The Salmo nella typhimurium Locus mviA Regulates Virulence in Ity⁴ but Not Ity⁺ 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 Ity⁴ mice, but not in Ity⁺ mice. A specific BglII site, mviA4185, between omz 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 × 10⁷ 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. cholerensis), 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, 8a). 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⁻ (resistant) and Ity⁺ (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⁺ but not Ity⁻ 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⁺ but not Ity⁻ 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 Ity⁺ but not Ity⁻ 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⁺ mice but not Ity⁻ mice and appears to be distinct from the genes responsible for the virulence of strain SR-11.
Materials and Methods

Mice. The mice used in this study included lty BALB/cAnPt and lty C.D2-ldh-t′ (N20) (25), raised from a stock obtained from M. Potter at the National Cancer Institute (NIH, Bethesda, MD). C.D2-ldh-t′ mice are congenic with BALB/cAnPt mice for a 20-cm region of DNA containing the lty locus. The C.D2-ldh-t′ mice were prepared by backcrossing the 20-cm portion of DBA/2 chromosome 1 containing the pep3 and ldh-t′ loci 20 times into lty BALB/cAnPt mice. It was found that the lty locus was also transferred as a passenger gene (25). lty C57BL/6J and lty LAF1, (C57L/J × A/J)F1, 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 JL340, 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 lty 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 Wl18-2 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. Supercopied 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 Gene cleaners (Bio 101 Inc, La Jolla, CA) to recover the DNA in a form suitable for ligation. Subclones were made by ligating the BgIII fragments into the low copy number vector pGB2, which has a pSC101 origin and codes for resistance to streptomycin and spectinomycin. 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′ km′ colonies were streaked for isolation, and large colonies were patched into 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 opp::3 TnlO insert. As more inserts were found, we tested for linkage of km′ and cm′ to each other. This allowed us to determine, for example, that all zde4093 inserts were 100% linked to each other and also to zde4185.

A chloramphenicol (cm′) 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, Sall, Acl, 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 TnlO and zde4185::TnlO 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 lty and lty 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 lty
BALB/cAnPt and Ity C.D2-ldh1+ (N20) mice as well as Ity C57BL/6J and Ity LAFl mice. As shown in Fig. 1, high levels of mviA salmonella were recovered from the Ity mouse strains 6 d post-infection: >10^6 salmonella were recovered from BALB/c mice and five of five C57BL/6 mice were already dead (>10^6 CFU). About 1/10,000 as many mviA^- than mviA salmonella were recovered from the two Ity strains. Quite different results were observed after infection of the Ity' mice. Both of the Ity' mouse strains showed high resistance to mviA' and mviA^- salmonella. Only three- to fourfold more salmonella were recovered from Ity' 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' mice but little if any effect in Ity' mice. Even though mviA^- salmonella were recovered in only low numbers from Ity' mice, there were several-fold more mviA^- salmonella in Ity' than Ity' 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 locate 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 Tn10 closely linked to osmZ (zde4005::Tn10) and a Tn10 inserted in opp (opp3::Tn10) (Fig. 2). Orientation of this region on the chromosome was determined from linkage of opp3::Tn10 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 BglIII 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 BglIII 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 BglIII site of pWB4184 and then forced the cassette into the chromosome of the mviA^- strain WB335. Two independent inserts were used to infect mice. As seen in Fig. 4, these mutants were as virulent in Ity' mice as WB600, indicating that interruption of mviA^- resulted in virulence. This BglIII 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 (pWB418EH 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 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' mice, even though the recipient strain WB4169 was virulent in Ity' 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 |
|---------|------------------|--------------------|
| ATCC14028 | Virulent | (26) |
| BS167 | *Shigella flexneri* galU::Tn10 | 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::Tn10 (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::Tn10 | J. Roth (University of Utah) (33) |
| W118-2 | Virulent | T. Eisenstein (Temple University) (34) |
| WB43 | mviA Δtrp-opp24 | WB600 trpD::Tn10 (FA' triornithine' [24]) |
| WB101 | gyrA mviA* | P22 (LTZ) × JL3404 |
| WB166-1 | Δtrp-opp24 opp3::Tn10 mviA* | P22 (WB167-1) × WB43 |
| WB167-1 | opp3::Tn10 mviA* | P22 (WB167-1) × WB43 |
| WB170 | opp3::Tn10 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::Tn10 mviA | P22 (WB170) × WB290 |
| WB600 | mviA | P22 (LT2-Z) × TT289 |
| WB3023 | galU::Tn10 trp/pWB3007 Trp* Gal* | WB600 pULB113 → WB9945 |
| WB3024 | galU::Tn10 trp/pWB3024 Opp* Gal* | Spontaneous Δ of pWB3007 |
| WB3096 | LE392 pWB3096 | Origin of replication pSC101 km' |
| WB3097 | LE392 pWB3097 | Origin of replication pSC101 cm' |
| WB9944 | E. coli trp | P1 (LE392) × JC3272 |
| WB9945 | E. coli galU::Tn10 trp rpsL | P1 (BS167) × WB9944 |

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. typhimu-

*rim strain WB500, which was virulent in *Ity* but not *Ity* mice (23). Since LT2-Z was avirulent in *Ity* and *Ity* mice, the observation demonstrated that WB500 had acquired a gene(s) from SR-11 required to exploit the salmonella resistance defect of *Ity* 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

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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/510::Tn10 mviA * | P22 (NH337) × WB600 |
| WB4095-2 | WB600 zde/510::Tn10 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 | WB600 zde4093::km  | Antibiotic insert, this study |
| WB4168 | WB600 zde4093::km  | Antibiotic insert, this study |
| WB4169 | WB600 zde4093::cm  | Antibiotic insert, this study |
| WB4170 | WB600 zde4089::km  | Antibiotic insert, this study |
| WB4171 | WB600 zde4089::cm  | Antibiotic insert, this study |
| WB4175 | WB600 zde4173::km  | Antibiotic insert, this study |
| WB4187 | WB600 zde4185::km  | Antibiotic insert, this study |
| WB4188 | WB600 zde4185::km  | Antibiotic insert, this study |
| WB4227 | WB600 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 Δ*trp-opp24*. Selection was for repair of Δ*trp-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*.

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 Δ*trp-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 Δ*trp-opp24*. This finding also suggested that the SR-11 gene(s) that increased the virulence of WB500 in *Itv* but not *Itv* mice (23) was not *mviA*.

![Figure 1. Effect of mviA and mviA † in Itv and Itv † 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 10⁸ for calculation of geometric means. All C57BL/6 mice died.](image-url)
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* (*mvi*A4093::cm) WB600 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 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::Tn10 | 7/11 (clockwise)                                                      |
| WB166-1†       | WB600     | opp3::Tn10 - mviA       | 7/12 (clockwise)                                                      |
| WB290‡         | WB170     | opp3::Tn10 - *zde4050::km | 7/12 (clockwise)                                                      |
| NH337†         | WB600     | *zde/5410::Tn10 - mviA  | 1/10                                                                  |
| WB4175§        | WB4169    | *zde4173::km* - *zde4093::cm | 0/4 *zde4093* (innvA?)*                                                |

* 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                                          |
| zde4093::cm    | pWB4093                              | BglII          | Yes                                         |
| zde4173::km    | pWB4173                              | BstXI          | No                                          |
| zde5410::Tn10  | 99% linked to osmZ                   |                |                                             |

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

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

Transductants that were km+ but cm- were expected to have replaced the recipient mviA with donor mviA. The km+ transductants that retained the cm- 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+ cm- transductants were all as virulent as the mviA recipient, whereas the km+ cm+ 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: SK-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.
‡ I1y mice were infected with 10² salmonella and killed 6 d later. Virulent strains were those that were recovered from the mice at 10⁷ or greater. Avirulent strains were those that were recovered from mice at 10⁵ or less.

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 Clal 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 Clal-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 transferred to nylon membrane when the probe was hybridized to Clal-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.

**Figure 6.** Plasmid expression of mviA +. Analysis of mviA + of four virulent strains of S. typhimurium. Virulent donor strains were prepared by electrottransformation 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-deleted salmonella (WB4169) with selection for km +. For each donor, two km + cm + and two km + cm - transductants were selected. Each circle represents the geometric mean CFU recovered from two or three mice. Open circles depict data from km + cm + transductants, expected to express the recipient mviA. Filled circles depict data from km + only transductants, expected to express the recipient mviA. Filled circles depict data from

km + cm - 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 10⁶ to 4 × 10⁷), and the km transductant of SR-11 with the highest numbers of CFU (range 10⁶ to 7 × 10⁷).
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+ mice, but almost no effect on the virulence of salmonella in Ity− mice. Any locus affecting salmonella growth rate would be expected to have a larger effect in Ity+ than Ity− mice, because salmonella grow faster in Ity+ than Ity− mice. However, the almost total absence of an effect of the mviA locus on virulence in Ity− 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 araA, 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+ mice 6 d post-infection (19). MviA, by comparison, had less than a fourfold effect on the numbers of salmonella in Ity− 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+ and Ity− mice is a difference in growth rate (19, 20). Since mviA salmonella show greater net growth in Ity+ than Ity− mice, it is likely that mviA regulates the rate of in vivo salmonella growth in Ity− mice. The fact that slightly more mviA+ salmonella were recovered from Ity+ than Ity− mice may reflect the slightly greater killing of salmonella that has been observed in Ity+ vs. Ity− mice (19) and in vitro in Ity+ vs. Ity− macrophages (12).

When we examined six other virulent strains of S. typhimurium that have been used in a number of different laborato-

ries in studies of salmonella pathogenesis, we observed that all carry mviA+. These six non-WB600-virulent strains all are more virulent in Ity+ than Ity− 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+, but not Ity− 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+ mice, does not preclude the possibility that the mechanism used by the non-WB600 strains to exploit Ity− 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+ vs. Ity− 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 is dominant over Ity+ could be that the microenvironment of growing salmonella in Ity+, but not Ity−, 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 leprae (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.
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