

Invasion Genes Are Not Required for *Salmonella enterica* Serovar Typhimurium To Breach the Intestinal Epithelium: Evidence That *Salmonella* Pathogenicity Island 1 Has Alternative Functions during Infection

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***Salmonella enterica* serovar Typhimurium invasion genes are necessary for bacterial invasion of intestinal epithelial cells and are thought to allow salmonellae to enter and cross the intestinal epithelium during infection. Many invasion genes are encoded on *Salmonella* pathogenicity island 1 (SPI1), and their expression is activated by HilA, a transcription factor also encoded on SPI1. We have studied the role of *Salmonella* invasion genes during infection of mice following intragastric inoculation. We have found that strains containing a mutation in *hilA* or *invG* were recovered from the intestinal contents, intestinal tissues, and systemic tissues at a lower frequency than their parental wild-type strain. In contrast, a strain in which SPI1 is deleted was recovered from infected mice at a frequency similar to that of its parental wild-type strain. The Δ SPI1 phenotype indicates that *S. enterica* does not require invasion genes to cross the intestinal epithelium and infect systemic tissues. This result has forced us to reconsider the long-held belief that invasion genes directly mediate bacterial infection of the intestinal mucosa and traversal of the intestinal barrier during infection. Instead, our results suggest that *hilA* is required for bacterial colonization of the host intestine. The seemingly contradictory phenotype of the Δ SPI1 mutant suggests that deletion of another gene(s) encoded on SPI1 suppresses the *hilA* mutant defect. We propose a model for *S. enterica* pathogenesis in which *hilA* and invasion genes are required for salmonellae to overcome a host clearance response elicited by another SPI1 gene product(s).**

Infection with *Salmonella enterica* serovar Typhimurium can cause a systemic, typhoid-like disease in mice. Following ingestion, bacteria can colonize the intestinal tract, penetrate the intestinal epithelium, and access systemic sites such as the spleen and liver through the lymphatic and blood circulation (7). Passage of the bacteria through the intestinal wall is believed to be initiated by bacterial invasion into enterocytes and M cells (7, 22, 26, 43). The ability of salmonellae to penetrate the intestinal mucosa has been correlated with their ability to invade cultured nonphagocytic cells (14, 17, 26). *Salmonella* invasion into cultured epithelial cells is mediated by a bacterial type III secretion system (25). Secretion of bacterial proteins such as SopE and SptP into the host cell cytosol reorganizes the cytoskeleton, leading to membrane ruffling and bacterial uptake (12, 13, 19, 28, 46). The type III secretion system is encoded by genes on *Salmonella* pathogenicity island 1 (SPI1) (33). Expression of the SPI1 secretion system as well as many of its secreted effectors are coordinately regulated by HilA, a transcriptional activator encoded on SPI1 (1–3, 11).

Invasion genes appear to be important for serovar Typhimurium infection of mice via the gastric route. Ligated loop assays have shown that bacterial strains with mutations in *hilA* and other invasion genes have a reduced ability to enter and disrupt M cells compared to their wild-type (WT) parental strain (10, 26, 36). Studies of the dose of bacteria required to kill 50% of infected mice (50% lethal dose [LD₅₀]) also suggest that invasion genes are important for infection via the gastric

route. The LD₅₀ of invasion mutants administered intraperitoneally (i.p.) is the same as that of the WT, whereas the LD₅₀ of invasion mutants administered intragastrically (i.g.) is increased at least 20-fold over that of the WT (1, 4, 14, 26, 36). In these studies, i.p. inoculation is thought to deliver bacteria directly to systemic sites and bypass the need for invasion genes to traverse the intestinal wall. The decrease in virulence of a *hilA* mutant and other invasion mutants when inoculated i.g. but not i.p. suggests that invasion genes are important for entering and crossing the intestinal epithelium.

Invasion genes also contribute to the ability of salmonellae to induce migration of neutrophils (PMNs) into and across the intestinal epithelium (15, 27, 31). A hallmark of gastroenteritis, this transmigration response requires *Salmonella* adhesion to the epithelial apical membrane. Bacterial contact causes the epithelial cells to express and secrete chemokines that are chemotactic for PMNs (30). In vitro studies have shown that *hilA* and other SPI1 invasion genes are required for serovar Typhimurium to induce cytokine expression and transepithelial migration of PMNs (16). Invasion genes also appear to be involved in nitric oxide production in cultured colonic epithelial cells. A *Salmonella enterica* serovar Dublin mutant defective in *invA*, which encodes a component of the SPI1 secretion system, does not induce NO production compared to a WT strain. NO production increases in response to stimulation with a combination of gamma interferon and interleukin-1 or tumor necrosis factor alpha (45 and references within). Thus, invasion factors may enhance NO production by inducing the expression of these cytokines. Interestingly, serovar Typhimurium may induce invasion and the production of certain cytokines via the same mechanism. The delivery of SopE, one of the type III secretion effectors, into COS-1 cells activates a

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CDC42-dependent signal transduction cascade (19) which appears to stimulate both membrane ruffling and expression of interleukin-8 (21). Invasion genes have also been shown to be important for serovar Typhimurium to induce macrophage apoptosis *in vitro* (8, 34). SipB, an SPI1-secreted protein, directly interacts with and activates the proapoptosis enzyme caspase-1 in cultured macrophages (20).

In summary, invasion genes have the potential to contribute to many aspects of *Salmonella* pathogenesis by allowing the bacteria to directly and indirectly alter the behavior of host cells, such as epithelial cells, PMNs, and macrophages. Previous studies have primarily focused on investigating the role of invasion genes in allowing salmonellae to enter and cross intestinal epithelial cells during infection. We have explored the role of invasion genes during *Salmonella* pathogenesis by studying three mutant strains—one mutant containing a deletion of SPI1, one mutant containing an insertion mutation in *hilA*, and one mutant containing an insertion mutation in *invG*, a component of the type III secretion apparatus encoded on SPI1. We have compared the levels of mutant and WT bacteria recovered from different tissues following intragastric inoculation of mice. Results from these infection assays together with LD₅₀ determinations and other published results suggest that invasion genes may contribute to *Salmonella* pathogenesis by mediating bacterial interactions with nonepithelial cell types. We propose a model in which SPI1 is involved in a complex cross-talk scheme between the bacteria and the host.

MATERIALS AND METHODS

Bacterial strains and growth conditions. RM12, a clone of SL1344, was tested for virulence in mice and used as the WT serovar Typhimurium strain in this study (23). P22 was used to transduce mutations into the parental RM12 strain. RM40 contains the *hilA::Tn5lacZY-080 (tet)* mutation which was transduced from EE658 (3). RM69 contains the *Δspi1::kan* mutation in which SPI1 was deleted and replaced by a kanamycin resistance marker (41). The *Δspi1::kan* mutation was transduced from SD11 (41). RM50 contains the *invG::cam* mutation, which was transduced from SB201 (gift of Jorge Galán). All strains were grown in Luria-Bertani (LB) according to Sambrook et al. (40) without pH adjustment to saturation by rolling culture tubes overnight at 37°C. CFU were determined on LB-agar plates (40) containing 5 g of NaCl per liter plus streptomycin (100 mg/liter), tetracycline (25 mg/liter), kanamycin (50 mg/liter), or chloramphenicol (25 mg/liter) when appropriate.

Animal infections. Female BALB/c mice 6 to 8 weeks old (Taconic Farms, Inc.) were given drinking water containing streptomycin (5 mg/ml) for 24 h prior to infection. Fresh drinking water containing antibiotic was supplied every 4 days for the duration of the experiment. Animals were starved overnight prior to inoculation. Bacterial inocula were prepared by centrifuging broth cultures and resuspending the bacteria in phosphate-buffered saline (PBS); 0.1 ml of the inoculum was delivered i.g. using a feeding needle (Harvard Instruments). The CFU in each inoculum was determined by plating dilutions on agar plates containing appropriate antibiotics.

LD₅₀ and mean day to death. To determine LD₅₀s, groups of four animals were infected with the specified inocula (10, 100, 1,000, and 10,000 CFU) for each strain. Animal deaths were recorded for 3 weeks, and the LD₅₀ was estimated by the Reed and Muench method (39). The mean day to death refers to the average time it took animals to die.

C.I.'s and single infections. For competitive indices (C.I.'s) and total bacterial counts (CFU), infected tissues were harvested and homogenized in PBS, and dilutions were plated on LB-agar plates containing streptomycin, tetracycline, kanamycin, or chloramphenicol. Specifically, a fragment of the intestine approximately corresponding to the ileum, three Peyer's patches proximal to the cecum, mesenteric lymph nodes, and the spleen were removed from infected animals. The intestinal segment was further divided into tissue (intestine) and nontissue (contents) fractions by extruding the luminal material within each segment. All bacterial strains used are streptomycin resistant. All mutant strains used are resistant to an additional antibiotic, such as kanamycin, tetracycline, or chloramphenicol. Therefore, the WT CFU was estimated by subtracting the sum of CFU on kanamycin, chloramphenicol, and tetracycline (sum of CFU for mutant strains) from the CFU on streptomycin (CFU for all strains). C.I.'s were determined for each tissue sample by dividing mutant CFU by WT CFU.

Statistical analysis. All statistical tests were done using the Prism statistical analysis package (GraphPad Software, San Diego, Calif.). C.I.'s were logarithmically transformed, and arithmetic means were calculated and used for analysis. The 95% confidence interval for the log₁₀ C.I. of the mutants was compared to

0 (one-sample *t* test). For the CFU comparisons, paired and unpaired Student *t* tests were done for each tissue (mixed and single infections, respectively). Mean day to death for the WT and *hilA::tet* strains was compared by the generalized Wilcoxon test.

RESULTS

The ability of salmonellae to infect experimental animals via the i.g. route can be inconsistent (4). One explanation for this unreliability is inhibition of *Salmonella* colonization by the resident intestinal flora. Germ-free mice have been seen to have an LD₅₀ 1,000-fold lower than non-germ-free mice when infected with serovar Typhimurium (35). Reduction of microflora by treatment with streptomycin also increases the susceptibility of mice to infection by increasing the ability of salmonellae to colonize the intestinal tract and translocate to the mesentery, spleen, and liver (5, 32, 38). Similarly, we found that streptomycin treatment of mice prior to i.g. inoculation increases the reproducibility of infection and dramatically reduces the dose required for infection (data not shown).

The numbers of bacteria recovered from mice following infection can vary greatly from animal to animal. One way of reducing this variability is to perform mixed infections (4, 14). This approach allows direct comparison of mutant strains and the WT strain in the same animal. In addition to using the WT strain as an internal standard, mixed infections have the advantage of reducing the number of animals necessary for determining the infection phenotype of a mutant bacterial strain.

Mixed infections. Eight mice were infected with a total of 10⁴ CFU containing a mixture of WT and three mutant strains, a *hilA::tet*, a *Δspi1::kan*, and an *invG::cam* mutant, in an approximately 1:1:1:1 ratio (Fig. 1). Tissues were harvested 5 days postinoculation, homogenized, diluted, and plated on LB-agar plates containing antibiotics. All strains are streptomycin resistant; therefore, to determine the WT CFU, the sum of the mutant CFUs, as determined on agar plates containing tetracycline, kanamycin, or chloramphenicol, was subtracted from the total streptomycin-resistant CFU. The C.I. was determined as mutant CFU/WT CFU and compared to 1 (one-sample *t* test).

The mean C.I. for the *hilA::tet* and *invG::cam* mutants was lower than 1 for all of the tissues analyzed. However, the mean C.I. was not statistically significantly different from 1 for the *invG::cam* mutant in the spleen and for the *hilA::tet* mutant in the Peyer's patches and spleen ($P > 0.05$, one-sample *t* test). Our explanation for why statistical significance was not achieved is that these three groups of C.I.'s included a single outlier that represents tissues from the same animal.

The *Δspi1::kan* mutant and WT strains were recovered at a similar frequency from all tissues at all times postinoculation ($P > 0.05$, one-sample *t* test). Although the recovery of the *Δspi1::kan* mutant was not significantly different from that of the WT, the calculated mean C.I. was greater than 1 in the intestine, Peyer's patches, mesenteric lymph nodes, and spleen.

Mixed versus single infections. We were concerned that the presence of different bacterial strains in the infected mice could alter their virulence phenotypes. For example, the presence of WT bacteria might suppress a virulence defect of the *Δspi1::kan* mutant. In fact, complementation of the invasion defect of an *invE* mutant strain by WT bacteria has been reported to occur *in vitro* (18). In addition, the WT and/or the *Δspi1::kan* strain could compete for host factors and reduce the recovery of the *hilA::tet* mutant from a mixed infection. To investigate these possibilities, we compared results from mixed and single infections. Groups of four animals were inoculated with 10⁵ CFU of a single strain or 10⁵ CFU of a 1:1 mixture of strains. Tissues were harvested 5 days postinoculation.

Statistical analyses showed that the *hilA::tet* mutant was re-

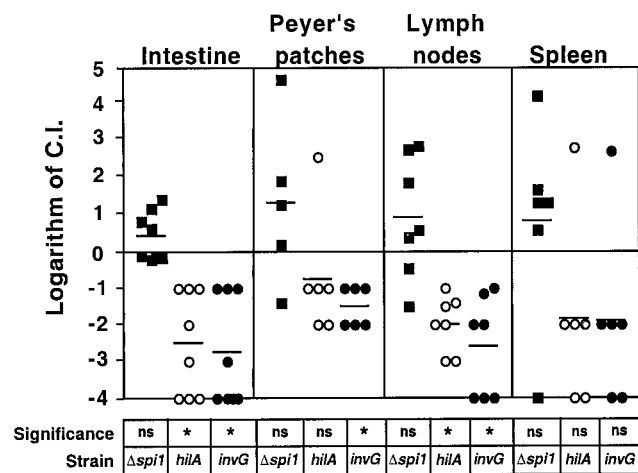


FIG. 1. Mean C.I. of mutant strains for different tissues at 5 days postinoculation. Eight mice were orally infected with a mixture of WT and mutant strains in equal proportions. Tissues were harvested at 5 days postinoculation, homogenized, and plated on streptomycin, tetracycline, kanamycin, or chloramphenicol. Mean C.I.'s were determined as described in Materials and Methods. Each symbol is the logarithm of C.I. Solid squares, C.I.'s for the $\Delta spi1::kan$ mutant; open circles, C.I.'s for the *hilA::tet* mutant; solid circles, C.I.'s for the *invG::cam* mutant. Horizontal lines are the mean of the logarithm of C.I.'s. ns, mean C.I. not significantly different from 1 (log C.I. = 0) ($P > 0.05$, one-sample t test). An asterisk indicates that a mean C.I. is significantly different from 1 (log C.I. = 0) ($P < 0.05$, one-sample t test).

covered at a reduced frequency compared to WT from Peyer's patches and mesenteric lymph nodes in both the single and mixed infections ($P < 0.05$, unpaired t test and paired t test, respectively) (Fig. 2A). The *hilA::tet* mutant was also recovered at a reduced frequency from the intestine and spleen in both infections; however, this reduction was only statistically significant in the case of mixed infections ($P < 0.05$, paired t test). The $\Delta spi1::kan$ mutant, on the other hand, was recovered at a similar frequency to the WT from all tissues in both single and mixed infections ($P > 0.05$, unpaired t test and paired t test, respectively) (Fig. 2B). These results suggest there is no trans-complementation of the $\Delta spi1::kan$ mutant by WT bacteria during a mixed infection and that the reduced recovery of the *hilA::tet* mutant is not dependent on coinfection with the WT strain.

Intestinal wall versus content. We were interested in knowing if the reduced recovery of the *hilA::tet* mutant in the intestinal wall (Fig. 1 and 2A) might be due to reduced colonization in the luminal fraction of the intestine. We also wanted to know if the $\Delta spi1::kan$ mutant was able to colonize the intestinal lumen as well as it could colonize the intestinal tissue. Groups of four animals were infected with a mixture of 10^5 CFU of WT and a mutant (the *hilA::tet* or $\Delta spi1::kan$ mutant) in an approximately 1:1 ratio. A fragment of the small intestine corresponding to the ileum was harvested at 5 days postinoculation. The intestinal contents and wall were homogenized separately and plated. Figure 3 shows the C.I.'s for both the *hilA::tet* and $\Delta spi1::kan$ mutants in these samples. The mean C.I. for the *hilA::tet* mutant is significantly less than 1 for both the intestinal wall and contents ($P < 0.05$, one-sample t test). The mean C.I. for the $\Delta spi1::kan$ mutant is not significantly different from 1 for both the intestinal wall and contents ($P > 0.05$, one-sample t test). There was no significant difference between the C.I.'s for each mutant strain in the intestinal wall versus the intestinal contents ($P > 0.05$, paired t test).

LD₅₀ and mean day to death. We obtained LD₅₀ and mean day to death values for the WT, $\Delta spi1::kan$, and *hilA::tet* strains using the Reed-Muench method (39) (Table 1). We infected four groups of four animals with 10, 100, 1,000, and 10,000 CFU and recorded deaths for 3 weeks. Mean day to death was determined as the average time it took mice to die at each dose.

As shown in Table 1, the LD₅₀s for the WT and the *hilA::tet* strains are similar (<10 CFU). It is possible that the LD₅₀ for the *hilA* mutant is actually higher than that for the WT strain, but 8 to 10 CFU was the lowest dose tested. In contrast, the LD₅₀ for the $\Delta spi1::kan$ mutant is at least 1,000-fold higher than the LD₅₀ for the WT strain. This result was surprising in light of our C.I. data showing that the $\Delta spi1::kan$ mutant is able to infect mice as efficiently as WT at 5 days postinoculation.

The mean day to death at the LD₅₀ for the *hilA::tet* mutant was higher than that for the WT strain (14 versus 9 days), although this difference was not statistically significant ($P > 0.05$, generalized Wilcoxon test). The mean day to death at the LD₅₀ for the $\Delta spi1::kan$ mutant was approximately the same as that for the *hilA::tet* mutant.

DISCUSSION

We have shown that invasion genes encoded on SPI1 are not necessary for serovar Typhimurium to enter and cross the intestinal wall. In addition, we have shown that invasion genes are important for bacterial colonization of the intestinal lumen. Our results suggest that deletion of a gene(s) on SPI1 suppresses the infection defect of the *hilA* and *invG* mutants. As discussed below, we propose that an SPI1-encoded factor stimulates host clearance responses in the intestine. In this model, invasion genes allow salmonellae to colonize the intestine by counteracting this host clearance response, possibly by causing the apoptosis of luminal phagocytes.

After i.g. inoculation, *hilA* and *invG* mutants are less able than a WT strain to infect intestinal and systemic tissues. By examining the contents of the intestinal lumen, we found that a mutation in *hilA* also reduces the recovery of salmonellae from the nontissue fraction of the ileum. Thus, *hilA* appears to be important for serovar Typhimurium to colonize the extracellular, luminal compartment of the intestine. Therefore, the reduced numbers of the *hilA* mutant found in the intestinal wall and systemic tissues might be the direct result of the reduced numbers of mutant bacteria present in the intestinal lumen.

Ligated loop assays have shown that invasion genes can be important for *Salmonella* entry into enterocytes, M cells, and Peyer's patches (9, 26, 36). Unfortunately, because ligated loop assays are so short, certain processes that affect bacterial colonization in vivo, such as bacterial replication and host clearance responses, cannot be assessed in these assays. Therefore, our finding that a *hilA* mutant exhibits an intestinal lumen colonization defect suggests that further work must be done to determine the relevance of the events observed during ligated loop assays for *Salmonella* pathogenesis in the murine model.

Several mechanisms might account for the *hilA* colonization defect. The *hilA* mutant might not adhere to or replicate at the mucosal surface. Another possibility is that the *hilA* mutant does not colonize the intestine because it is susceptible to bactericidal host factors such as phagocytes. Invasion genes have been found to be important for the ability of salmonellae to kill phagocytes in vitro (8, 20, 34). As discussed below, we favor the idea that the *hilA* mutant is defective in colonization because it is killed by phagocytes in the intestine.

Since we observed an infection defect in the *hilA::tet* mutant

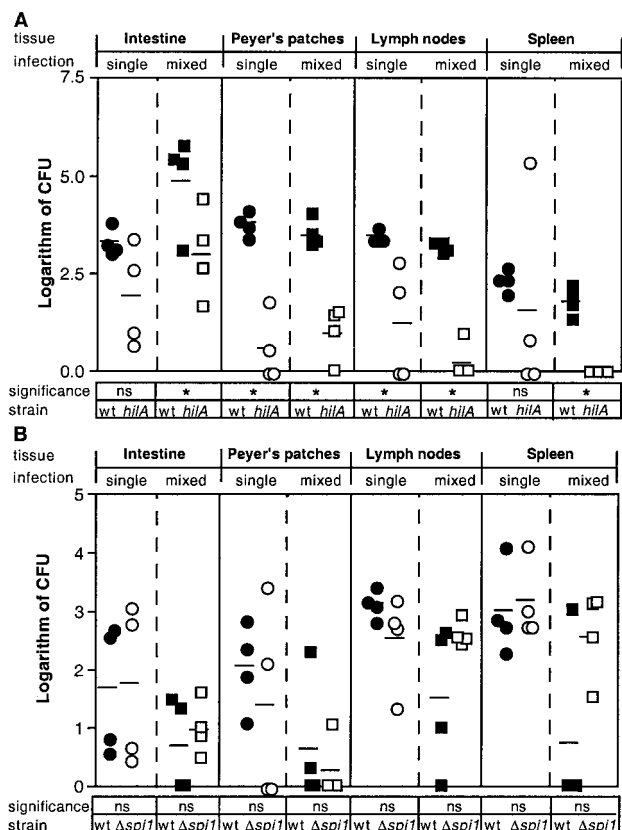


FIG. 2. Comparison of mixed and single infections. (A) Infection with WT and *hilA* mutant strains; (B) infection with WT and Δ *spi1* mutant strains. Groups of four mice were orally infected with 10^5 total CFU. Circles represent single infections with WT or mutant strains. Squares represent mixed infections in a 1:1 ratio of WT to mutant. Tissues were recovered 5 days postinoculation, homogenized, and plated on streptomycin, tetracycline, or kanamycin. Each symbol is the logarithm of CFU for each animal tissue. Solid symbols, WT CFU; open symbols, mutant CFU. Horizontal lines represent means of the logarithm of CFU. ns, no significant difference between the mutant and the WT logarithm of CFU ($P > 0.05$, nonpaired *t* test for single infections, paired *t* test for mixed infections). An asterisk indicates that there is a significant difference between the mutant and the WT logarithm of CFU ($P < 0.05$, nonpaired *t* test for single infections, paired *t* test for mixed infections).

5 days postinoculation, it was surprising that the LD₅₀ for this mutant was indistinguishable from that for the WT strain. However, although the *hilA* mutant is defective for infection at early times postinoculation, we have observed that after a delay, the *hilA* mutant can be recovered from systemic tissues at levels similar to those of the WT strain (data not shown). Invasion gene-independent mechanisms may ultimately allow the *hilA* mutant to enter nonphagocytic cells, infect systemic sites, and kill mice. In vitro studies suggest that salmonellae possess invasion factors in addition to those encoded on SPI1. Stone et al. have described two classes of mutations that reduce *Salmonella enterica* serovar Enteritidis entry into nonphagocytic cells and that do not map to SPI1 (42). Hong and Miller have reported that SigD, which is normally secreted by the SPI1 type III secretion system, contributes to a serovar Typhimurium invasion mechanism that can operate independently of *invA* (24). Ligated loop assays with *invA* and *invG* mutant strains suggest that under certain circumstances, invasion genes are not necessary for invasion into M cells (10). In addition, serovar Typhimurium might enter enterocytes and M cells via a receptor, in a similar fashion to CFTR-dependent

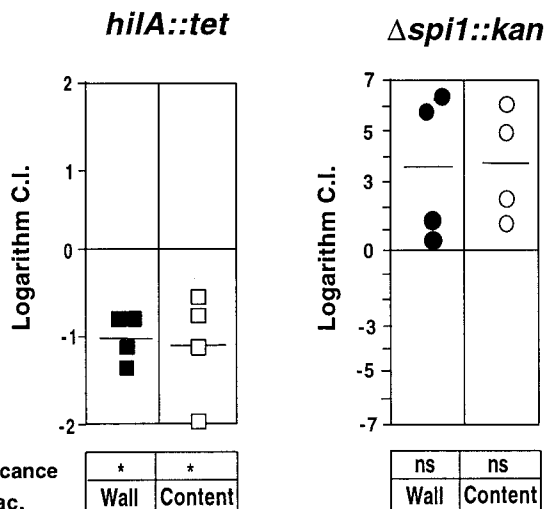


FIG. 3. C.I.'s for intestinal wall and contents of the *hilA::tet* and Δ *spi1::kan* mutant strains from individual animals, 5 days after oral inoculation. Symbols represent the logarithm of C.I., which was determined as described in Materials and Methods. Solid symbols, C.I.'s for the intestinal wall for both mutant strains; open symbols, C.I.'s for the intestinal contents for both mutant strains. Horizontal lines represent means of logarithm of C.I.'s. ns, mean C.I. not significantly different from 1 (log C.I. = 0) ($P > 0.05$, one-sample *t* test); an asterisk indicates that the mean C.I. is significantly different from 1 (log C.I. = 0) ($P < 0.05$, one-sample *t* test). Int. frac., intestinal fraction.

entry of *Salmonella enterica* serovar Typhi into epithelial cells (37). Alternatively, we favor the idea that bacterial entry into epithelial and M cells may be unnecessary for infection of the intestinal mucosa and systemic sites. In fact, in the absence of invasion genes, salmonellae appear to be shuttled from the intestine to the bloodstream within CD18-expressing phagocytes (44).

Our LD₅₀ results contrast with other findings showing that the i.g. LD₅₀ for a *hilA* mutant strain is 10- to 50-fold higher than that for the WT strain (1, 36). One possible explanation for the difference between our LD₅₀ results and previous reports is that we used a streptomycin-treated mouse model. While the LD₅₀ for WT salmonellae has been reported to be between 10^4 and 10^5 CFU (1, 14, 36), streptomycin treatment reduces the LD₅₀ to 10. We speculate that killing of resident intestinal microflora by streptomycin might enhance phagocyte migration into the intestinal lumen and therefore shuttling of bacteria from the intestine to systemic sites (44). Thus, streptomycin treatment may affect *Salmonella* infection in two ways—by reducing competition with the intestinal microflora and by increasing the contribution of phagocytes to systemic infection.

The most intriguing results we have obtained are from our infection studies with the Δ *spi1* mutant. This mutant strain was

TABLE 1. LD₅₀ and mean day to death for WT and mutant strains^a

Strain	Genotype	LD ₅₀ (CFU)	Mean day of death
RM12	WT	<10	9
RM40	<i>hilA::tet</i>	<10	14
RM69	Δ <i>spi1::kan</i>	3.4×10^3	13

^a Groups of four animals were infected with 10, 100, 1,000, or 10,000 CFU of each strain and followed for 3 weeks. LD₅₀ was determined by the Reed-Muench formula.

recovered at a frequency similar to that of the WT strain from all tissues. This result is surprising because the $\Delta spi1$ mutant lacks the *hilA* gene and so was expected to have an infection defect similar to that of the *hilA* mutant. Our results suggest that deletion of an SPI1 gene(s) in the $\Delta spi1$ strain suppresses the *hilA* infection defect and allows the salmonellae to colonize and infect their host in an SPI1-independent manner. We favor a model in which a *hilA*-independent SPI1 factor(s) stimulates a host clearance response that the bacteria can overcome by expressing a *hilA*-dependent factor(s).

It is also surprising that the $\Delta spi1$ strain can infect the intestinal mucosa and spread to systemic sites efficiently because, like other invasion mutants, the $\Delta spi1$ strain is not able to enter HEp-2 cells in vitro (S. Damrauer and C. Lee, unpublished results). However, as discussed above, invasion gene-independent mechanisms for bacterial penetration of the intestinal epithelium might account for the ability of the $\Delta spi1$ mutant to infect intestinal and systemic tissues.

Our result showing an increased LD₅₀ for the $\Delta spi1$ mutant was surprising considering the high recovery of this mutant in systemic tissues 5 days postinoculation. The number of *Salmonella* bacteria present at systemic sites is usually directly correlated with disease and death. One explanation for our results is that the $\Delta spi1$ strain might not induce certain host responses and consequently might not damage the host to the same degree as the WT strain. Interestingly, Khan et al. have reported that a *waaN* mutant strain defective in lipid A acylation is recovered at very high numbers in the spleens and livers of infected mice but does not induce cytokine expression, tissue damage, or death (29). In addition, the *waaN* mutant strain is ultimately cleared from the spleen and liver of infected animals. The $\Delta spi1$ mutant might similarly be cleared at later times postinoculation. In this case, SPI1 genes might be important for persistence of salmonellae at systemic sites.

Model for the contribution of SPI1 genes to virulence. We propose a working model to explain why invasion genes are important for bacterial colonization and persistence in the intestine (Fig. 4). Our model attempts to consider current ideas about the role of invasion genes in protein translocation and stimulation of host cell signal transduction as well as their potential role in altering epithelial cell, neutrophil, and macrophage biology. The infection phenotypes of the WT and *invG*, *hilA*, and $\Delta spi1$ mutant strains can be explained by speculating that three important events occur when serovar Typhimurium infects its murine host (Fig. 4). Analogous to elicitors and avirulence factors of plant pathogens (6, 47), *Salmonella* spp. express a *hilA*-independent SPI1 product that elicits a defense response in its host. The SPI1 elicitor may recruit or activate intestinal phagocytes which can clear the infection. However, expression of SPI1 invasion genes allows the bacteria to overcome the host clearance response, possibly by triggering apoptosis of the phagocytes in the intestinal lumen. Our results suggest that the host response is stimulated by the expression of an SPI1 elicitor gene(s) that is present in the WT, *invG*, and *hilA* strains but deleted in the $\Delta spi1$ strain. Although the WT strain and the *hilA* and *invG* mutants trigger the host defense mechanism, only the WT strain can successfully infect the host because it can express invasion genes and counteract the host response in the third step. In this scenario, the *hilA* and *invG* mutants are cleared by the recruited or activated phagocytes. In contrast, even in the absence of *hilA*, the $\Delta spi1$ strain can infect the host because it expresses no SPI1 elicitor and does not trigger host clearance mechanisms.

Although it may not seem beneficial to serovar Typhimurium to stimulate a host clearance response, there may be situations in which such an activity promotes pathogenesis, for

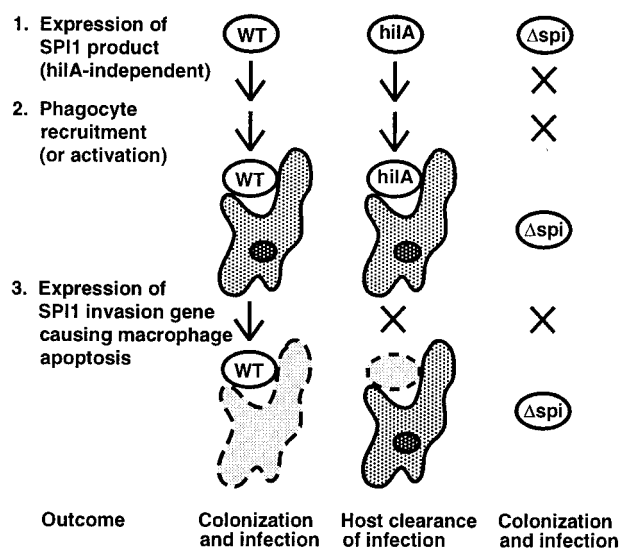


FIG. 4. Model for the role of SPI1 in colonization of the intestine by serovar Typhimurium. The left-hand column lists the three events that lead to colonization and infection by the WT strain. The bottom row shows the outcome for each bacterial strain. X's show which processes (arrows) are blocked for the mutant strains. Live bacteria (white) and host cells (shaded) are outlined in solid lines, whereas dashed outlines around bacteria or host cells (pale grey) indicate that they have been killed.

example, by inducing inflammatory diarrhea and thus enhancing bacterial transmission to new hosts. Future experiments will be aimed at identifying the SPI1 elicitor gene(s) that must be deleted to suppress the *hilA* infection defect. Such studies may shed light on the mechanisms by which mammalian hosts sense and defend against *Salmonella* infections.

Our in vivo studies of three SPI1 mutants have revealed new details about the interaction between serovar Typhimurium and its murine host. First, the contribution of SPI1 genes to pathogenesis may be more complex than previously thought. Our results indicate that invasion genes play a role in serovar Typhimurium colonization of the intestinal wall and lumen in mice. Second, our results suggest that an SPI1 gene product induces a host clearance response during *Salmonella* infection. Previous in vivo analyses had not revealed the possible contributions of SPI1 genes to bacterial colonization of the ileal lumen or to induction and evasion of a host clearance response. Our work emphasizes the importance of using several in vivo approaches and bacterial mutants to study the role of bacterial genes in pathogenesis.

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