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mig-14 Is a Horizontally Acquired, Host-Induced Gene Required for *Salmonella enterica* Lethal Infection in the Murine Model of Typhoid Fever

RAPHAEL H. VALDIVIA,[1]* DANIELA M. CIRILLO,[1]* ANTHEA K. LEE,[1]
DONNA M. BOULEY,[2] AND STANLEY FALKOW[1]

Department of Microbiology and Immunology[1] and Department of Comparative Medicine,[2] Stanford University School of Medicine, Stanford University, Stanford, California 94305

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We have characterized a host-induced virulence gene, *mig-14*, that is required for fatal infection in the mouse model of enteric fever. *mig-14* is present in all *Salmonella enterica* subspecies I serovars and maps to a region of the chromosome that appears to have been acquired by horizontal transmission. A *mig-14* mutant replicated in host tissues early after infection but was later cleared from the spleens and livers of infected animals. Bacterial clearance by the host occurred concomitantly with an increase in gamma interferon levels and recruitment of macrophages, but few neutrophils, to the infection foci. We hypothesize that the *mig-14* gene product may repress immune system functions by interfering with normal cytokine expression in response to bacterial infections.

There are six subspecies of *Salmonella enterica* that are capable of colonizing both warm- and cold-blooded animals (1, 9). *S. enterica* subspecies I serovars are strictly associated with infection of warm-blooded animals and can cause a wide a range of diseases, including gastroenteritis, bacteremia, and typhoid fever (11). *S. enterica* serovar Typhimurium (from here on referred to as serovar Typhimurium) is the causative agent of gastroenteritis in humans and a typhoid-like disease in mice (11). Serovar Typhimurium survives and replicates within phagocytic cells of the reticuloendothelial system, resulting in the release of proinflammatory cytokines in response to bacterial compounds such as lipopolysaccharide (LPS) and peptidoglycan (11, 16, 17, 20). Two of these inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), are required for host clearance of *Salmonella* infections (11, 12, 16, 18). Treatment of infected animals with IFN-γ decreases the numbers of bacteria found in the spleen early in infection, and injection of anti-TNF-α or anti-IFN-γ abolishes the ability of mice to clear sublethal doses of serovar Typhimurium (12, 26, 27). Recently, it has been reported that modified *Salmonella* lipid A (an LPS component) can reduce the LPS-mediated expression of TNF-α by human monocytes and E-selectin by endothelial cells (14). These lipid A modifications are regulated by the PhoP/PhoQ virulence regulon (7, 13), suggesting a potential role for PhoP/PhoQ-activated genes not only in intracellular survival but also in lowering cytokine and chemokine production.

In the present study we characterized the virulence properties and evolutionary history of a host-induced serovar Typhimurium factor with potential immunomodulatory functions. A previous study described a PhoP/PhoQ-dependent, macrophage-inducible promoter, *fmi-14*, which exhibited a 22-fold induction within murine macrophages (36). To identify the open reading frame (ORF) associated with *fmi-14*, we isolated an adjacent 2.2-kb serovar Typhimurium DNA fragment by recombinational cloning (6). Sequence analysis of this fragment revealed a single ORF (*mig-14*) encoding a putative 298-amino-acid (aa) soluble polypeptide with limited homology to *Bacillus subtilis* RecG, an ATP-dependent DNA helicase (27% identity over a 102-aa overlap), and the LysR-like activator AppY from *Escherichia coli* (30% identity over 59 aa). To disrupt *mig-14*, a 1.6-kb *Cla*I fragment containing the 5′ end of *mig-14* was cloned into the allelic-exchange vector pRTP-1 (34), and an ΩKan^r (*aph*) cassette (8) was inserted at a unique *Bcl*I site within the *mig-14* coding sequence. The resulting plasmid was transformed into serovar Typhimurium strain SL1343R (*trp rpsL*) and gene replacement events were identified by Southern blot hybridization. The *mig-14*::*aph* mutation was mobilized into the virulent strain SL1344 (*xyl hisG rpsL*) by P22-HT-mediated transduction to create the strain RVY-5.

Virulence defects of *mig-14* mutants. We characterized the virulence defects of RVY-5 by determining the competitive index (CI) of the mutant strains at various time points. The CI is the ratio of RVY-5 to SL1344 present in different organs after challenge with a 1:1 ratio of wild-type to mutant bacteria. Groups of four female BALB/c mice were injected intraperitoneally with an equal mixture of SL1344 and RVY-5 (5×10^2 bacteria total). The mice were killed at days 3 and 5 postinfection; the spleens and livers were collected, homogenized, and plated on selective media to determine the number of CFU of each input strain. At day 3, RVY-5 and SL1344 were equally efficient at colonizing the liver (mean CI = 0.49 ± 0.29) and spleen (mean CI = 0.81 ± 0.27). However, by day 5, RVY-5 showed an ~10- to 50-fold reduction in its ability to compete with SL1344 (liver mean CI = 0.09 ± 0.07; spleen mean CI = 0.17 ± 0.16). To determine whether the *mig-14* mutation would impair the ability of serovar Typhimurium to colonize the gut-associated lymphoid tissue, we performed mixed infections and calculated the CI of RVY-5 after oral inoculation (Fig. 1A). Groups of four mice were inoculated intragastrically with equal numbers (10^6 bacteria total) of SL1344 and RVY-5. The animals were killed at days 3 and 5

* Corresponding authors. Present address for Raphael H. Valdivia: Department of Molecular and Cell Biology, University of California–Berkeley, 401 Barker Hall, Berkeley, CA 94720. Phone: (510) 642-5756. Fax: (510) 642-7846. E-mail: valdivia@uclink4.berkeley.edu. Present address for Daniela M. Cirillo: Lab. Microbiologia Clinica, Ospedale Molinette, Cso Bramante 88, 10126 Torino, Italy. Phone: 39-011-6335247. Fax: 39-011-6335194. E-mail: danielamc@mclink.it.

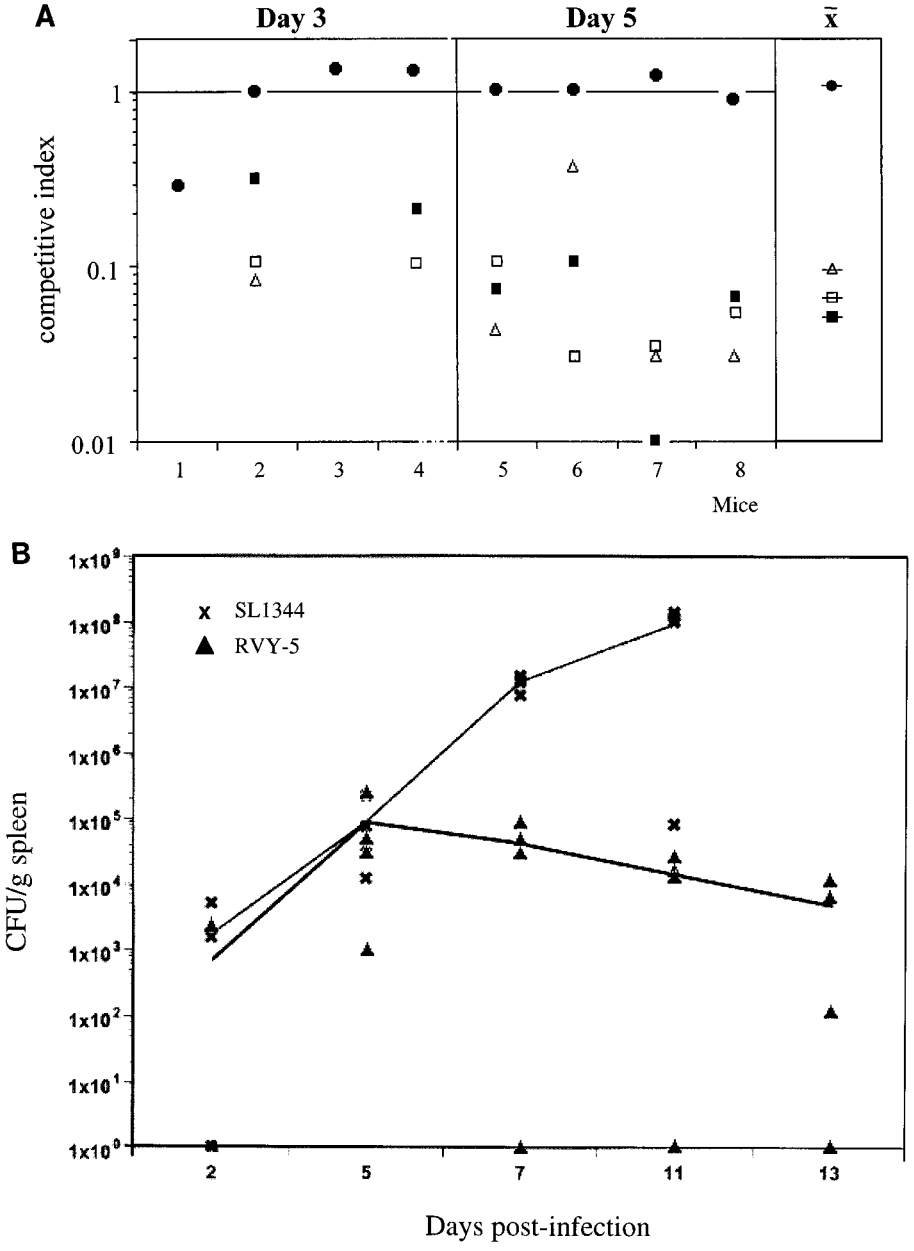

FIG. 1. Virulence defects of *mig-14* mutants. (A) Growth defects of RVY-5 in mixed infections. Groups of four mice were infected orally with a 1:1 mixture of SL1344 (wild type) and RVY-5 (*mig-14*::*aph*) (10^6 CFU per mouse). At days 3 and 5 postinfection, PP (●), MLN (□), spleens (S) (■), and livers (L) (△) were collected and the number of CFU for each strain was determined. The CI for RVY-5 was calculated as the ratio of RVY-5 to SL1344 recovered from the various organs. At day 3 postinfection, both strains colonized the PP, but RVY-5 was less efficient at colonizing the MLN, S, and L. At day 5, the CI for RVY-5 in the PP was about 1, while the CI in the MLN, L, and S ranged from 0.1 to 0.01 (x = mean CI at day 5). (B) In vivo growth kinetics of RVY-5. Groups of four mice were infected orally with either SL1344 or RVY-5 (5×10^6 organisms per mouse) and the numbers of CFU per gram of spleen were determined at days 2, 5, 7, 11, and 13 (see text). For days 5, 7, and 11 the statistical difference in the mean $\log_{10}$ CFU per gram of spleens infected with either SL1344 or RVY-5 was determined with the Student *t* test. At day 5, the mean CFU per gram of spleen for SL1344 and RVY-5 were not significantly different ($P > 0.2$). At day 7 and day 11, the differences in mean CFU per gram of spleen were significant ($P < 0.001$). Data shown are representative of at least three independent experiments.

postinfection, and bacteria from the Peyer's patches (PP), mesenteric lymph nodes (MLN), spleen, and liver were recovered on selective plates. At day 3 postinfection, RVY-5 colonized the PP as efficiently as SL1344 (at this time point only two out of the four mice showed bacterial spread beyond the MLN). At day 5, the bacterial load of RVY-5 in the PP was still similar to that of SL1344, yet we observed a 15- to 30-fold decrease in RVY-5's CI in the spleen and liver (Fig. 1A). Since it is possible that the RVY-5 CI in the spleen and liver reflects a delayed kinetics in the seeding of these organs rather than survival defects, we compared the growth kinetics of RVY-5 in the spleen and liver. Mice were infected orally with either 5×10^6 SL1344 organisms or 5×10^6 RVY-5 organisms (four mice per strain per time point). Animals were killed at days 2, 5, 7, 11, and 13 postinfection, and the numbers of CFU per gram of tissue were determined. Early during infection (day 2 and day 5) the bacterial load in the liver and spleen increased at similar rates in mice infected with both RVY-5 and SL1344. At day 5

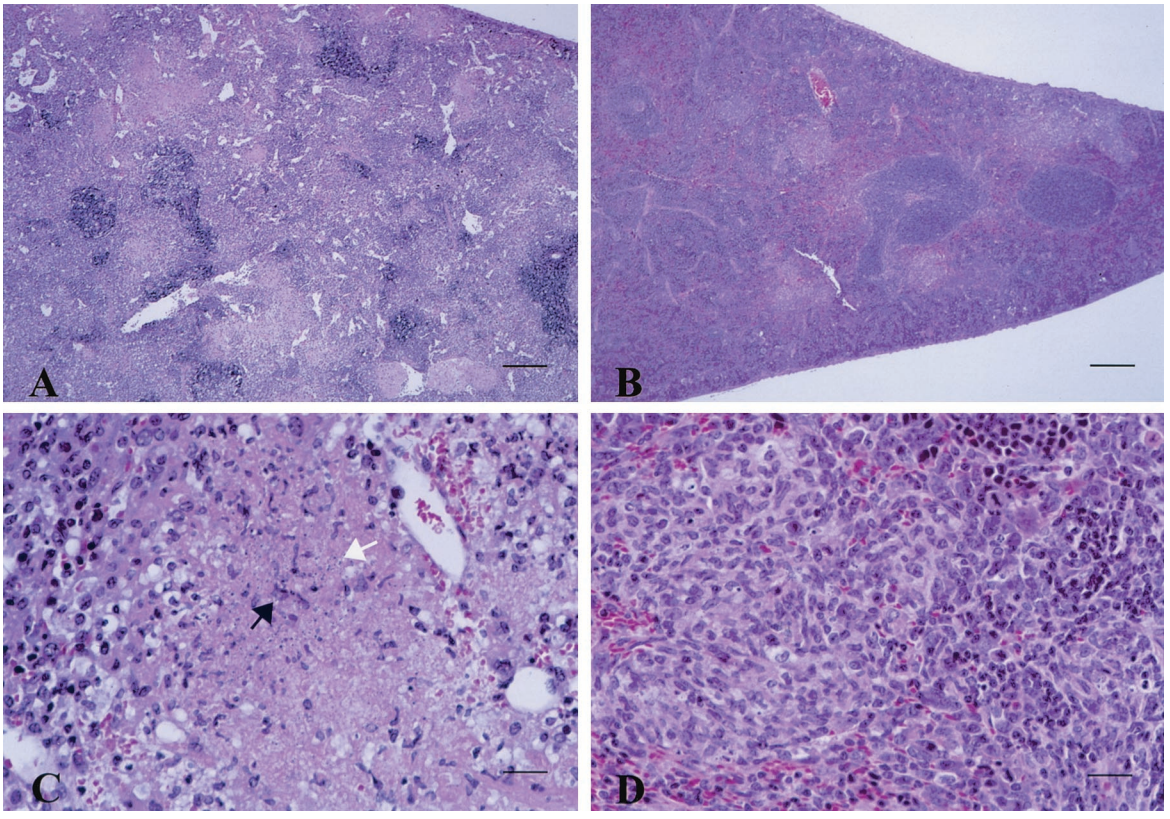

FIG. 2. Histopathology of mice infected with SL1344 and RVY-5. At day 6 postinfection, the overall splenic architecture is disrupted in mice infected with SL1344 (A). Necrotic cell debris (white arrow) predominates and rare viable neutrophils (black arrow) are present (C). In contrast, at 6 days postinfection, normal splenic architecture is largely maintained in the spleens of RVY-5-infected mice (B) and the affected areas contain chronic inflammatory cells consisting of mononuclear cells (lymphocytes, macrophages, and plasma cells), fibroblasts, few neutrophils, and minimal necrosis (D). Spleens were removed from infected mice, fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned, and stained with hemotoxylin-eosin. (Bars = 200 μm in panels A and B [×10], and 25 μm in panels C and D [×80].)

the mean $\log_{10}$ CFU of SL1344 was 4.71 ± 0.53 (n = 4) and that of RVY-5 was 4.39 ± 1.01 (n × 4). After day 5, the number of CFU per gram of tissue began to decrease in RVY-5-infected mice, and by day 13, the animals displayed a low-level chronic infection with no outward symptoms of disease (spleen $\log_{10}$ CFU of 4.71 ± 0.18 [n = 3] at day 7, 4.24 ± 0.16 [n = 3] at day 11, and 3.89 ± 1.29 [n = 3] at day 13). In contrast, SL1344 replicated exponentially until the deaths of the animals occurred, between days 7 and 11 ($\log_{10}$ CFU of 7.06 ± 0.12 [n = 4] at day 7 and 7.28 ± 1.38 [n = 4] at day 11). These experiments indicated that RVY-5 was capable of replicating in mice early during infection but was unable to overcome the host defenses at later stages. The bacterial load in mice infected with RVY-5 continued to decrease, so that 45 days after the oral inoculation with $>10^6$ CFU, no bacteria could be recovered from infected tissues (data not shown), suggesting that RVY-5 was cleared from tissues rather than persisting as a chronic infection. The oral 50% lethal dose (LD_{50}) for RVY-5 in BALB/c mice was determined by infection with 10-fold dilutions of either SL1344 or RVY-5 and monitored for 45 days. The oral LD_{50} (31) for RVY-5 was 1.21×10^8 bacteria per mouse, while the LD_{50} for SL1344 was less than 1×10^3 bacteria per mouse, suggesting that the competition experiments had underestimated the virulence defects of *mig-14* mutants. One possible explanation for the discrepancy between the CI and the LD_{50} measurements is that the *mig-14* mutation may be partially complemented in *trans* by coinfection with wild-type serovar Typhimurium. In support of this, we have observed that in mixed infections performed with higher infectious doses, *mig-14* mutants replicate at rates similar to those of wild-type bacteria (data not shown).

We confirmed that the virulence defect of RVY-5 was not due to polar effects of the Ω interposon on the expression of other genes by introducing *mig-14* on an episomal element into RVY-5. *mig-14* was amplified by PCR and inserted as an *Eco*RI fragment into the miniF-derived vector pBDJ121 (Amp^r) (gift of B. Jones). The resulting plasmid, pMIG14, was transformed into RVY-5 by electroporation. Groups of five mice were infected intragastrically with 10^6 CFU of SL1344, RVY-5, or RVY-5(pMIG14). SL1344 and RVY-5(pMIG14) killed all mice within 6 to 10 days, whereas all mice infected with RVY-5 survived.

Pathology and cytokine profiles of RVY-5-infected mice. We assessed the virulence properties of RVY-5 in tissue culture models of infection and have determined that *mig-14* is not required for invasion or replication within cultured or primary macrophages (data not shown). However, RVY-5 has a survival defect in the spleens and livers of infected animals during the later stages of infection, suggesting that RVY-5 may be more susceptible to clearance by the host after the immune system has been stimulated. We investigated the pathology of RVY-5 infections by collecting spleens, livers, MLN, and PP from mice infected with 10^6 RVY-5 or SL1344 organisms at 2, 6, and 11 days after oral inoculation. The tissues were fixed in 10% buffered neutral formalin solution, processed for routine histology, and examined by light microscopy. In both groups of

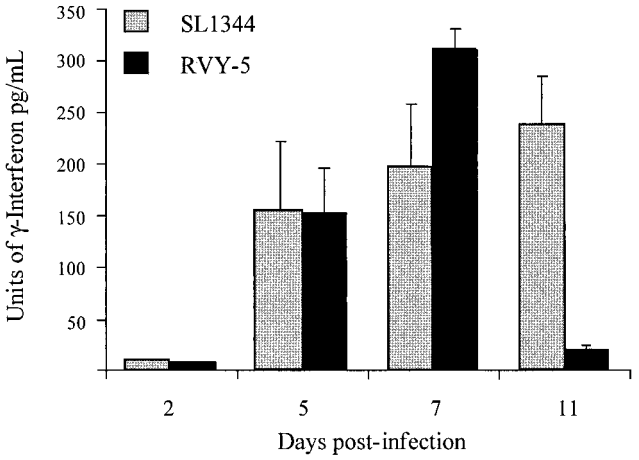

FIG. 3. Serum IFN-γ profiles of infected mice. Blood was collected from the hearts of infected mice on days 2, 5, 7, and 11 after the oral infection, and cytokine enzyme-linked immunosorbent assay for IFN-γ was performed on the serum. Spleens were removed and homogenized and CFU per gram of spleen were determined. On day 2 all animals displayed serum cytokine levels largely below the threshold of sensitivity of the detection kit. By day 5, mice infected with RVY-5 and SL1344 had similar levels of cytokines and bacterial loads in the spleen ($\log_{10}$ CFU, 3.48 ± 0.9 [n = 4] for SL1344 and 3.31 ± 0.81 [n = 4] for RVY-5). At day 7, RVY-5-infected mice displayed significantly higher levels of IFN-γ than mice infected with SL1344 even though the bacterial loads present in lymphoid organs were significantly lower ($\log_{10}$ CFU per gram of spleen, 5.75 ± 0.5 [n = 4] for SL1344 and 2.75 ± 0.06 [n = 4] for RVY-5). By day 11, concomitantly with clinical recovery, there was a drastic decrease in cytokine production in mice infected with RVY-5.

mice, the most consistent and striking lesions were found in the spleen (Fig. 2) and liver (not shown). At day 2 postinfection, rare to scattered neutrophil accumulations surrounding central necrotic cell debris were found in the splenic red pulp in four out of five mice infected with SL1344 and in three out of five mice infected with RVY-5 (data not shown). However, at day 6, the splenic lesions induced by RVY-5 were strikingly different from lesions induced by SL1344. SL1344-infected mice had severe coalescing splenic necrosis, while RVY-5-infected mice had lower numbers of inflammatory foci composed of mononuclear and fibroblastic cells, few neutrophils, and minimal necrosis (Fig. 2). The severe parenchymal necrosis and the lack of resolution of splenic and liver abscesses in the SL1344-infected mice most likely contributed to the mortality observed after day 6 postinoculation.

We examined the role of host response to serovar Typhimurium infection by examining the serum cytokine profiles of mice infected with either RVY-5 or SL1344 (Fig. 3). Blood was collected in a terminal bleed from the hearts of infected animals on days 2, 5, 7, and 11 postinfection. The number of CFU in the spleen and the levels of inflammatory cytokines (IFN-γ and TNF-α) in the circulating blood were determined with a capture cytokine enzyme-linked immunosorbent assay (Genzyme). The levels of TNF-α in the sera of RVY-5- and SL1344-infected mice were not significantly different between days 2 and 7 postinfection (data not shown). In contrast, mice infected with SL1344 and RVY-5 displayed a differential IFN-γ response. As previously reported (19, 30), SL1344-infected mice showed steadily increasing levels of IFN-γ until the deaths of the animals occurred (days 7 and 11). Interestingly, mice infected with RVY-5 showed IFN-γ levels significantly higher than those of serum from SL1344-infected mice (day 7), even though the bacterial load of RVY-5 was 100- to 1,000-fold lower than that of SL1344. These results suggest that infected mice were able to mount a stronger IFN-γ response to RVY-5 than to SL1344.

IFN-γ enhances the macrophage's capacity to generate respiratory bursts, increases the rate of lysosomal fusion with bacterium-containing phagosomes, and stimulates the production of nitric oxide (NO) from inducible NO synthase (iNOS) (39). Innate immune responses to intracellular pathogens, such as serovar Typhimurium, begin with interleukin-12 (IL-12) production by infected cells, leading to IFN-γ production by NK cells, which results in iNOS activation in macrophages (4). In vivo depletion of IL-12 with anti-IL-12 antibodies or inhibition of NO production with NG-monomethyl-L-arginine augments serovar typhimurium growth in infected tissues (18, 21–24, 35). NO has been reported to have bacteriostatic properties but is also required for proper macrophage and neutrophil migration into the infected spleen (23). We hypothesize that a robust IFN-γ response to RVY-5 may explain why the mice contained the growth of this mutant but not of its isogenic wild-type parent. It is possible that by interfering with the ability of the host to mount a full IFN-γ response, serovar Typhimurium enhances its survival during chronic infections. Future experiments will be aimed at determining which cytokine/chemokine response pathway may be compromised in mice infected with serovar Typhimurium.

***mig-14* is part of a horizontally acquired set of genes.** The 2.2-kb DNA fragment containing *mig-14* has a 39.9% GC content. This is in marked contrast with the GC content of the *Salmonella* chromosome (52%) (1). Since virulence genes with a low GC content are often part of larger clusters of virulence genes (pathogenicity islands), we isolated a 9-kb *Eco*RI fragment of the *Salmonella* chromosome containing *mig-14* and adjacent genes. DNA sequence analysis of this region indicated that *mig14* maps to centisome 61 (*smpB-nrdE* intergenic region) in the *S. enterica* chromosome. This region of the *Salmonella* chromosome (centisomes 59 to 61) contains several genetic markers that are absent from the *E. coli* chromosome and which are responsible for some of *Salmonella*'s unique physiological and biochemical characteristics (Fig. 4). The 9-kb *Eco*RI fragment contained 10 ORFs adjacent to *mig-14*. One ORF, present at the 5′ border of *mig-14*, encodes a putative protein with significant homology to the *Shigella flexneri* VirK. VirK is required for the posttranslational processing of the intracellular spreading factor IcsA (28). The nine ORFs at the 3′ border of *mig-14* encode NxiA, a putative Ni^{2+}-containing hydrogenase; TctE and TctD, two previously described regulators of tricarboxylate transport in *S. enterica* (38); three ORFs encoding a putative periplasmic membrane protein and two integral membrane proteins that are likely responsible for tricarboxylate transport (37); and three ORFs with high nucleotide homology to the *E. coli* genes *o360*, *ygaF*, and *gabD* (5) (Fig. 4). *mig-14* appears to be the only virulence factor gene in this region since serovar Typhimurium strains bearing mutations in *virK*, *nxiA*, and the structural (*tctI*) and regulatory (*tctDE*) genes of the *tct* locus were able to kill mice after an oral challenge with 10^6 bacteria per animal (reference 2 and data not shown). This was not unexpected since we have observed, through the use of *gfp* fusions, that neither the *tct* locus nor *nxiA* is induced in host cells (data not shown).

In *E. coli*, *o360* is adjacent to small ORFs with homology to phage components and *ileY*, encoding a tRNA (5). Since several phages and pathogenicity islands have been described to insert at tRNA sites (1, 9, 10), it is possible that the acquisition of *S. enterica*-specific genes at this locus may have begun by multiple insertions at *ileY*. Bäumler and Heffron have described this region of the *Salmonella* chromosome as containing a "mosaic-like structure" in which different segments are

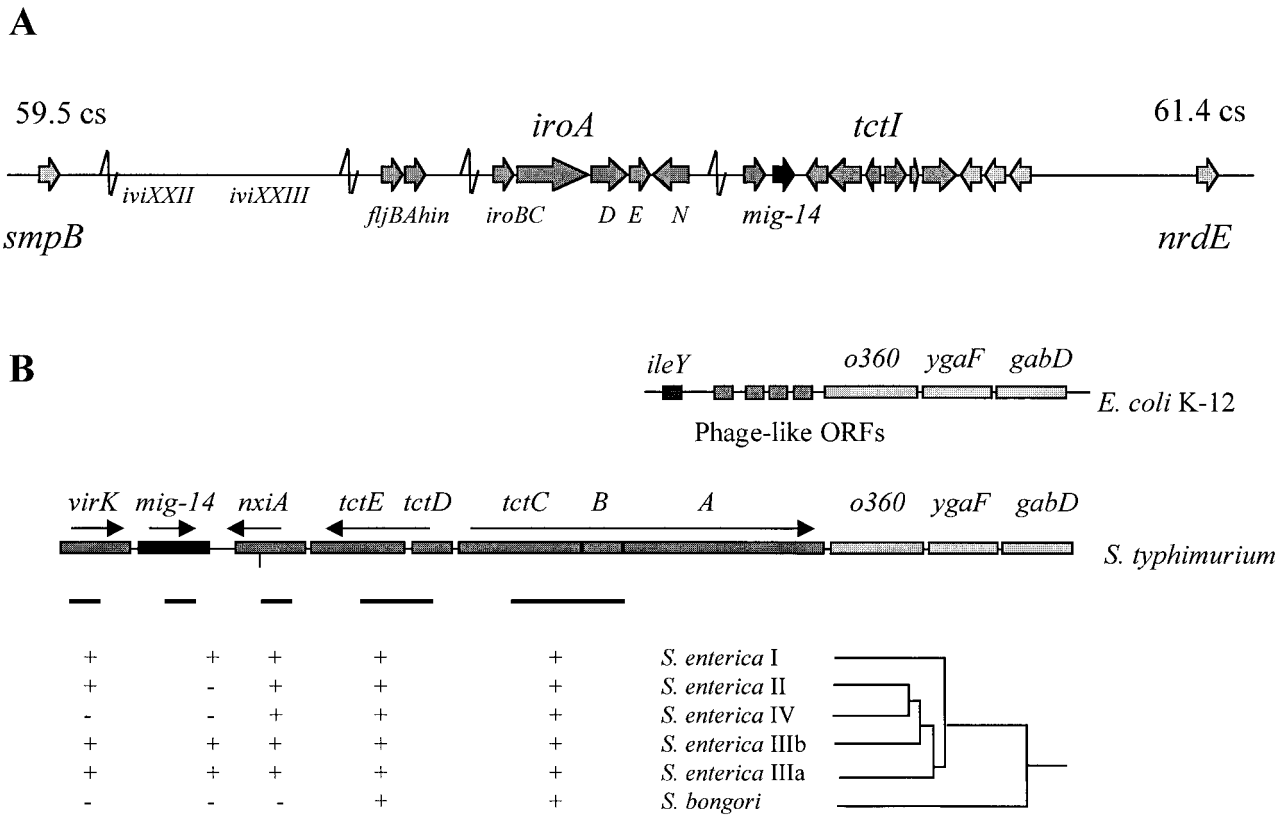

FIG. 4. The *mig-14* locus. (A) *mig-14* is located in a region of the *Salmonella* chromosome known to harbor horizontally acquired virulence genes and other *Salmonella*-specific genes. The map location of *ivi* genes, *iroN*, and *virK* has been previously described (2, 3, 15, 33). (B) *mig-14* is flanked by genes encoding a putative nickel transporter (*nxiA*) and a tricarboxylate transport apparatus (*tctI*) and homologues of the *S. flexneri virK* gene and the *E. coli* ORFs *o360*, *ygaF*, and *gabD*. In *E. coli*, *o360* and *ygaF* are adjacent to phage-like ORFs and the tRNA *ileY* (5). DNA hybridization experiments with PCR-generated probes (black bars) spanning internal regions of *virK*, *mig-14*, *nxiA*, *tctDE*, and *tctC* indicated that *mig-14* is present only in *S. enterica* subtypes I, IIIa, and IIIb. In contrast, the adjacent gene *nxiA* is present in all *S. enterica* subtypes but not *S. bongori*, while the *tctI* locus is found in all *Salmonella* species tested. The dendrogram (not to scale) shows the evolutionary relationship between varied *Salmonella* serovars as determined by DNA hybridization and multilocus enzyme electrophoresis (32). Representative serovars tested were *S. bongori* 48:z35:− and 44:r:− and *S. enterica* subgroup I serovars Typhi, Enteriditis, Choleraesuis, Dublin, and Gallinarum, subgroup II serovars Phoenix and 50:b:z6, subgroup IIIa serovars 48:g1z51:− and 41:z41z23:−, subgroup IIIb serovars 50:k:z and 61::c:z35, and subgroup IV serovars Marina and Chameleon.

present (or absent) in different *S. enterica* and *Salmonella bongori* serovars (2). This is in contrast to pathogenicity islands, where large clusters of genes appear to have been acquired as a unit. Sequence analysis of the region spanning *virK*, *mig-14*, *nxiA*, the *tctI* operon, and the *S. enterica* homologues of *o360* and *ygaF* indicated the presence of directed and inverted repeats between *mig-14*, *nxiA*, and *virK*. For example, the 3′ end of *mig-14* displays two tandem 35-bp direct repeats approximately 300 bp downstream of the putative transcriptional terminator. To test the possibility that each ORF was acquired (or deleted) independently during the evolution of *S. enterica*, we probed a collection of *S. enterica* and *S. bongori* isolates for the presence of different segments of the *mig-14* locus (Fig. 4). These DNA hybridization experiments indicated that *mig-14* was acquired by *S. enterica* after its split from the *S. bongori* lineage but was subsequently deleted in *S. enterica* subspecies II (Salamae) and IV (Houtenae). Furthermore, the acquisition or deletion of *mig-14* appears to have occurred independently of its neighboring genes *virK* and *nxiA*.

Mig-14 appears to be a unique virulence factor. Unlike the bulk of virulence genes, *mig-14* has no apparent role in the primary metabolism or housekeeping functions of the bacterium, it has been acquired as a mobile genetic element, and its expression is restricted to the intracellular environment of host cells (36). More importantly, *mig-14* appears to be a late-acting factor, because the phenotype of the mutant is not apparent until several days post-colonization. The molecular function of Mig-14 is unclear. The limited homology of Mig-14 to DNA-interacting proteins could indicate a potential role in the regulation of gene expression. If this is the case, it can be expected that the expression of other late-acting virulence factors would be under the control of Mig-14 and, indirectly, of PhoP/PhoQ. Future experiments will be aimed at determining the molecular mechanism of Mig-14 function and its potential role in immunomodulation.

A recurrent theme in *Salmonella* pathogenesis is the presence of mobile genetic elements that enhance the bacterium's pathogenic properties by conferring a broader host range or resistance to the host's immune system (1, 25, 29). A region of genetic "plasticity" in the chromosome, such as that encountered at centisome 61, may have permitted the rapid acquisition and deletion of virulence factors, such as Mig-14, as *S. enterica* species adapted to new warm-blooded hosts.

Nucleotide sequence accession number. The *Eco*RI fragment of the *Salmonella* chromosome containing *mig-14* and adjacent genes has been deposited in GenBank under accession no. AF020810.

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R.H.V. and D.M.C. contributed equally to this work.

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