

## Growth Phase-Regulated Induction of *Salmonella*-Induced Macrophage Apoptosis Correlates with Transient Expression of SPI-1 Genes

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Received 16 July 1998/Accepted 30 March 1999

**Invasive *Salmonella* has been reported to induce apoptosis in a fraction of infected macrophages within 2 to 14 h from the time of infection by a mechanism involving the type III secretion machinery encoded by the *Salmonella* pathogenicity island 1 (SPI-1). Here, we show that bacteria in the transition from logarithmic to stationary phase cause 90% of the macrophages to undergo phagocytosis-independent, caspase-mediated apoptosis within 30 to 60 min of infection. The ability of *Salmonella* to induce this rapid apoptosis was growth phase regulated and cell type restricted, with epithelial cells being resistant. Apoptosis induction was also abrogated by disruption of the *hilA* gene (encoding a regulator of SPI-1 genes) and by the expression of a constitutively active PhoPQ. *hilA* itself and a subset of SPI-1 genes were transiently expressed during aerobic growth in liquid medium. Interestingly, however, *hilA* was found to be required only for the expression of the *prgH* gene, while *sipB*, *invA*, and *invF* were expressed in a *hilA*-independent manner. The expression of SPI-1 genes and the secretion of invasion-associated proteins correlated temporally with the induction of apoptosis and are likely to represent its molecular basis. Thus, growth phase transition regulates the expression and secretion of virulence determinants and represents the most efficient environmental cue for apoptosis induction reported to date.**

Infection of mice with *Salmonella typhimurium* results in a systemic disease similar to typhoid. In humans, *S. typhimurium* is one of the most common causes of food poisoning. Otherwise-healthy individuals develop a gastroenteritis which might become chronic (the infected persons continue to shed bacteria for more than 1 year), but in immunocompromised patients *S. typhimurium* can cause a serious systemic disease. In the host's intestine, *Salmonella* adheres to specialized epithelial cells (M cells [6, 17, 29]). The bacteria cause cytoskeletal and membrane rearrangements that result in their uptake by the host cells (10). By destroying the infected M cells (29), the bacteria gain access to the mesenteric lymph follicles, where they encounter and infect macrophages. Recently, the induction of macrophage cytotoxicity and/or apoptosis by invasive *Salmonella* has been documented in vitro (4, 21, 25) and in vivo (32). In two cases, components of the SPI-1-encoded type III secretion system have been shown to be necessary for efficient apoptosis induction (4, 25). The type III secretion system is a specialized, evolutionarily conserved protein secretion apparatus required for the export of virulence determinants which plays an important role in the invasion and destruction of the epithelial cells by *Salmonella* (12, 29). Several genes coding for putative components of type III secretion systems map to a

contiguous region at centisome 63 of the *Salmonella* chromosome, the *Salmonella* pathogenicity island 1 (SPI-1). SPI-1 genes encode secreted proteins required for epithelial cell invasion, like SipA, -B, -C, and -D, SpaO, and SpaN, as well as proteins regulating the expression of invasion genes, like HilA (1, 2). Environmental signals, like oxygen concentration, osmolarity, and the growth state of the bacteria, influence the expression of HilA and the secretion of invasion-associated proteins (1). HilA expression can be regulated by two members of the phosphorylated response regulator superfamily, namely, SirA (the positive regulator [16]) and PhoP (the negative regulator [1]).

In this paper, we show that changes in growth phase have a profound effect on the ability of *Salmonella* to induce apoptosis in macrophages. The ability to induce apoptosis is restricted to a narrow, specific bacterial growth phase, namely, the transition from the exponential to the stationary phase. In this particular growth phase, *Salmonella* induces the most rapid and complete apoptosis ever reported (more than 90% of the macrophages are dead 30 min after infection). Transposon insertion mutagenesis and analysis of existing mutants confirm the pivotal importance of the invasion genes for this process. Furthermore, we find that the expression of several invasion genes is also growth phase regulated and correlates with apoptosis induction.

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### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Unless otherwise stated, the bacteria were generally grown overnight (16 to 20 h) at 37°C in 5 ml of Luria-Bertani (LB) broth with shaking at 150 rpm. For reinoculation, 50 µl of an overnight culture was added to 5 ml of fresh LB broth and incubated for the time periods indicated.

**Transposon-induced mutagenesis.** Transposon mutagenesis was performed as described by Tsois and Heffron (36). For screening of generated mutants for loss of cytotoxicity, single colonies were grown in 100 µl of LB broth in microtiter plates for 5 h after reinoculation from an overnight culture. One microliter of the reinoculated culture was used to infect macrophages as described below.

**Characterization of the transposon insertion sites.** Isolated *S. typhimurium* mutants unable to kill macrophages were further analyzed by PCR. Genomic DNA was isolated from the mutants and digested with *EcoRV*, which cleaves once in Tn10d(Tc). *EcoRV*-digested DNA was ligated at a low concentration to generate circular DNA. The genomic DNA flanking the transposon on either side was amplified by using two different sets of primers homologous to the Tn10d(Tc) transposon. The amplified fragments were sequenced with the AmpliCycle sequencing kit from Perkin-Elmer.

**Analysis of gene expression by RT-PCR.** RNA was isolated from *S. typhimurium* strains at different growth stages. One microgram of RNA was used in the reverse transcription (RT) with Superscript at 42°C for 60 min in the presence of 1 pmol of downstream primer. One-twentieth of the RT mixture was used in the PCR. Different amounts of chromosomal DNA were used to ensure that the amplification was in a linear range.

**Analysis of the profile of secreted proteins.** The bacterial cultures (3 ml) were chilled on ice for 30 min before the bacteria were pelleted. The supernatants were filtered through 0.45- $\mu$ m-pore-size filters prior to precipitation with trichloroacetic acid. The precipitate was dissolved in sample buffer and run on a sodium dodecyl sulfate–10% polyacrylamide gel. Proteins were visualized with Coomassie brilliant blue R-250.

**Cell culture and infection.** BAC-1.2F5 cells (26) and bone marrow-derived macrophages from C57BL/6J mice were cultured in minimum essential medium (MEM)- $\alpha$  supplemented with 10% fetal calf serum (FCS) and 20% L-cell conditioned medium as a source of colony-stimulating factor 1 (35). J774A.1 cells (30), MDCK cells (31), and Caco-2 cells (9) were grown in Dulbecco's modified minimal essential medium with 10% FCS, and HeLa cells (13) were grown in RPMI containing 10% FCS. The cells (50,000/well) were seeded into 96-well plates 16 h prior to infection with bacteria diluted in cell culture medium without antibiotics (with a constant multiplicity of infection [MOI] of 25 in all experiments). The plates were centrifuged at 300  $\times$  g for 5 min to synchronize the infection and incubated for 30 min at 37°C (5% CO<sub>2</sub>).

In selected experiments, a peptide inhibitor of caspases (general inhibitor) (Z-VAD-CH<sub>2</sub>F; Enzyme System Products, Dublin, Calif.) was added to the cells 45 min prior to infection with *Salmonella*.

**Assessment of host cell viability and detection of *Salmonella*-induced apoptosis.** Host cell viability was determined either by trypan blue dye exclusion or by crystal violet staining of viable cells. For crystal violet staining, the cells were washed three times with phosphate-buffered saline prior to the addition of 100  $\mu$ l of crystal violet solution (0.2% crystal violet in 20% methanol). The cells were then incubated for 15 min at 37°C and washed extensively with water to remove excess dye. The absorbance was measured at 620 nm. The absorbance of uninfected cells was set to 100%. The absorbance of cells infected with bacteria deficient in apoptosis induction or treated with inhibitors of apoptosis prior to infection exceeds the 100% value due to the staining of the infecting bacteria.

## RESULTS AND DISCUSSION

**Growth phase-dependent induction of macrophage apoptosis by *Salmonella*.** Infection of BAC-1.2F5 cells with *S. typhimurium* grown under different conditions resulted in the death of the macrophages (data not shown). To determine whether the ability of some of the cultures to induce macrophage cytotoxicity correlated with a specific growth phase, the bacteria were grown at 37°C with agitation. Aliquots were taken at different time points and used to infect BAC-1.2F5 cells in 96-well plates with a constant MOI of 25. The survival of the macrophages was assessed 30 min after infection by crystal violet staining. Interestingly, *S. typhimurium* acquires and retains the capability of inducing macrophage death only in a narrow growth phase, namely, in the transition between logarithmic and stationary growth phases (Fig. 1A). A culture in this specific growth phase will be referred to from now on as activated. Marked cellular damage was already morphologically evident 10 min after infection (data not shown). Previous reports of *Salmonella*-induced macrophage apoptosis (4, 21, 25) described cellular damage and DNA fragmentation in a fraction of the infected cells (20 to 60%) 2 to 14 h after incubation with invasive *S. typhimurium*. To determine the time course and the extent of the cytotoxic effect in our system, BAC-1.2F5 cells were infected with an activated *S. typhimurium* culture and trypan blue was added to the cells at different time points after infection. Cell death proceeded extremely rapidly, and 80 to 90% of the macrophages were stained with trypan blue 30 min after infection (Fig. 1B).

The cytotoxic effect of *S. typhimurium* on BAC-1.2F5 cells is

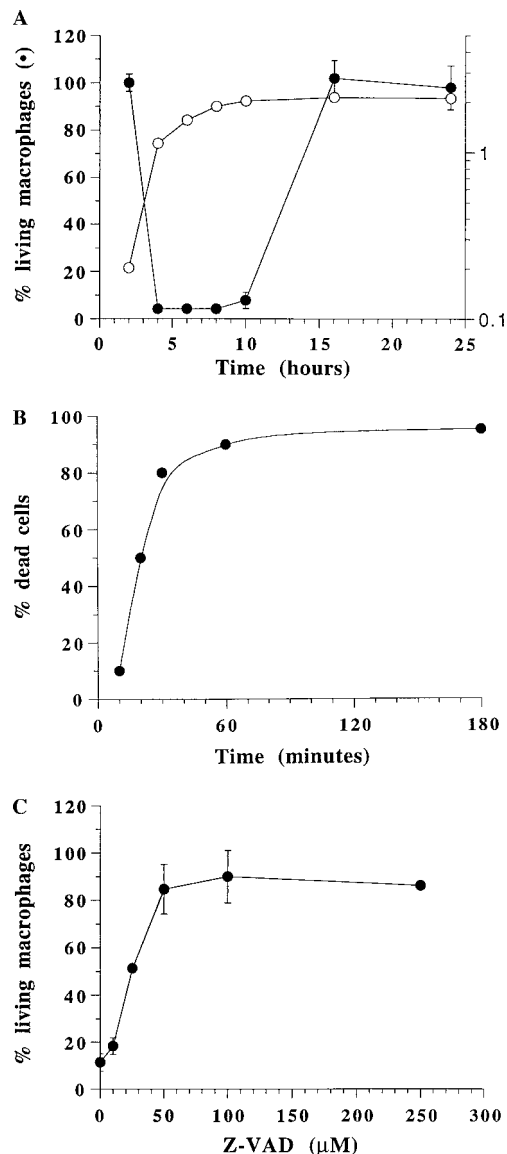


FIG. 1. Effect of bacterial growth phase on *Salmonella*-induced macrophage apoptosis. BAC-1.2F5 cells were seeded into 96-well plates (50,000 cells/well) and either left uninfected or infected with *S. typhimurium* LT2 (34) in different growth phases. Macrophages were infected with an activated culture with a constant MOI of 25. The bacteria were spun onto the macrophages to synchronize infection. (A) Thirty minutes after infection, the number of viable cells was determined by crystal violet staining. The percentage of viable cells (uninfected controls = 100%) and the optical density at 600 nm (OD<sub>600</sub>) of the bacterial culture used to infect the cells are plotted. The assays were carried out in triplicate. The vertical bars represent the standard deviations of the mean. (B) Time course of macrophage death induced by infection with an activated culture. The percentage of dead cells was determined by trypan blue dye exclusion. (C) *Salmonella*-induced apoptosis is caspase dependent. Macrophages were infected with *Salmonella* after preincubation (45 min) with different concentrations of a caspase inhibitor (Z-VAD-CH<sub>2</sub>F). Thirty minutes after infection, the number of viable cells was determined by crystal violet staining.

the fastest reported to date. To determine its nature, we preincubated the macrophages with different concentrations of a cell permeant general caspase inhibitor (Z-VAD-CH<sub>2</sub>F). Caspases are a family of cysteine proteases which play a central role in apoptosis but not in necrosis (20, 27). The inhibitor blocked *Salmonella*-induced cell death by 85% at the concentration of 50  $\mu$ M (Fig. 1C). Protection of the macrophages

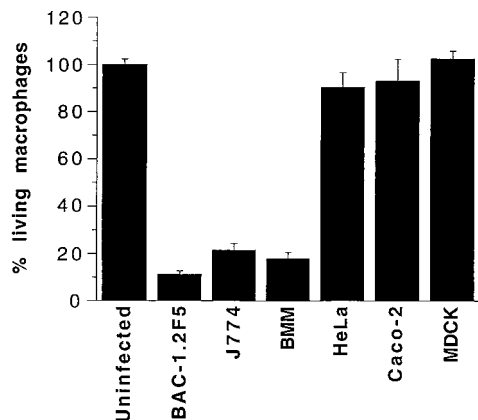


FIG. 2. *Salmonella* induces apoptosis in macrophages but not in epithelial cells. Macrophages (BAC-1.2F5 and J774 cells and bone marrow-derived macrophages [BMM]) or epithelial cells (HeLa, MDCK, or Caco-2 cells) were seeded into 96-well plates (50,000/well) and infected with activated *Salmonella* cultures in the appropriate growth phase (MOI of 25). Thirty minutes after infection, the number of viable cells was determined by crystal violet staining. The results are expressed as percentage of viable cells (uninfected controls = 100%). The assays were carried out in quadruplicate. The vertical bars represent the standard deviations of the mean.

from the cytotoxic effect of *S. typhimurium* by the caspase inhibitor unequivocally establishes that cell death in our system occurs via apoptosis. *Yersinia enterocolitica* (33)- and *Shigella flexneri* (5)-induced macrophage apoptosis can also be blocked by caspase inhibitors, indicating that bacterial components must be able to cross talk with the mechanisms of activation of this group of enzymes.

Macrophages undergoing *Salmonella*-induced apoptosis showed the characteristic morphological features of this process (nuclear shrinkage and chromatin condensation in the typical crescent shape [data not shown]); in addition, their DNA was fragmented and displayed the nucleosomal ladder that correlates with apoptosis (data not shown).

*Salmonella*-induced apoptosis was not restricted to BAC-1.2F5 cells. A second mouse macrophage-like cell line, J774, and primary bone marrow-derived macrophages were also efficiently killed, with the same fast kinetics. In contrast, three epithelial cell lines, HeLa (human), Caco-2 (human), and MDCK (canine), did not undergo apoptosis upon infection with an activated *S. typhimurium* culture (Fig. 2). Epithelial cells are resistant to *Salmonella*-, *Shigella* (22)- and *Yersinia* (24)-induced apoptosis (Fig. 2), although they are able to undergo caspase-mediated apoptosis when treated with anti-Fas antibodies or tumor necrosis factor alpha (7). It is possible that epithelial cells, in contrast to macrophages, might lack a component of the signal transduction pathway targeted by the putative *Salmonella* factor(s) triggering apoptosis (e.g., the surface receptors). Alternatively, they might contain an inhibitor(s) of this specific apoptotic pathway. Several *Salmonella* strains (*Salmonella dublin*, *Salmonella enteritidis*, and *Salmonella bongori*) were tested to determine their ability to induce apoptosis. All tested strains were cytotoxic for BAC-1.2F5 cells when grown to the appropriate growth phase (data not shown). Cytotoxicity was independent of the presence of the virulence plasmid in two different strains: ST12/75, compared with its plasmid-cured derivative, STM8c (14); and *S. dublin* SD2229, compared with its plasmid-cured derivative, SDM173c (reference 14 and data not shown). In addition, the two strains of *S. bongori* from the *Salmonella* Reference Collection C (3) efficiently induced macrophage apoptosis (data not shown), de-

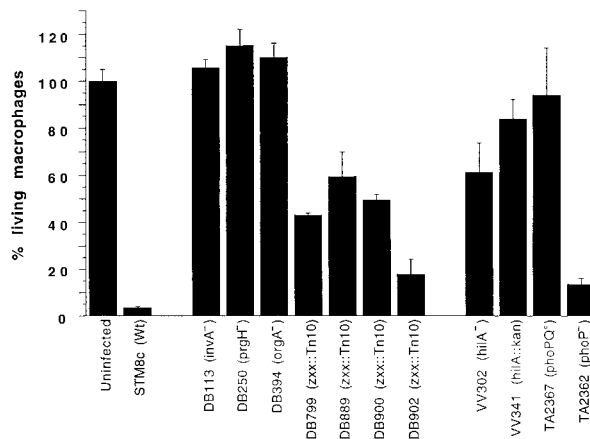


FIG. 3. Effects of different *Salmonella* mutant strains on macrophage viability. BAC-1.2F5 cells were seeded into 96-well plates (50,000/well) and either left uninfected or infected with different mutant strains of *Salmonella* in the appropriate growth phase. Infection was performed as described in the legend to Fig. 1A. Thirty minutes after infection, the number of viable cells was determined by crystal violet staining. The results are expressed as percentage of viable cells (uninfected controls = 100%). The assays were carried out in quadruplicate. The vertical bars represent the standard deviations of the mean. Wt, wild type.

spite their lack of at least part of the recently described SPI-2 (28).

**Mutations affecting *Salmonella*-induced macrophage apoptosis.** Recent reports have indicated the importance of the *ompR-envZ* regulon (21) and of the components of the type III secretion system (4, 25) in *Salmonella*-induced macrophage cytotoxicity. In our system, the *ompR* mutant (CJD359 [8]) was still cytotoxic, whereas a mutation in the *invA* gene (SB111 [11]) abrogated the effect (data not shown), indicating that a functional type III secretion apparatus was necessary for macrophage killing.

In support of this, 6 of 10 apoptosis-deficient mutants identified by Tn10d(Tc) insertion mutagenesis mapped to three SPI-1 genes (*invA*, *prgH*, and *orgA*). The remaining four mutants did not map to the sequenced portions of SPI-1 or SPI-2 (data not shown) and displayed some residual cytotoxic activity (Fig. 3). These mutants are currently being characterized in more detail.

The results described above predicted that loss of function of the invasion gene regulator HilA would abrogate apoptosis induction (Fig. 3). Two independent *hilA* mutants were tested and found to induce apoptosis less efficiently than the wild-type strain. The *phoPQ*(Con) mutation (strain TA2367 [18]), which has been shown to inhibit the expression of HilA and of HilA-regulated genes (17), was also severely impaired in apoptosis induction; by contrast, *phoP* mutant bacteria (strain TA2362 [18]) behaved like the wild-type strain (Fig. 3). The experiments shown in Fig. 3 reaffirm the importance of the gene products encoded by the SPI-1 and of their regulators in *Salmonella*-induced macrophage apoptosis. It should be noted, however, that the effects of both the *hilA* and the *phoPQ*(Con) mutations on apoptosis induction were less severe than those of the *prgH*, *invA*, and *orgA* mutations.

**Expression of a subset of SPI-1 genes and secretion of invasion-associated proteins are growth phase regulated.** The experiments summarized above prompted us to determine whether the expression pattern of the genes implicated in the induction of apoptosis was influenced by the growth phase. Gene expression was monitored by RT-PCR in RNA samples

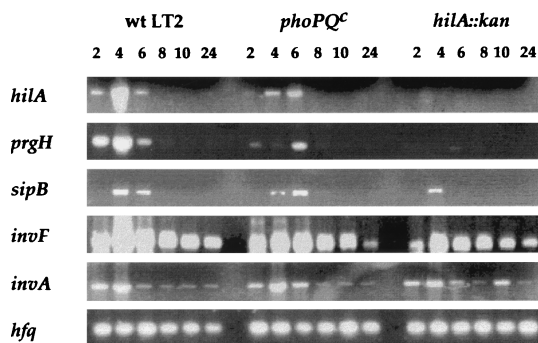


FIG. 4. Analysis of invasion gene expression in wild-type (wt), *phoPQ*(Con) (*phoPQ*<sup>c</sup>) and *hilA::kan* *Salmonella* strains. Wild-type, *phoPQ*(Con), and *hilA*-deficient *Salmonella* strains were grown at 37°C under continuous agitation. At the time points indicated, RNA was extracted and subjected to RT-PCR analysis with the appropriate primers (see Materials and Methods).

isolated at different time points of the growth curve. A subset of genes related to the SPI-1-encoded type III secretion system (*hilA*, *prgH*, and *sipB*) were shown to be tightly growth regulated, with maximum expression coinciding with the apoptosis-inducing phenotype. The expression of *prgH* and *sipB* followed that of *hilA*, confirming its role as a master regulator of genes needed for invasion (17). In contrast, the *invA* and *invF* transcript could be detected throughout the growth curve. The expression of *hfq* was constant throughout the growth curve and is shown as a control (Fig. 4, wild type).

Analysis of the profiles of secreted, invasion-associated proteins revealed at least four protein bands that were absent in the *invA* and *hilA* mutants but could be routinely identified in the supernatants of the wild-type cultures. In good agreement with the growth phase-regulated expression of a subset of invasion genes, these proteins (87, 64, 42, and 36 kDa, respectively) were much more abundant in the supernatant of the activated culture than in those of the stationary-phase culture (Fig. 5). Thus, both secretion of invasion-associated proteins and expression of a subset of invasion genes are growth phase regulated.

The sharp peak of *hilA* expression could result either from a positive regulation of the transcript during its induction or from its repression at the other time points or from a combination of both. The mRNAs of the *hilA* regulators *sirA* and *phoPQ* are present throughout the growth curve (data not shown). Therefore, if one of these genes is responsible for the behavior of the *hilA* transcript, they must be regulated post-

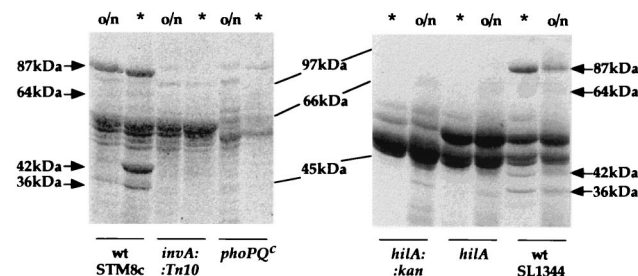


FIG. 5. Secreted-protein profiles of wild-type and mutant *Salmonella* strains. Trichloroacetic acid-precipitated proteins from supernatants (3 ml) of activated (\*) or overnight (o/n) cultures were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie blue staining for each of the strains indicated. The molecular masses of invasion-associated proteins are indicated next to the arrows. The *hilA* mutations were in SL1344 background (15). wt, wild type.

transcriptionally or, assuming that the proteins behave like the respective transcripts, posttranslationally. PhoP, PhoQ, and SirA are members of two-component systems and are therefore expected to be subjected to posttranslational regulation (16, 23). Contrary to our expectations, the *phoPQ*(Con) mutation that suppresses *hilA* induction by low oxygen, low pH, and high osmolarity (17) did not block growth phase-regulated *hilA* expression. A reduction of *hilA* expression could, however, be observed, and peak induction occurred 6 instead of 4 h after reinoculation of this mutant (Fig. 4); consistent with a role of *hilA* in *prgH* expression (1), the amount of this transcript was reduced in the *phoPQ*(Con) mutant, and its kinetics of accumulation paralleled those observed for *hilA*. The causal link between *prgH* and *hilA* was further confirmed by the finding that *prgH* expression was abrogated in an *hilA*-deficient strain (Fig. 4). The PrgH protein is a component of the needle complex, essential for the transfer of secreted proteins into eukaryotic cells (19). The decrease [in the case of the *phoPQ*(Con) strain] or the absence (in the case of the *hilA*-deficient strain) of *prgH* expression therefore likely accounts for the severe reduction in apoptosis induction (Fig. 3) and in the secretion of invasion-associated proteins observed in these two mutants (Fig. 5). In agreement with this hypothesis, but surprising in view of the dominant role ascribed to HilA in previous publications, the expression of other invasion genes was only marginally (*sipB*, coding for a secreted protein) or not at all (*invA* and *invF*) affected by either the *phoPQ*(Con) or the *hilA* mutation (Fig. 4). Thus, our data confirm that the *phoPQ*(Con) mutation can negatively influence *Salmonella* virulence by repressing *hilA* expression (1). However, in contrast to previous reports (1, 2), under our culture conditions an absolute requirement for HilA exists in the case of the *prgH* gene but not in the cases of the *sipB*, *invA*, or *invF* genes.

Whether this discrepancy is due to differences in the assay used (expression of invasion gene-*lacZY* fusions was used in the previous studies, while we assay mRNA level directly) or to the conditions used to induce expression (low oxygen, low pH, and high osmolarity were the stimuli used previously) remains to be determined.

*Salmonella*-induced macrophage cytotoxicity, with some features of apoptosis, has been reported by several groups (4, 21, 25). In all cases, induction of the cytotoxicity depended on the use of specific bacterial culture conditions. Lindgren et al. (21) and Monack et al. (25) grew the bacteria under reduced aeration, while Chen et al. (4) used hyperosmolarity (0.3 M NaCl) to stimulate apoptosis induction by *Salmonella*.

We show here that the transition from logarithmic to stationary phase alone, which all bacteria are likely to undergo, represents the most potent stimulus for apoptosis induction reported to date (Fig. 1).

*Shigella* (5), *Yersinia* (33), and *Salmonella* (Fig. 1C) require the caspase cascade to effect macrophage apoptosis. The unusual rapidity of the apoptotic processes indicates that *Salmonella*, rather than suppressing the activity of molecules that normally prevent apoptosis, triggers the cell death pathway of the macrophages directly, possibly by interacting with the caspase cascade. The task will now be to identify the *Salmonella* virulence determinants which interact with the caspase cascade and to unravel their mechanism of activation.

#### ACKNOWLEDGMENTS

Ursula Vinatzer and Daniela Berdnik made equal contributions to this work.

The skillful technical assistance of Julia Katzenbeisser is gratefully acknowledged. We are indebted to Thomas Decker, Andreas Meinke, and Pavel Kovarik, Vienna Biocenter, for many helpful discussions and

for critically reading the manuscript. We also thank Alistair Lax (Institute for Animal Health, Compton, Nr Newbury, United Kingdom), Catherine Lee (Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Mass.), Kenneth Sanderson (Department of Biological Sciences, University of Calgary), and Mikael Rhen (Center for Tumor Biology, Karolinska Institute) for their generous gifts of strains.

This work was supported by grant P10766-MED of the Austrian Research Fund (to M.B.) and by grant 70008/2-Pr/4/95 of the Austrian Federal Ministry of Science, Transport and the Arts (to A.V.G.).

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