INFECTION AND IMMUNITY, Oct. 2000, p. 5702–5709 Vol. 68, No. 10
0019-9567/00/$04.00+0


# *Salmonella* Pathogenicity Island 1-Independent Induction of Apoptosis in Infected Macrophages by *Salmonella enterica* Serotype Typhimurium

ADRIANUS W. M. VAN DER VELDEN,[1] SUSANNE W. LINDGREN,[1,2]
MICAH J. WORLEY,[1] AND FRED HEFFRON[1]*

*Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201-3098,[1] and Department of Biology, California State University, Sacramento, California 95819-6077[2]*

Received 14 April 2000/Accepted 30 June 2000

**The enteric pathogen *Salmonella enterica* serotype Typhimurium induces apoptosis in infected macrophages. This process is rapid, specific, and depends on the type III protein secretion system encoded within *Salmonella* pathogenicity island 1 (SPI1). Here, we demonstrate that serotype Typhimurium can activate programmed macrophage cell death independently of SPI1. SPI1 independent induction of apoptosis in infected macrophages is observed as early as 12 to 13 h postinfection, even in the absence of intracellular bacterial replication. Delayed activation of programmed macrophage cell death is not observed with serotype Typhimurium strains mutated in *ompR* or SPI2. Even though SPI2 mutants have a defect in intracellular proliferation, our results indicate that long-term intracellular survival and growth are not required for delayed macrophage killing per se, since *Salmonella* mutants that are severely defective in intracellular growth still induce delayed apoptosis. Inactivation of genes required for either rapid or delayed induction of apoptosis results in a conditional noncytotoxic phenotype, whereas simultaneous inactivation of genes required for both rapid and delayed induction of apoptosis renders serotype Typhimurium noncytotoxic under all conditions tested. Our hypothesis is that differential activation of programmed macrophage cell death by serotype Typhimurium occurs under discrete physiological conditions at distinct locations within an infected host.**

*Salmonella enterica* serotype Typhimurium is a facultative intracellular pathogen that causes a typhoid like disease in mice. Following oral infection, bacteria actively invade the intestinal mucosa and enter the bloodstream via the gut-associated lymphoid tissue (GALT). Subsequent residence within professional phagocytes of the liver and spleen is required for a persistent infection, which ultimately leads to the death of the mouse. Growth and survival of *Salmonella* within macrophages is supported by numerous studies, including the direct observation of *Salmonella* within hepatic phagocytes (45), comparative infection studies in genetic strains of mice that produce macrophages with varying resistance to *Salmonella* (38, 40), and the persistence of infection in mice treated with gentamicin, an antibiotic that primarily kills extracellular bacteria (10, 18). Finally, genetic studies indicate that *Salmonella* mutants that are attenuated for intramacrophage survival are also attenuated for systemic infection in mice (20). While all of these studies demonstrate that *Salmonella* survives and replicates within macrophages, several groups have recently shown that *Salmonella* is also able to kill these host cells (3, 13, 35, 39).

Contradictory results have been reported for *Salmonella* genes required for the induction of apoptosis as well as the timing at which it takes place. One study showed that serotype Typhimurium kills macrophages as late as 18 h postinfection (35). This process depends on the two-component regulatory system *ompR-envZ*, as *ompR* was the only gene identified in a stringent selection to find *Salmonella* mutants that are unable to kill macrophages. InvA is an essential structural component of the *Salmonella* pathogenicity island 1 (SPI1)-encoded type III export apparatus, whereas SipB is a SPI1-secreted effector molecule (22, 30). Null mutations in either *invA* or *sipB*, two genes within SPI1, had no effect on the ability of serotype Typhimurium to kill infected macrophages in this study (35). However, other studies appear to contradict these observations and demonstrate that within a few hours upon contact, serotype Typhimurium induces apoptosis in infected macrophages in an *invA* (and thus SPI1)-dependent process (13, 36, 39). SipB is both necessary and sufficient for the rapid activation of this apoptotic pathway (29).

Here, we resolve this apparent controversy by demonstrating that serotype Typhimurium kills macrophages via two independent processes. It is demonstrated that SPI1 gene expression accounts for rapid induction of apoptosis, whereas SPI1-independent, delayed induction of apoptosis is abrogated in strains mutated in *ompR* and SPI2. These results have important implications for understanding *Salmonella* pathogenesis, which are discussed.

## MATERIALS AND METHODS

**Bacterial strains, bacteriophages, and recombinant DNA techniques.** Bacteria were grown overnight in Luria-Bertani (LB) broth at 37°C. Antibiotics, when required, were used at the following concentrations: nalidixic acid (Nal), 50 μg/ml; chloramphenicol (Cam), 30 μg/ml; kanamycin (Kan), 60 μg/ml; and ampicillin (Amp), 100 μg/ml. Recombinant DNA techniques and Southern hybridizations were performed using standard protocols (4, 37). Analytical-grade chemicals were purchased from Sigma (St. Louis, Mo.) or Roche Biochemicals/Boehringer Mannheim (Indianapolis, Ind.).

Mutations in the *ompR*, *invA*, *spiB*, and *prc* genes have been described previously (20, 23, 35, 49) and were used to construct a set of isogenic serotype Typhimurium mutants (Table 1). Bacteriophage KB1*int* was used to transduce the *ompR*::Mud*J* allele of SWL350 (35) into SR-11 χ3041 (wild type [wt]), yielding strain AWM405 (*ompR*). Bacteriophage P22HT*int* was used to transduce the *invA*::Tn*phoA* allele of AJB75 (7) into AWM501 (*sipB*, see below) and AWM527 (*ssrB*, see below), yielding AWM544 (*invA sipB*) and AWM545 (*ssrB*

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Rd., L220, Portland, OR 97201-3098. Phone: (503) 494-6738. Fax: (503) 494-6862. E-mail: heffronf@ohsu.edu.

TABLE 1. Bacterial strains used in this study

| Strain | Genotype | Source or reference |
|---|---|---|
| *E. coli* | | |
| DH5α | *endA*1 *hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80 d*lac* Δ(*lacZ*)M15] | Laboratory collection |
| S17λ*pir* | *pro thi recA hsdR*::chromosomal RP4-2 (Tn*1*::ISR1 *tet*::Mu Km::Tn*7*); λ*pir* | 31 |
| *S. enterica* serotype Typhimurium | | |
| AJB3 | SR-11 χ4252 (Nal$^r$) | 51 |
| AJB75 | ATCC 14028 *invA*::Tn*phoA* (Kan$^r$) | 7 |
| ATCC 14028 | wt | ATCC |
| BA715 | ATCC 14028 *rpsL* (Str$^r$) | 1 |
| MJW129 | ATCC 14028 *ssrB*::*cat* (Cam$^r$) | This study |
| MS4290 | ATCC 14028 *prc*::Tn*10* (Tet$^r$) | 20 |
| SR-11 χ3041 | wt | R. Curtiss III |
| STN119 | IR715 *spiB*::mTn*5* (Kan$^r$) | 49 |
| SWL350 | SR-11 *ompR*::Mud*J* (Kan$^r$) | 35 |
| SWL2020 | SR-11 *invA*::*cat* | This study |
| SWL2025 | SR-11 *sipB*::*cat* | This study |
| AWM405 | SR-11 χ3041 *ompR*::Mud*J* | This study |
| AWM472 | SR-11 χ3041 *invA*::*cat* | This study |
| AWM501 | SR-11 χ3041 *sipB*::*cat* | This study |
| AWM498 | SR-11 χ3041 *ompR*::Mud*J invA*::*cat* | This study |
| AWM499 | SR-11 χ3041 *ompR*::Mud*J sipB*::*cat* | This study |
| AWM527 | SR-11 χ3041 *ssrB*::*cat* | This study |
| AWM543 | SR-11 χ3041 *ompR*::Mud*J ssrB*::*cat* | This study |
| AWM544 | SR-11 χ3041 *sipB*::*cat invA*::Tn*phoA* | This study |
| AWM545 | SR-11 χ3041 *ssrB*::*cat invA*::Tn*phoA* | This study |
| AWM568 | SR-11 χ3041 *spiB*::mTn*5* | This study |
| AWM664 | SR-11 χ3041 *prc*::Tn*10* | This study |

*invA*), respectively. Bacteriophage P22HT*int* was used to transduce the *sipB*::mTn*5* allele of STN119 (49) into SR-11 χ3041 (wt), yielding strain AWM568 (*sipB*). Bacteriophage P22HT*int* was also used to transduce the *prc*::Tn*10* allele of MS4290 (20) into SR-11 χ3041 (wt), yielding strain AWM664 (*prc*).

Allelic exchange was performed to disrupt the serotype Typhimurium *invA* gene. An internal fragment of the *invA* gene was amplified from serotype Typhimurium ATCC14028 (wt) using primers 5′-GCATGAATTCGCAGAACAG CGTCG-3′ and 5′-GTTGTCTAGATCTTTTCCTTAATTAAGCC-3′, which generated a PCR fragment with unique 5′ *Eco*RI and 3′ *Xba*I sites, respectively. This PCR product was cloned into the *Eco*RV site of pBluescript II SK(+) and sequenced. Subsequently, the *invA* allele was inactivated by insertion of a chloramphenicol resistance gene (a 1.2-kb *Sma*I fragment from pCMXX [7]) into a unique internal *Sna*BI site and cloned into suicide plasmid pKAS32 (48). The resulting plasmid was electroporated into *Escherichia coli* SM10λ*pir* and conjugated to serotype Typhimurium ATCC 14028 derivative BA715 (*rpsL*) (1). A double crossover at the *invA* allele was obtained via homologous recombination. A chloramphenicol- and streptomycin-resistant exconjugant was selected and named SWL2020 (*invA*). Bacteriophage KB1*int* was used to transduce the *invA*::*cat* mutation into SR-11 χ3041 (wt), yielding strain AWM472 (*invA*).

Allelic exchange was performed to disrupt the serotype Typhimurium *sipB* gene. A fragment of the *sipB* gene was amplified from serotype Typhimurium SR-11 (wt) using primers 5′-GAAGGTACCGAAGATGAGTCTCTGCGG-3′ and 5′-GAGCTCTTCTCAACAGAATGAT-3′, which generated a PCR fragment with unique 5′ *Kpn*I and 3′ *Sac*I sites, respectively. The resulting PCR product was blunt-end ligated into the *Eco*RV site of pBluescript SK(+) and sequenced to verify its accuracy. Subsequently, the *sipB* allele was inactivated by insertion of a chloramphenicol resistance gene (a 1.2-kb *Sma*I fragment from pCMXX [7]) into a unique *Sma*I site. This plasmid was restricted with *Kpn*I and *Sac*I, and the insertionally mutagenized *sipB*::*cat* allele was cloned into suicide plasmid pJP5603 (42). The resulting plasmid was electroporated into *E. coli* S17λ*pir* (31) and conjugated to AJB3, a nalidixic acid-resistant derivative of serotype Typhimurium SR-11 (51). A chloramphenicol- and nalidixic acid-resistant exconjugant was selected and named SWL2025 (*sipB*). Bacteriophage KB1*int* was used to transduce the *sipB*::*cat* mutant allele into SR-11 χ3041 (wt) and AWM405 (*ompR*), yielding strains AWM501 (*sipB*) and AWM499 (*ompR sipB*), respectively.

Allelic exchange was performed to disrupt the serotype Typhimurium *ssrB* gene. An 853-bp fragment of the *ssrB* allele was amplified from serotype Typhimurium ATCC14028 (wt) using primers 5′-CTTAATTTTCGCGAGGGCAG C-3′ and 5′-TAGAATACGACATGGTAAAGCCCG-3′. This PCR product was cloned into pCR-Blunt (Invitrogen, Carlsbad, Calif.). The *ssrB* allele was inactivated upon insertion of a chloramphenicol resistance gene (a 1.2-kb *Sma*I fragment from pCMXX [7]) into a unique *Ssp*I site. This plasmid was digested with *Eco*RI, and the disrupted *ssrB* allele was ligated into suicide vector pKAS32 (48). The resulting plasmid (pMJW99) was transformed into *E. coli* SM10λ*pir* and conjugated to serotype Typhimurium ATCC 14028 derivative BA715 (*rpsL*) (1). A double crossover at the *ssrB* allele was obtained via homologous recombination. A chloramphenicol- and streptomycin-resistant exconjugant was selected and named MJW129 (*ssrB*). Bacteriophage P22HT*int* was used to transduce the *ssrB*::*cat* mutant allele into SR-11 χ3041 (wt) and AWM405 (*ompR*), yielding strains AWM527 (*ssrB*) and AWM543 (*ompR ssrB*), respectively.

**Macrophage assays.** The murine derived macrophage cell lines J774 (American Type Culture Collection [ATCC], Manassas, Va.) and RAW264.7 (ATCC) were cultured (37°C, 5% $CO_2$) in Dulbecco modified Eagle medium (DMEM; Gibco-BRL, Rockville, Md.), supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), glutamine (Gibco-BRL), sodium pyruvate (Gibco-BRL), and nonessential amino acids (Gibco-BRL). Bone marrow-derived macrophages were isolated from C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) and cultured for 6 days (37°C, 5% $CO_2$) in DMEM supplemented with 10% FBS, 20% L929 supernatant (a generous gift from H. G. A. Bouwer, Immunology Research, VAMC, Portland, Oreg.), and glutamine and sodium pyruvate (Gibco-BRL).

Macrophage survival assays (gentamicin protection assays) were performed as described by Fields et al. (20). In brief, $10^5$ J774 macrophages were infected with stationary-phase cultures (below) at a multiplicity of infection (MOI) of ≤1. At 18 h postinfection, monolayers were washed three times with phosphate-buffered saline (PBS) and lysed with Triton X-100 (Sigma). Bacterial viability was determined by plating for CFU at various times postinfection. Similar results were obtained using RAW264.7 macrophages (data not shown).

The percentage of macrophage cytotoxicity was determined by measuring the release of host cytoplasmic lactate dehydrogenase (LDH). J774 and RAW264.7 macrophages were infected with bacterial cultures grown to either late-log phase or stationary phase (below) at an input MOI of ~60. At 1 h postinfection, infected monolayers were washed three times with PBS and lysed with Triton X-100 (Sigma), after which bacterial uptake was determined by plating for viable intracellular CFU. Differences between strains were observed and taken into account by normalizing to the number of internalized bacteria (approximately 1% of input bacteria). At 6 and 18 h postinfection, the release of LDH was quantified colorimetrically using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wis.). The absorbance ($A_{490}$) was determined on a microplate reader (Dynatech Laboratories, Inc., Chantilly, Va.), after which the percentage of cytotoxicity was calculated using the following formula: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. The spontaneous release is the amount of LDH released from the

cytoplasm of uninfected macrophages, whereas the maximum release is the amount of LDH present in whole-cell lysates from uninfected macrophages.

In addition to measuring the release of LDH, quantitative macrophage cytotoxicity assays were performed as described by Lindgren et al. (35; data not shown). In brief, to determine the MOI at which 50% of the infected macrophages are killed ($MOI_{CD50}$), $10^5$ J774 macrophages were infected with twofold serial dilutions of bacterial cultures ($31 \leq MOI \leq 1{,}000$, the limits of detection), as verified by plating for CFU. At 6 and 18 h postinfection, the remaining viable macrophages were fixed in a 10% formalin solution (10 to 15 min) and stained in a 0.13% crystal violet solution (>2 h). The absorbance ($A_{595}$) was determined on a microplate reader (Dynatech Laboratories); the MOI for the well that gave 50% of the absorbance recorded for uninfected wells was considered the $MOI_{CD50}$ (i.e., 50% of the cytotoxic dose). Similar results were obtained using RAW264.7 macrophages (data not shown).

The Cell Death Detection ELISA$^{PLUS}$ Assay (Roche Diagnostics Corp.) was used to determine whether serotype Typhimurium-infected macrophages were undergoing apoptosis. This assay has been used successfully to study *Pseudomonas aeruginosa*-induced apoptosis in eukaryotic cells (26). Macrophages were infected with bacterial cultures grown to either late-log phase (data not shown) or stationary phase (below) at an infection rate of 1.5 bacteria per macrophage. The amount of cytoplasmically located histones bound to fragmented DNA was quantified colorimetrically at 18 h postinfection, after which the absorbance ($A_{410nm}$) was determined on a microtiter plate reader. An enrichment factor indicative of apoptosis was calculated using the following formula: ($A_{410}$[experimental])/($A_{410}$[uninfected]).

Bacterial cultures were grown under various conditions. To obtain stationary-phase cultures, bacteria were grown aerobically in LB broth (3 ml) for 15 h at 37°C. To obtain late-log phase cultures, bacteria were grown overnight (aerobically, 15 h at 37°C) in LB broth (3 ml), subcultured 1:20 in LB broth (3 ml), and grown to late-log phase (3 h) under the same culture conditions. Using a Mud*J* transcriptional fusion to *sipB*, optimal transcription of SPI1 genes in late-log phase cultures was confirmed since under these culture conditions high levels of β-galactosidase were produced (data not shown).

## RESULTS

**Serovar Typhimurium kills macrophages independently of SPI1.** Conflicting reports on macrophage killing (13, 35, 39) prompted us to investigate the effect of bacterial growth phase on the ability of serotype Typhimurium to kill macrophages. Throughout this study, two complementary methods were used to determine *Salmonella*-induced cell death in both J774 and RAW264.7 macrophages. In addition to measuring the release of cytoplasmic LDH, macrophage killing was calculated using a quantified macrophage cytotoxicity assay (data not shown) (35). Strikingly similar results were obtained with these two independent assays. *Salmonella*-induced macrophage cell death was determined by measuring the release of LDH at infection rates of about 0.7 and 1.5 bacteria per macrophage. Other MOIs were also tested, with identical results (data not shown).

Under SPI1-inducing conditions (see Materials and Methods) (13), rapid, SPI1-dependent macrophage killing was observed (Fig. 1A). In contrast, bacterial cultures grown to stationary phase, while unable to rapidly kill infected macrophages, induced a delayed cytotoxic effect (Fig. 1B). Delayed induction of macrophage cell death required neither *invA* nor *sipB* (Fig. 1B) and was observed as early as 12 to 13 h postinfection (Fig. 1C). These results suggest that serotype Typhimurium induces delayed macrophage cell death independently of SPI1.

**SPI2 and *ompR* are required for delayed macrophage killing.** Delayed cytotoxicity was dependent on a functional *ompR* locus, since *ompR* mutant bacteria were unable to kill infected macrophages (Fig. 2A). Recent evidence suggests that OmpR activates transcription of the SPI2 encoded regulon *ssrAB* (32). This operon is essential for the transcription of SPI2 genes (14), which are highly induced inside macrophages (16, 50). To test whether, in addition to *ompR*, SPI2 is required for delayed induction of macrophage cell death, serotype Typhimurium strains mutated in *ssrB* and *spiB* were tested. These genes encode a transcriptional activator and a structural component of the SPI2 encoded type III protein export apparatus, respectively (41). As shown in Fig. 2B, serotype Typhimurium strains mutated in *ompR*, *ssrB*, or *spiB* were unable to kill infected macrophages when grown to stationary phase prior to infection. However, these strains were fully cytotoxic under SPI1 inducing conditions (Fig. 2C), indicating that *ompR* and SPI2 are not required for rapid induction of macrophage cell death. Cumulatively, these results suggest that delayed, SPI1-inde-

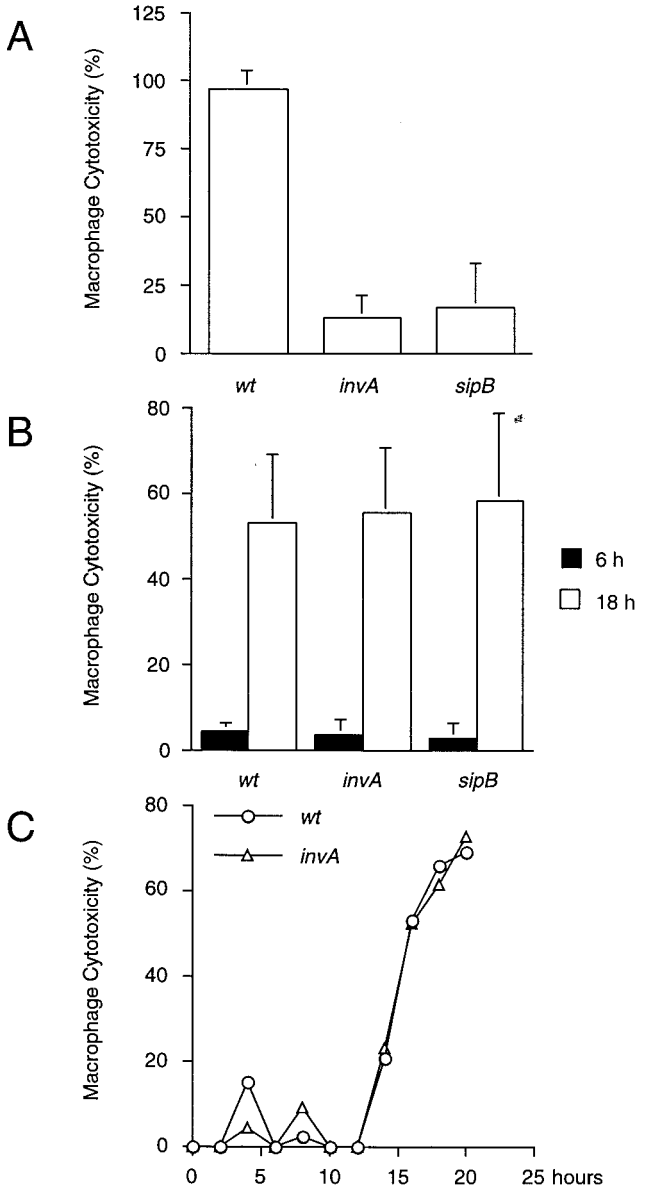


FIG. 1. Serovar Typhimurium kills macrophages independently of SPI1. J774 macrophages were infected with late-log-phase (A) or stationary-phase (B) cultures of wt serotype Typhimurium or strains carrying null mutations in *invA* or *sipB*. Bacterial growth was monitored by measuring optical density at 600 nm (see Materials and Methods; also, data not shown). (A and B) Macrophage cell death was quantitated at 6 h (A) and 18 h (B) postinfection by measuring the release of LDH. (C) Using stationary-phase cultures of either wild-type serotype Typhimurium or an *invA*-deficient strain, macrophage cytotoxicity was monitored for 20 h and quantitated at 2-h intervals by measuring the release of LDH. Data from the graphs in panels A and B are arithmetic means of at least three independent experiments. Error bars indicate the standard deviations of the mean. The data from graph C are representative of two independent experiments.

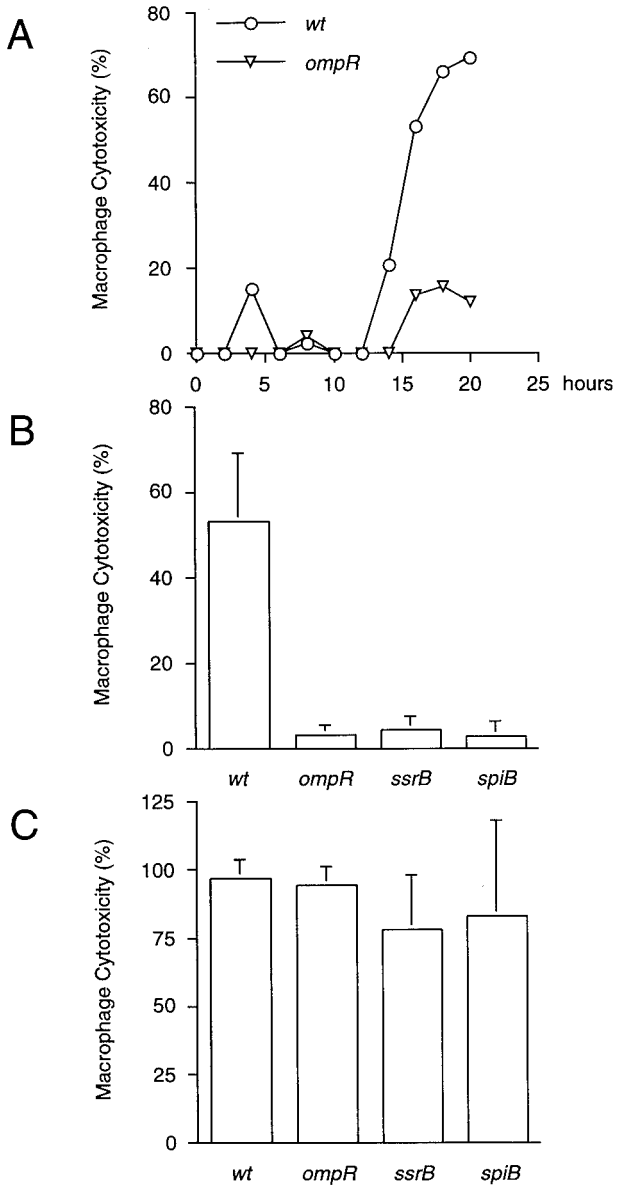


FIG. 2. SPI2 and *ompR* are required for delayed macrophage killing. (A) J774 macrophages were infected with stationary-phase cultures of either wt serotype Typhimurium or an *ompR*-deficient strain, after which macrophage cytotoxicity was monitored for 20 h and quantitated at 2-h intervals by measuring the release of LDH. (B and C) In addition, J774 macrophages were infected with stationary-phase (B) or late-log-phase (C) cultures of wild-type serotype Typhimurium or strains carrying null mutations in either *ompR*, *ssrB*, or *spiB*. Macrophage cell death was quantitated at 18 h (B) and 6 h (C) postinfection by measuring the release of LDH. Data from the graph in panel A are representative of two independent experiments. The data from the graphs in panels B and C are arithmetic means of at least three independent experiments. The error bars indicate the standard deviations of the mean.

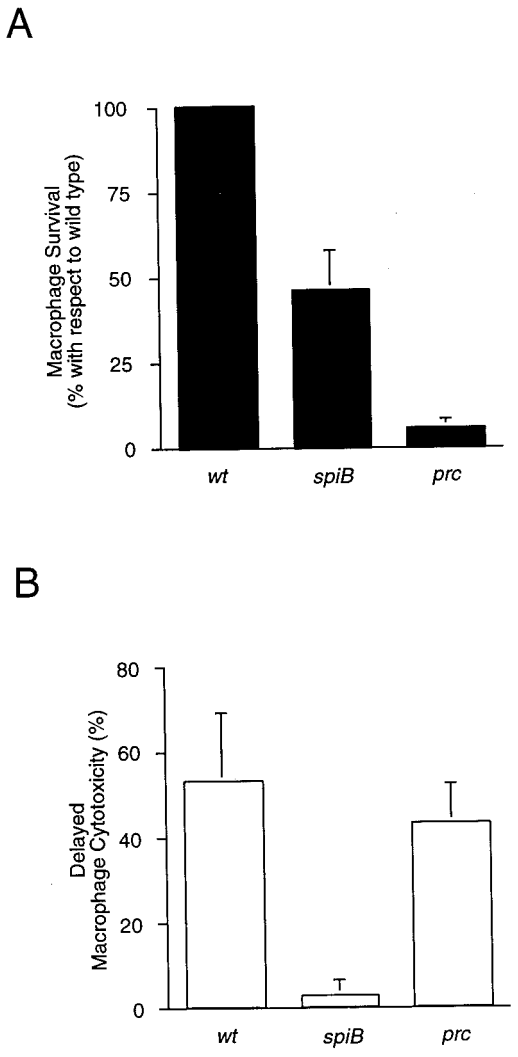


FIG. 3. Long-term intracellular survival and growth is not required for delayed macrophage killing. (A) J774 macrophages were infected with stationary-phase cultures (conditions shown to turn off SPI1-dependent rapid induction of macrophage cell death) of either wild-type serotype Typhimurium, a *spiB* mutant strain, or a macrophage-sensitive *prc*-deficient strain, after which macrophage survival was determined at 15 and 18 h postinfection (three times each) by measuring the viable intracellular CFU. (B) Macrophage cytotoxicity was quantitated at these times by measuring the release of cytoplasmic LDH. The data are arithmetic means of at least three independent experiments from 15-h time points. The error bars indicate the standard deviations of the mean.

pendent cytotoxic effects are masked under conditions that turn on SPI1 gene expression.

In agreement with the literature, we observed a defect (2- to 10-fold) in intracellular proliferation for SPI2 mutant strains at 15 h postinfection (14, 27, 28, 41, 46). However, long-term intracellular survival and proliferation is not required for delayed macrophage killing per se, since a *prc* mutant, encoding a periplasmic protease (6, 20) required for intracellular survival and growth (Fig. 3A) (11, 21), kills infected macrophages as efficiently as the wild type (Fig. 3B). Thus, despite a profound macrophage survival defect, the *prc* mutant was fully cytotoxic. In fact, the *prc* mutant strain was representative of a large panel of serotype Typhimurium mutants that are defective in intramacrophage survival and yet were still cytotoxic (data not shown). Collectively, these observations suggest that long-term intramacrophage survival and growth are not required for delayed, *ompR*- and SPI2-dependent macrophage killing. However, an indirect effect can not be ruled out until we have identified the SPI2 secreted effector(s) involved.

**Rapid and delayed macrophage killing processes are independent.** To determine whether rapid and delayed macrophage killing were independent of one another, doubly deficient mutant strains were constructed. Double mutants carried

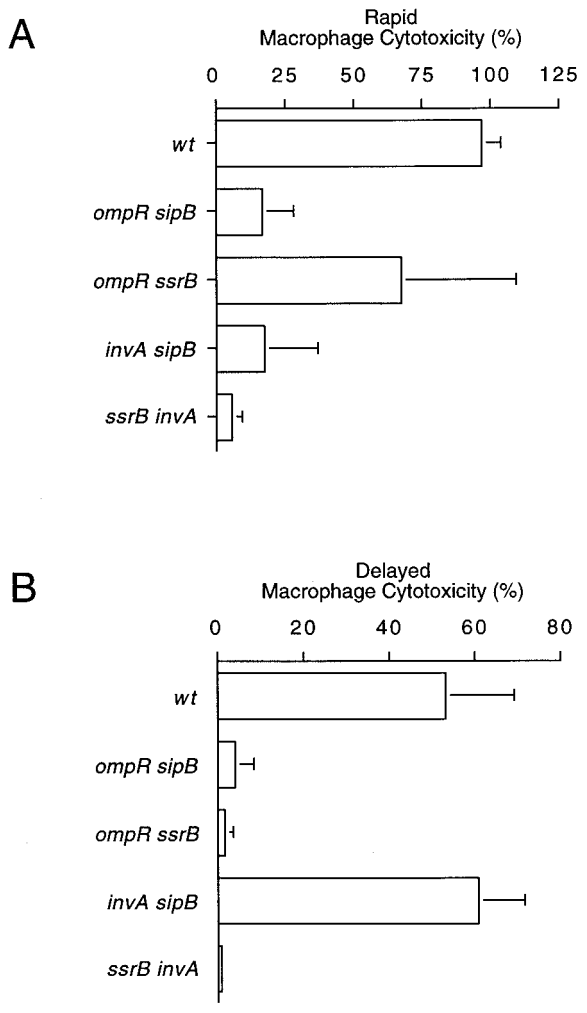


FIG. 4. Rapid and delayed macrophage killing processes are independent. J774 macrophages were infected with wt serotype Typhimurium or *ompR sipB*, *ompR ssrB*, *invA sipB*, or *ssrB invA* double mutants. (A and B) Bacterial cultures were grown to either late-log phase (A) or stationary phase (B) prior to infection. Macrophage cell death was quantitated at 6 h (A) and 18 h (B) postinfection by measuring the release of LDH. The data from each graph are arithmetic means of at least three independent experiments. The error bars indicate the standard deviations of the mean.

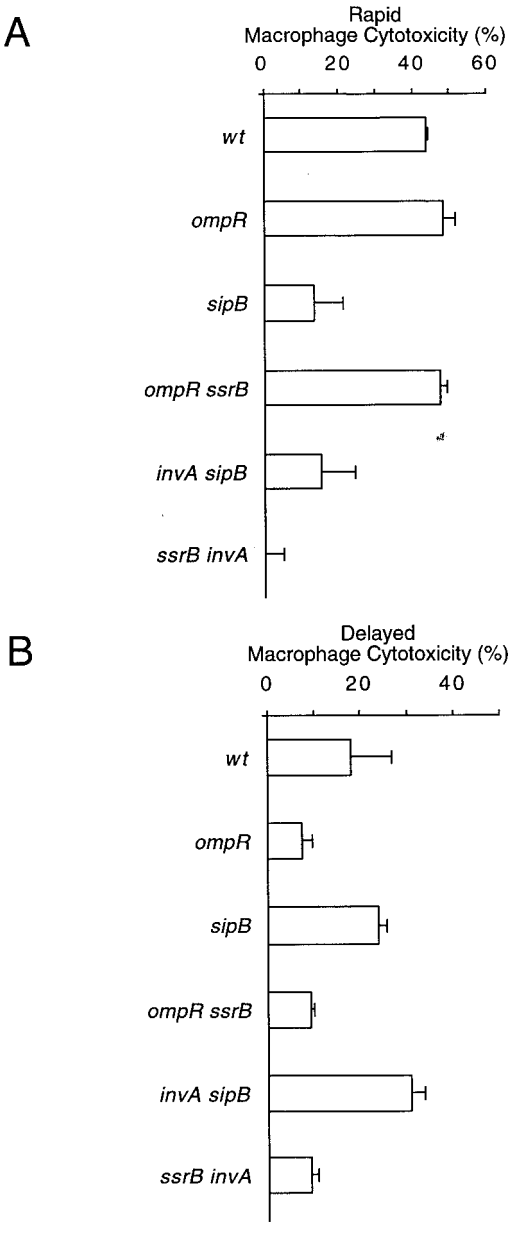


FIG. 5. *S. typhimurium* induces rapid and delayed macrophage cell death in bone marrow-derived macrophages. To demonstrate that serotype Typhimurium induced rapid and that delayed macrophage cell death was not specific to J774 macrophages, these results were repeated in RAW264.7 macrophages (data not shown). In addition, bone marrow-derived macrophages were established from C57BL/6 mice and infected with mutant strains defective in inducing either rapid macrophage cell death (*sipB*, *invA sipB*) or delayed macrophage cell death (*ompR*, *ompR ssrB*) or with a mutant strain defective in both rapid and delayed macrophage killing (*ssrB invA*). (A and B) Bacterial strains were grown to either late-log phase (A) or stationary phase (B) prior to infection. Macrophage cell death was quantitated at 6 h (A) and 30 h (B) postinfection by measuring the release of LDH. The data from each graph are arithmetic means of three independent experiments. The error bars indicate the standard deviations of the mean.

null mutations in genes required for either rapid macrophage killing only (*invA sipB*), delayed macrophage killing only (*ompR ssrB*), or genes required for both rapid and delayed macrophage killing (*ompR sipB* and *ssrB invA*). Under SPI1 inducing conditions, *ompR sipB*, *invA sipB*, and *ssrB invA* doubly deficient mutants were noncytotoxic, whereas an *ompR ssrB* double mutant was as cytotoxic as the wt (Fig. 4A). Under conditions that favored delayed macrophage killing, an *invA sipB* doubly deficient strain was fully cytotoxic, whereas *ompR sipB*, *ompR ssrB*, and *ssrB invA* double mutants were unable to kill infected macrophages (Fig. 4B). To demonstrate that these observations were not specific to J774 macrophages, these results were confirmed using RAW264.7 macrophages (data not shown) and bone marrow-derived macrophages (Fig. 5).

Collectively, these results indicate that bacterial strains mutated in genes required for either rapid or delayed induction of macrophage cell death are noncytotoxic only under specific pregrowth conditions. However, bacterial strains mutated in loci that affect both rapid and delayed macrophage killing are noncytotoxic under all conditions tested. These observations are evidence that rapid and delayed macrophage killing processes act independently of one another.

***ompR* and SPI2, but not SPI1, are required for delayed induction of apoptosis in infected macrophages.** Next, we in-

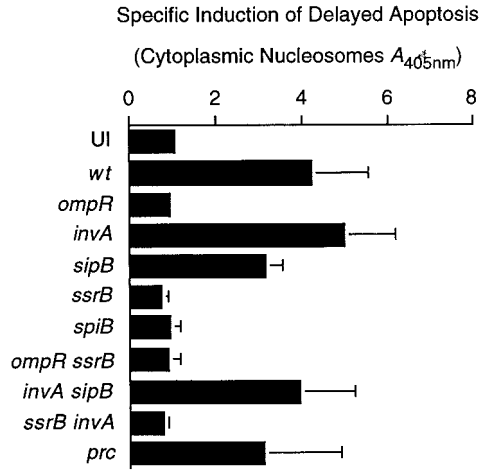


FIG. 6. *ompR* and SPI2, but not SPI1, are required for delayed induction of apoptosis in infected macrophages. J774 macrophages were infected with wt serotype Typhimurium or with mutant strains defective in either rapid killing (*sipB*, *invA*, *invA sipB*) or delayed killing (*ompR*, *ssrB*, *spiB*, *ompR ssrB*) with a mutant strain defective in both rapid and delayed macrophage killing (*ssrB invA*) or with a strain defective in macrophage survival (*prc*). The ability of these strains to induce apoptosis was determined at 18 h postinfection by measuring the amount of cytoplasmically located histones bound to fragmented DNA. The data from this graph are the arithmetic means of three independent experiments. The error bars indicate the standard deviations of the mean.

vestigated the nature of serotype Typhimurium-induced rapid and delayed macrophage cell death. Thus far, a nonspecific method, measuring the release of host cytoplasmic LDH, was used to calculate macrophage cytotoxicity. To determine whether macrophages were undergoing apoptosis upon infection with serotype Typhimurium, the amount of cytoplasmically located histones bound to fragmented DNA was quantified. Under SPI1 inducing conditions, serotype Typhimurium rapidly induced apoptosis via an SPI1-dependent process (data not shown). Under conditions that favored delayed macrophage cytotoxicity, killing was independent of SPI1 (Fig. 6). Delayed induction of apoptosis was abrogated in strains defective in either *ompR* or SPI2 (Fig. 6). These results indicate that serotype Typhimurium induces either rapid or delayed apoptosis in infected macrophages. Rapid activation of programmed cell death depends on SPI1, whereas delayed induction of apoptosis is SPI1 independent. Furthermore, our observations suggest that *ompR* and SPI2 are required for delayed activation of programmed macrophage cell death.

## DISCUSSION

In this study, we demonstrate that macrophages undergo either rapid or delayed apoptosis upon infection with serotype Typhimurium. Delayed activation of programmed cell death is masked when SPI1 genes are expressed. Mutations that affect either rapid or delayed induction of apoptosis result in noncytotoxic phenotypes only under specific growth conditions. However, mutants defective in both rapid and delayed macrophage killing are unable to induce apoptosis under any condition tested, even at a high MOI (data not shown). Rapid activation of programmed macrophage cell death depends on SipB and the SPI1 encoded type III protein export machinery, whereas delayed induction of apoptosis is SPI1 independent. Our results indicate that *ompR* and a functional SPI2 encoded type III protein secretion system are required for delayed induction of apoptosis. However, a nonspecific effect cannot be excluded until we have identified an SPI2 effector(s) that is both necessary and sufficient for the activation of delayed programmed macrophage cell death.

In agreement with the literature, we observed a defect (2- to 10-fold) in intracellular proliferation for SPI2 mutants at 15 and 18 h postinfection (14, 27, 28, 41, 46). However, *prc*, *htrA*, and 11 other macrophage-sensitive mutants tested are fully cytotoxic and yet are more severely defective in their ability to survive and grow inside phagocytic cells (Fig. 3A) (11, 20). In fact, MS4290 (*prc*) was the most sensitive mutant isolated in an extensive search for *Salmonella* mutants that cannot survive inside macrophages (11, 20). Despite this substantial defect, *prc* mutant bacteria, as well as a large panel of other macrophage-sensitive serotype Typhimurium mutants, induced both rapid (data not shown) and delayed apoptosis in infected macrophages (Fig. 3B and Fig. 6). These results strongly support an additional role for SPI2 in delayed induction of apoptosis in infected macrophages.

Our observations indicate that rapid and delayed activation of programmed macrophage cell death are independent of one another, since mutations in SPI1 do not affect delayed induction of apoptosis and mutations in SPI2 do not affect rapid induction of apoptosis. Recent studies support this view by demonstrating that these two specialized protein secretion systems are controlled by distinct regulatory circuits. For example, substrates for the SPI1 encoded type III protein export apparatus are secreted under mildly alkaline conditions (15), whereas substrates for the type III protein export system encoded within SPI2 are secreted at pH 5.0 (9). Furthermore, numerous studies suggest that, once inside a phagocytic host, serotype Typhimurium represses SPI1 gene expression and turns on genes that are important for long-term residence, growth, and survival inside these host cells (2, 5, 8, 14, 16, 19, 24, 25, 33, 34, 43, 44, 50). It is therefore unlikely that substrates for SPI1 and SPI2 encoded type III protein export systems are secreted simultaneously.

Our hypothesis is that serotype Typhimurium induces rapid and delayed apoptosis in infected macrophages under discrete physiological conditions at distinct times and locations during the natural course of infection in the host (Fig. 7). Accumulating evidence suggests that the SPI1 encoded type III protein secretion system is important primarily during the intestinal phase of infection, since SPI1 mutants are significantly attenuated only when administered to mice orally (reference 22 and references therein and reference 23). In contrast, *ompR* and SPI2 are absolutely required during the systemic phase of infection (12, 16, 17, 41, 47, 50). In fact, SPI2 has been implicated in growth inside phagocytic cells at systemic sites of infection (12, 16, 17, 41, 47, 50). A possible consequence of the rapid, SPI1-dependent induction of apoptosis in macrophages of the GALT is that additional phagocytic cells are attracted to the site of inflammation. Our model suggests that *Salmonella* represses the SPI1-dependent killing mechanism upon internalization by macrophages, allowing continued proliferation and systemic spread prior to *ompR*- and SPI2-dependent induction of delayed apoptosis at systemic sites of infection. Because apoptotic cells are ingested by neighboring phagocytes, we propose that delayed induction of apoptosis in infected macrophages may allow *Salmonella* to spread intercellularly within apoptotic bodies. This model is supported by a recent study in which it was demonstrated that serotype Typhimurium is transported from the intestine, via the bloodstream, to the liver and spleen by CD18-expressing monocytes in an SPI1-independent process (52), as well as by studies in which it was demonstrated that *Salmonella* virulence was un-

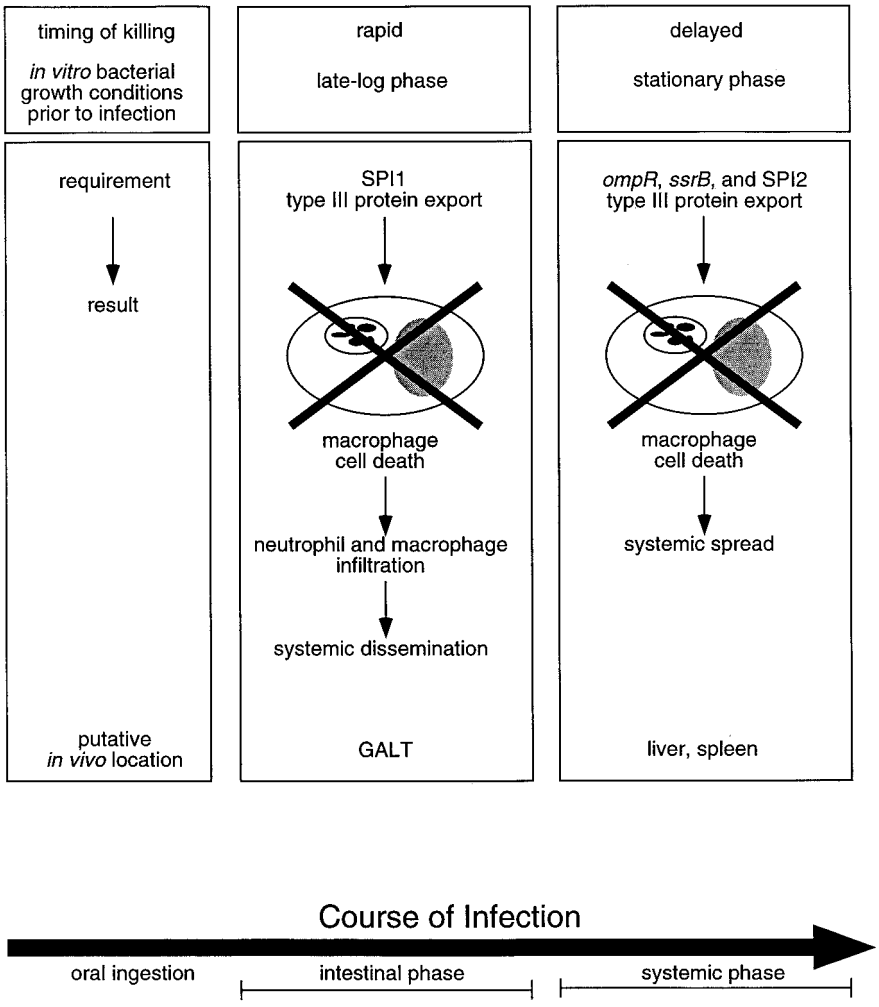


FIG. 7. Model of serotype Typhimurium-induced apoptosis in vivo. We propose that serotype Typhimurium induces rapid and delayed apoptosis in infected macrophages under discrete physiological conditions at distinct times and locations during the natural course of infection. Because the SPI1 encoded type III protein secretion system is important primarily during the intestinal phase of infection (23), we propose that rapid, SPI1-dependent induction of apoptosis in macrophages of the GALT results in increased inflammation and recruitment of phagocytes that may be required for systemic dissemination. Our model predicts that *Salmonella* represses the rapid macrophage killing mechanism upon internalization, permitting extensive intracellular proliferation and systemic spread prior to delayed, *ompR*- and SPI2-dependent induction of apoptosis at systemic sites of infection. In support of this view, *ompR* and SPI2, unlike SPI1, are required during the systemic phase of infection (12, 16, 17, 41, 47, 50). This model predicts that *Salmonella* induces delayed apoptosis in infected macrophages to spread intercellularly within apoptotic bodies.

affected by treatment with antibiotics that kill extracellular bacteria (10, 18).

## ACKNOWLEDGMENTS

We thank Renée Tsolis for providing strain STN119 prior to publication and Joanne Engel for helpful discussions. L929 supernatant was generously provided by Archie Bouwer. We thank members of the Heffron and So laboratories for critical comments on the manuscript.

This work was supported by Public Health Service grant AI37201 to F.H. from the National Institutes of Health.

## REFERENCES

1. **Ahmer, B. M., J. van Reeuwijk, P. R. Watson, T. S. Wallis, and F. Heffron.** 1999. *Salmonella* SirA is a global regulator of genes mediating enteropathogenesis. Mol. Microbiol. **31:**971–982.
2. **Alpuche-Aranda, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller.** 1992. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. Proc. Natl. Acad. Sci. USA **89:**10079–10083.
3. **Arai, T., K. Hiromatsu, H. Nishimura, Y. Kimura, N. Kobayashi, H. Ishida, Y. Nimura, and Y. Yoshikai.** 1995. Endogenous interleukin 10 prevents apoptosis in macrophages during *Salmonella* infection. Biochem. Biophys. Res. Commun. **123:**600–607.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1994. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
5. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703–714.
6. **Bäumler, A. J., J. G. Kusters, I. Stojiljkovic, and F. Heffron.** 1994. *Salmonella typhimurium* loci involved in survival within macrophages. Infect. Immun. **62:**1623–1630.
7. **Bäumler, A. J., R. M. Tsolis, P. J. Valentine, T. A. Ficht, and F. Heffron.** 1997. Synergistic effect of mutations in *invA* and *lpfC* on the ability of *Salmonella typhimurium* to cause murine typhoid. Infect. Immun. **65:**2254–2259.
8. **Behlau, I., and S. J. Miller.** 1993. A PhoP repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. **175:**4475–4484.
9. **Beuzon, C. R., G. Banks, J. Deiwick, M. Hensel, and D. W. Holden.** 1999. pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella typhimurium*. Mol. Microbiol. **33:**806–816.
10. **Bonina, L., G. B. Costa, and P. Mastroeni.** 1998. Comparative effect of gentamycin an pefloxacin treatment on the late stages of mouse typhoid. New Microbiol. **21:**9–14.
11. **Buchmeier, N. A., and F. Heffron.** 1989. Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse pop-

ulations of macrophages. Infect. Immun. **57:**1–7.
12. **Chatfield, S. N., C. J. Dorman, C. Hayward, and G. Dougan.** 1991. Role of *ompR*-dependent genes in *Salmonella typhimurium* virulence: mutants deficient in both OmpC and OmpF are attenuated *in vivo*. Infect. Immun. **59:**449–452.
13. **Chen, L. M., K. Kaniga, and J. E. Galan.** 1996. *Salmonella* spp. are cytotoxic for cultured macrophages. Mol. Microbiol. **21:**1101–1115.
14. **Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow.** 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. **30:**175–188.
15. **Daefler, S.** 1999. Type III secretion by *Salmonella typhimurium* does not require contact with a eukaryotic host. Mol. Microbiol. **31:**45–51.
16. **Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel.** 1999. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. Mol. Microbiol. **31:**1759–1773.
17. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated *in vivo*. Infect. Immun. **57:**2136–2140.
18. **Dunlap, N. E., W. H. Benjamin, Jr., A. K. Berry, J. H. Eldridge, and D. E. Briles.** 1991. A 'safe-site' for *Salmonella typhimurium* is within splenic polymorphonuclear cells. Microb. Pathog. **13:**181–190.
19. **Ernst, R. K., D. M. Dombroski, and J. M. Merrick.** 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. **58:**2014–2016.
20. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
21. **Gahring, L. C., F. Heffron, B. B. Finlay, and S. Falkow.** 1990. Invasion and replication of *Salmonella typhimurium* in animal cells. Infect. Immun. **58:**443–448.
22. **Galán, J. E.** 1996. Molecular genetic basis of *Salmonella* entry into host cells. Mol. Microbiol. **20:**263–271.
23. **Galán, J. E., and R. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
24. **Galán, J. E., and R. Curtiss III.** 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. **58:**1879–1885.
25. **Garcia-Vescovi, E., F. C. Soncini, and E. A. Groisman.** 1996. $Mg^{2+}$ as an extracellular signal: environmental regulation of *Salmonella* virulence. Cell **84:**165–174.
26. **Hauser, A. R., and J. N. Engel.** 1999. *Pseudomonas aeruginosa* induces type III-secretion-mediated apoptosis of macrophages and epithelial cells. Infect. Immun. **67:**5530–5537.
27. **Hensel, M., J. E. Shea, B. Rapauch, D. Monack, S. Falkow, C. Gleeson, T. Kubo, and D. W. Holden.** 1997. Functional analysis of *ssaJ* and the *ssaK/U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* pathogenicity island 2. Mol. Microbiol. **24:**155–167.
28. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden.** 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. **30:**163–174.
29. **Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky.** 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc. Natl. Acad. Sci. USA **96:**2396–2401.
30. **Kaniga, K., S. Tucker, D. Trollinger, and J. E. Galán.** 1995. Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella typhimurium* entry into cultured epithelial cells. J. Bacteriol. **177:**3965–3971.
31. **Kinder, S. A., J. L. Badger, G. O. Bryant, J. C. Pepe, and V. L. Miller.** 1993. Cloning of the *YenI* restriction endonuclease and methyltransferase from *Yersinia enterocolitica* serotype O:8 and construction of a transformable $R^-M^+$ mutant. Gene **136:**271–275.
32. **Lee, A. K., C. S. Detweiler, and S. Falkow.** 2000. OmpR/EnvZ regulates the two-component system SsrA-SsrB in *Salmonella* pathogenicity island 2. J. Bacteriol. **182:**771–781.
33. **Lee, C. A., and S. Falkow.** 1990. The ability of *Salmonella typhimurium* to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA **87:**4304–4308.
34. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. USA **89:**1847–1851.
35. **Lindgren, S. W., I. Stojiljkovic, and F. Heffron.** 1996. Macrophage killing is an essential virulence mechanism of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **93:**4197–4201.
36. **Lundberg, U., U. Vinatzer, D. Berdnik, A. v. Gabain, and M. Baccarini.** 1999. Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. J. Bacteriol. **181:**3433–3437.
37. **Maniatis, T., J. Sambrook, and E. F. Fritsch.** 1989. Molecular cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Mock, B. A., D. L. Holiday, D. P. Cerretti, S. C. Darnell, A. D. O'Brien, and M. Potter.** 1994. Construction of a series of congenic mice with recombinant chromosome 1 regions surrounding the genetic loci for resistance to intracellular parasites (Ity, Lsh, and Bcg), DNA responses (Rep-1), and cytoskeletal protein villin (Vil). Infect. Immun. **62:**325–328.
39. **Monack, D. M., B. Raupach, A. E. Hromockyj, and S. Falkow.** 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. USA **93:**9833–9838.
40. **O'Brien, A. D.** 1986. Influence of host genes on resistance of inbred mice to lethal infection with *Salmonella typhimurium*. Curr. Top. Microbiol. Immunol. **124:**37–48.
41. **Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman.** 1996. Identification of a pathogenicity island for *Salmonella* survival in host cells. Proc. Natl. Acad. Sci. USA **93:**7800–7804.
42. **Penfold, R. J., and J. M. Pembert.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118:**145–146.
43. **Pfeifer, C. G., S. L. Marcus, O. Steele-Mortimer, L. A. Knodler, and B. B. Finlay.** 1999. *Salmonella typhimurium* virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. Infect. Immun. **67:**5690–5698.
44. **Pratt, L. A., and T. J. Silhavy.** 1995. Porin regulon of *Escherichia coli*, p. 105–27. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. American Society for Microbiology, Washington, D.C.
45. **Richter-Dahlfors, A., A. M. J. Buchan, and B. B. Finlay.** 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. J. Exp. Med. **186:**569–580.
46. **Shea, J. E., C. R. Beuzon, C. Gleeson, R. Mundy, and D. W. Holden.** 1999. Influence of the *Salmonella typhimurium* pathogenicity island 2 type III secretion system on bacterial growth in the mouse. Infect. Immun. **67:**213–219.
47. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **93:**2593–2597.
48. **Skorupski, K., and R. K. Taylor.** 1996. Positive selection vectors for allelic exchange. Gene **169:**47–52.
49. **Tsolis, R. M., S. M. Townsend, T. A. Ficht, L. G. Adams, and A. J. Bäumler.** 1999. Contribution of *Salmonella typhimurium* virulence factors to diarrheal disease in calves. Infect. Immun. **67:**4879–4885.
50. **Valdivia, R. H., and S. Falkow.** 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. Mol. Microbiol. **22:**367–378.
51. **van der Velden, A. W. M., A. J. Bäumler, R. M. Tsolis, and F. Heffron.** 1998. Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice. Infect. Immun. **66:**2803–2808.
52. **Vasquez-Torres, A., J. Jones-Carson, A. J. Bäumler, S. Falkow, R. Valdivia, W. Brown, M. Le, R. Berggren, W. T. Parks, and F. C. Fang.** 1999. Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. Nature **401:**804–808.

*Editor:* A. D. O'Brien