *zde4005* of plasmid pWB4005. This plasmid did not contain *mviA* but contained DNA close to *mviA* (see Fig. 2). The recombinant plasmid was electroporated into the virulent strains SR-11, TML, SL1344, and C5, and chased into the chromosome of these strains with plasmid pWB3097. Because the selected marker, *zde4005*::km is inserted with a plasmid that does not contain *mviA*, these newly constructed donor strains should express their original *mviA* genotype. P22 transduction was

Table 2. *Mapping of mviA*

Donor	Recipient	Recombination between:	Frequency of recombination between *mviA* and selected marker (direction)
WB296*	WB335	*zde4005*::*km* – *opp3*::Tn*10*	7/11 (clockwise)
WB166-1‡	WB600	*opp3*::Tn*10* – *mviA*	7/12 (clockwise)
WB290§	WB170	*opp3*::Tn*10* – *zde4050*::km	7/12 (clockwise)
NH337‡	WB600	*zde/5410*::Tn*10* – *mviA*	1/10
WB4175‖	WB4169	*zde4173*::*km* – *zde4093*::cm	0/4 *zde4093* (in*mviA*?)

* Resistance to either kanamycin or tetracycline was selected; only the transductants that recombined between the two were tested for virulence in mice.
‡ Resistance to tetracycline was selected, and recombinants were tested for virulence in mice.
§ Resistance to kanamycin was selected, and only tetracycline susceptible recombinants were tested for virulence in mice.
‖ Resistance to kanamycin was selected, and only chloramphenicol susceptible strains were tested for virulence in mice.

Table 3. *Transfer of Virulence to an mviA⁺ Recipient by Chromosomal Insertion of Antibiotic Cassettes in Specific Restriction Sites of Cloned mviA DNA*

Insert	Donor plasmid or chromosomal linkage	Site*	Virulence when forced into mviA⁺ chromosome
zde4005::km	pWB4005	PvuII	No
zde4006::km	pWB4005	ClaI	No
zde4050::km	pWB4050	HindIII	No
zde4089::km	pWB4089	SalI	No
zde4089::cm	pWB4089	SalI	No
zde4093::km	pWB4093	BglII	Yes
zde4093::cm	pWB4093	BglII	Yes
zde4173::km	pWB4173	BstXI	No
zde5410::Tn*10*	99% linked to *osmZ*	No	

* The positions of these sites are depicted in Figs. 2 and 3.

carried out between the virulent km^r donors and the virulent cm^r recipients. Transductants were selected for km^r and patched for loss of *zde4093*::cm.

Transductants that were km^r but cm^s were expected to have replaced the recipient *mviA* with donor *mviA*. The km^r transductants that retained the cm^r of the recipient were expected to have resulted from a crossover between *zde4005* and *zde4093*, and were expected to have retained the virulent recipient *mviA*. From Fig. 6, it is apparent that the km^r cm^r transductants were all as virulent as the *mviA* recipient, whereas the km^r cm^s strains were all avirulent. This observation confirmed the previous data and indicated that the avirulence gene(s) of strains SR-11, TML, SL1344, and C5 are at, or very near, a site homologous to that of *mviA* of WB600, and are most likely *mviA*$^+$.

Use of the Cloned 3.5-kb mviA DNA as a Probe to Look for mviA in Other Strains of Salmonella. To further test the pos-

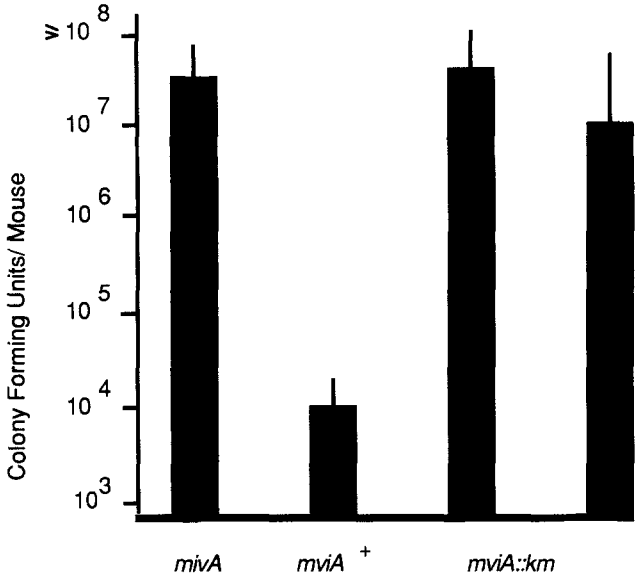

Figure 4. Effect on virulence of an insertion in *mviA*$^+$. WB335 is the isogenic *mviA*$^+$ derivative of WB600 (*mviA*). WB4187 and WB4188 are independently isolated derivatives of strain WB335, each containing an insertion mutation (a km cassette) in *mviA4185*. WB600, WB335, WB4187, and WB4188 were each injected intravenously into three to six mice. The mice were killed 6 d later, and the numbers of CFU in their combined spleen and liver were determined.

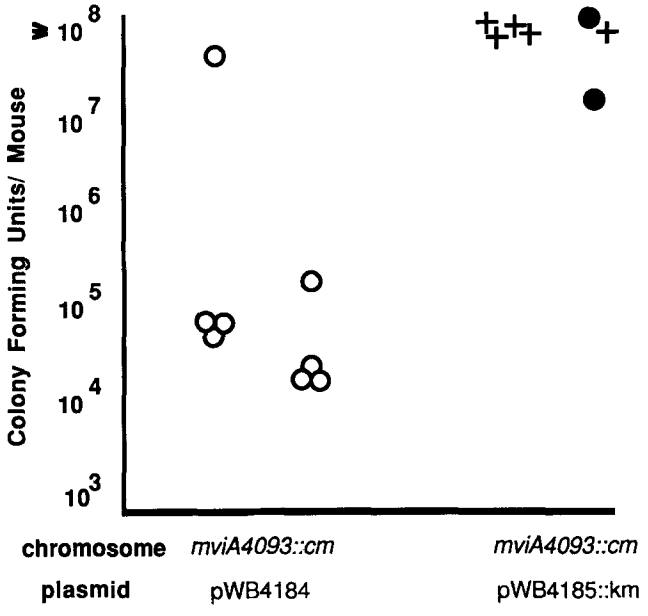

Figure 5. Mice were infected with 10^2 CFU intravenously of WB4169 with either of two plasmids, pWB4184 (3.5-kb ClaI *mviA*$^+$) or pWB4185 (pWB4184 with the *mviA4185*::km insert). Selection for the plasmids was maintained by feeding the mice streptomycin (resistance is coded for on the plasmids). Circles represent the number of combined CFU recovered from the liver and spleen of individual mice on day 6. Crosses indicate dead mice. Columns of circles and crosses represent results with independent plasmid transformants. One mouse infected with pWB4183 had $>10^7$ CFU. Analysis of 16 of 16 colonies recovered from that mouse indicated that they were no longer streptomycin resistant and had lost the plasmid.

Table 4. *Search for mviA among Mouse-virulent S. typhimurium by Transduction Repair of Δtrp-opp24 in the mviA Strain WB43*

Virulent donor*	Transductants‡ (avirulent/virulent§)
LT2-Z (*mviA*$^+$)	2/2
WB600 (*mviA*$^+$)‖	4/0
WB600 (*mviA*)	0/4
SR-11	6/2
TML	4/0
SL1344	3/1
C5	3/1
W118-2	3/0
ATCC14028	7/1

* All strains have been used for studies of salmonella pathogenesis in mice and all but LT2-Z and WB600 (mviA$^+$) are known to be mouse virulent: SR-11 (30, 31, 46, 47), TML (15, 48), SL1344 (29), C5 (49, 50), ATCC14028 (26), W118-2 (51), and WB600 (24).

‡ Selected for repair of *Δtrp-opp24*.

§ *Ity*^{s} mice were infected with 10^2 salmonella and killed 6 d later. Virulent strains were those that were recovered from the mice at 10^7 or greater. Avirulent strains were those that were recovered from mice at 10^5 or less.

‖ WB600 (mviA$^+$) is strain WB335.

sibility that the avirulence gene in the six virulent strains is an allele of *mviA*, we used the 3.5-kb fragment of WB600 that was able to transfer the *mviA* phenotype (pWB4093), to probe ClaI and HindIII cut genomic DNA from SR-11, TML, SL1344, C5, ATCC14028, W118-2, WB600, and WB335 after it had been run on a 0.6% agarose gel and transferred to nylon membrane. When the probe was hybridized to ClaI-cut DNA, we observed a single band from each of the eight strains at 3.5 kb. When the probe was used against HindIII-cut DNA, each strain yielded a single 7.5-kb band. These results make it very likely that an allele of *mviA* is probably located in the same map position in each strain. This result also indicates that the difference between *mviA* of WB600 and *mviA*$^+$ of the other strains is not due to a major deletion or rearrangement of DNA.

Determining the Relative In Vitro Growth Rate of mviA$^+$ *vs. mviA Salmonella.* We tested for the possibility that the difference in growth rate of *mviA*$^+$ and *mviA* salmonella in mice may also be found in vitro by comparing the growth rates of WB335 strains having insertionally inactivated *mviA* with isogenic strains having the same insertion cassette located in a closely linked site. The growth rates of two *mviA*$^+$ strains, WB4170 (4183::km) and WB4171 (4183::cm), and two *mviA* strains, WB4187 (4185::km) and WB4227 (4185::cm), were compared. Because the environmental conditions under which salmonella grow in vivo are not known, a number of different growth conditions were tested. In addition to culturing all four strains in LB broth under standard laboratory conditions, they were also cultured aerobically, anaerobically, in two different minimal media, at different osmolarities, at two different hydrogen ion concentrations (pH 4.5 and pH 7), and at two temperatures (37°C and 30°C). These conditions resulted in generation times from 18 min to >7 h. In no case did the difference in *mviA* affect the growth rate of salmonella (data not shown).

Discussion

These studies have examined the *mviA*$^+$ gene obtained from the avirulent LT2-Z strain JL3404 of *S. typhimurium*. Our data indicate that *mviA*$^+$ is a functional gene that

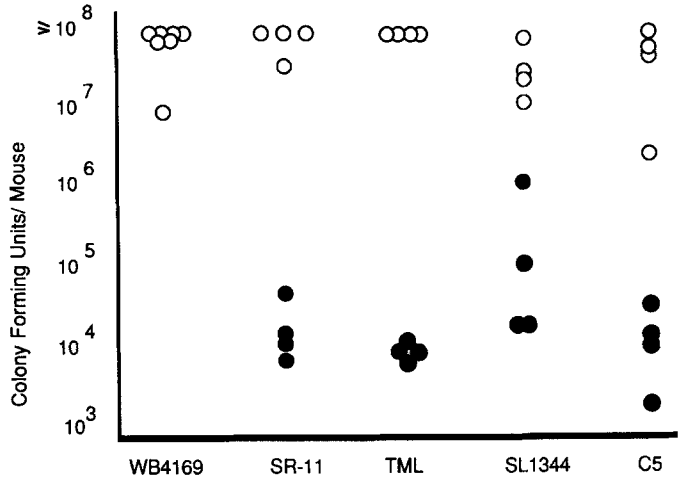

Figure 6. Plasmid expression of *mviA*$^+$. Analysis of *mviA* of four virulent strains of *S. typhimurium*. Virulent donor strains were prepared by electrotransformation into the virulent parents of pWB4005 containing km in its PvuII site and selection for insertion of the km cassette into the chromosome as described. Two independent km-containing strains of each donor were prepared. P22 lysates of these strains were then transduced into *mviA* WB4169 (WB600 background with a cm cloned into its *zde4093* site) with selection for km^r. For each donor, two km^r cm^r and two km^r cm^s transductants were selected. Each circle represents the geometric mean CFU recovered from two or three mice. Open circles depict data from km^r cm^r transductants, expected to express the recipient *mviA*. Filled circles depict data from km^r cm^s transductants expected to express the donor *mviA*. In all cases except two, the range of values for each average was <10-fold. The two exceptions were the km cm transductant of the C5 donors with the lowest numbers of CFU (range 10^5 to 4×10^7), and the km transductant of SR-11 with the highest numbers of CFU (range 10^4 to 7×10^5).

results in avirulence when placed in the background of otherwise virulent WB600. In the absence of a functional *mviA*$^+$ gene, strains of the WB600 background are virulent. We have cloned a fragment that expresses the functional *mviA*$^+$ gene and identified a restriction site, *mviA4185*, within the cloned DNA that permits the inactivation of *mviA*$^+$ by the insertion of an antibiotic cassette. In strains harboring both *mviA* and *mviA*$^+$, one on the chromosome and the other in a plasmid, *mviA*$^+$ is dominant regardless of whether it is borne by the chromosome or the plasmid. The difference between *mviA* (WB600) and *mviA*$^+$ (JL3404) is probably not the result of a large deletion or inversion of *mviA* DNA, since identical Southern blots were observed for these *mviA* and *mviA*$^+$ strains, when probed with a cloned *mviA* fragment.

The alleles *mviA* and *mviA*$^+$ were observed to have a large effect on the virulence of salmonella in *Ity*^{s} mice, but almost no effect on the virulence of salmonella in *Ity*^{r} mice. Any locus affecting salmonella growth rate would be expected to have a larger effect in *Ity*^{s} than *Ity*^{r} mice, because salmonella grow faster in *Ity*^{s} than *Ity*^{r} mice. However, the almost total absence of an effect of the *mviA* locus on virulence in *Ity*^{r} mice appears to be unique to *mviA* and suggests that there may be some direct genetic interaction between events controlled by the *mviA* and *Ity* loci. The mutation *aroA*, which almost completely blocks the growth of salmonella in vivo (19, 29), has at least a 100-fold effect on the numbers of salmonella recovered from *Ity*^{r} mice 6 d post-infection (19). *MviA*, by comparison, had less than a fourfold effect on the numbers of salmonella in *Ity*^{r}, mice under similar conditions. Because of the special interaction between the *mviA* and *Ity* loci, an eventual understanding of the mechanism of action of *mviA* may provide insight into the mechanism of action of *Ity*.

The major in vivo difference in the virulence of salmonella in *Ity*^{s} and *Ity*^{r} mice is a difference in growth rate (19, 20). Since *mviA* salmonella show greater net growth in *Ity*^{s} than *Ity*^{r} mice, it is likely that *mviA* regulates the rate of in vivo salmonella growth in *Ity*^{s} mice. The fact that slightly more *mviA*$^+$ salmonella were recovered from *Ity*^{s} than *Ity*^{r} mice may reflect the slightly greater killing of salmonella that has been observed in *Ity*^{r} vs. *Ity*^{s} mice (19) and in vitro in *Ity*^{r} vs. *Ity*^{s} macrophages (12).

When we examined six other virulent strains of *S. typhimurium* that have been used in a number of different laboratories in studies of salmonella pathogenesis, we observed that all carry *mviA*$^+$. These six non-WB600-virulent strains all are more virulent in *Ity*^{s} than *Ity*^{r} mice. We have previously demonstrated that one of these strains, SR-11, contains a gene or genes that can enable LT2-Z salmonella to become virulent in *Ity*^{s}, but not *Ity*^{r} mice (23). It is not known why the six non-WB600 mouse-virulent strains (all isolated independently) are *mviA*$^+$ rather than *mviA*. The fact that these highly virulent strains use a gene or genes, other than *mviA* to exploit *Ity*^{s} mice, does not preclude the possibility that the mechanism used by the non-WB600 strains to exploit *Ity*^{s} mice may be biochemically similar to the mechanism used by *mviA* WB600.

One way this might happen would be if *mviA*$^+$ was a regulated gene. The function of *mviA*$^+$ might be beneficial for growth in certain environments outside of the host, yet incompatible with necessary virulence in vivo. In mouse-virulent strains such as SR-11, *mviA*$^+$ expression may be suppressed by a regulator that can determine when the salmonella have reached an intracellular environment. This proposed regulator would permit SR-11 to preferentially exploit *Ity*^{s} vs. *Ity*^{r} mice. If the above hypothesis is correct, then the avirulence of most LT2 strains could be due to a mutation in the gene coding for the regulator. The virulence of LT2 strain, WB600, would be the result of a second mutation changing *mviA*$^+$ to *mviA*.

A possible explanation for the fact that the mouse gene *Ity*^{r} is dominant over *Ity*^{s} could be that the microenvironment of growing salmonella in *Ity*^{r}, but not *Ity*^{s}, mice contains a metabolite in high concentration that inhibits a salmonella pathway that would otherwise allow rapid growth in vivo. If this were the case, then *mviA*$^+$ might act by inhibiting the same salmonella pathway. This regulation might be accomplished directly by *mviA*$^+$ or its product MviA. Alternatively, *mviA*$^+$ might be involved in transport or synthesis of a metabolic inhibitor of the proposed pathway.

Since the *Ity* locus affects the resistance of mice to not only salmonella but also certain strains of *Mycobacterium bovis* (52), *Mycobacterium lepraemurium* (53), and *Leishmania donovani* (54), efforts to understand the action of *mviA* and other genes necessary to exploit the *Ity* locus may contribute to an understanding of the pathogenic mechanisms important in the resistance to a number of different intracellular pathogens.

We thank the many salmonella researchers who have generously supplied us with strains of *S. typhimurium*. Our studies have also benefited from the *Ity* congenic mouse stocks provided by Dr. Michael Potter at the National Cancer Institute, Bethesda, MD, and from helpful discussions with Patrick Higgins, Thomas Elliott, and Charles Turnbough of the University of Alabama at Birmingham.

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