Molecular Mechanisms of *Salmonella* Virulence

By

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A DISSERTATION

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Abstract

Successful initiation of infection with the enteric pathogen *Salmonella typhimurium* depends on bacterial adhesion to- and colonization of- the intestinal mucosa. Numerous *in vitro* studies suggest that these processes may depend on fimbrial adhesins. However, mutations in individual fimbrial biosynthetic genes only moderately alter mouse virulence, suggesting that fimbriae play only a minor role during infection *in vivo*. Here, an alternative interpretation of these observations is evaluated, namely that fimbrial adhesins of *S. typhimurium* are functionally redundant and coordinately facilitate bacterial attachment to the intestinal mucosa. Using a genetic approach, it is demonstrated that multiple fimbrial adhesins are required for full virulence in mice and that fimbrial operons act in synergy during infection *in vivo*. Together, our results imply that loss of a single attachment factor is compensated for by the production of alternate adhesins.

Following attachment, *S. typhimurium* penetrates the intestinal epithelium and enters the lamina propria, where resident macrophages ingest invading bacteria. Despite the fact that *S. typhimurium* has devised strategies to survive and replicate within this hostile environment, recent studies indicate that *S. typhimurium* can induce apoptosis in infected macrophages *in vitro*. This process is rapid, and depends on *Salmonella* pathogenicity island 1 (SPI1). Here, we report that *S. typhimurium* activates programmed macrophage cell death in the

absence of SPI1 gene expression. SPI1 independent induction of apoptosis in infected macrophages is delayed and depends on *ompR* and SPI2. Furthermore, it is demonstrated that rapid- and delayed activation of programmed macrophage cell death are independent processes. Our model predicts that *S. typhimurium* induces apoptosis in infected macrophages under discrete physiological conditions and at distinct locations within an infected host.

Chapter 1: Introduction

I. Epidemiology of *Salmonella* infections in humans

The enteric pathogen *Salmonella* is the leading cause of bacterial gastroenteritis in humans and a major cause of morbidity and mortality worldwide. Primary sources of infection with *Salmonella* are contaminated foods. Infections with *Salmonella* range in severity from self-limiting gastroenteritis and transient bacteremia to typhoid fever. Occasionally, *Salmonella* colonizes the gallbladder, thereby chronically infecting its host. This carrier state is characterized by continuous fecal shedding and increased frequency of transmission (85, 147, 197). Estimates predict that annually, *Salmonella* is the cause of 3.7 million cases of gastroenteritis in the US alone and 12.5 million cases of typhoid fever worldwide. As a result, over three million people die every year (147).

A. Taxonomy

Based on their 5S and 16S ribosomal RNA sequences, multi-locus enzyme electrophoresis, and antigenic serotyping, over 2,300 *Salmonella* species have been identified and categorized into two lineages, *S. enterica* and *S. bongori*. These two lineages are further divided in seven subspecies (*S. enterica* subspecies I-IV, VI, VII and *S. bongori* [subspecies V]) (11, 203). Most

Salmonella species infect warm-blooded vertebrate hosts, although important reservoirs include cold-blooded animals and birds (11, 203). The majority of clinical isolates (>99%) belong to *S. enterica* subspecies I (203), including *Salmonella enterica* serovars Typhimurium and Enteritidis (*S. typhimurium* and *S. enteritidis*, respectively), which cause self-limiting gastroenteritis in man, and *S. enterica* serovar Typhi (*S. typhi*), the causative agent of human typhoid fever (85, 147, 197, 203).

II. Evolution of *Salmonella* virulence

Salmonella virulence is a complex, multi-factorial process. Virulence genes are scattered throughout the chromosome and an estimated four percent of the genome is required for virulence (30). Recent studies indicate that a variety of genetic alterations, including the acquisition of colonization factors, epithelial cell invasion genes, and intracellular survival genes, contributed to the evolutionary divergence between *Salmonella* and *Escherichia coli*, its closest non-pathogenic relative (2, 11, 12, 16, 20, 83, 84, 86, 129, 166, 203, 210). *Salmonella* horizontally acquired at least five large virulence gene clusters (*Salmonella* pathogenicity island (SPI1-5) (27, 28, 72, 74, 90, 150, 167, 205, 238, 239), two of which will be discussed in greater detail below.

Evolutionary changes continue to expand the large, already existing arsenal of virulence determinants in *Salmonella* and may account for the

infection of (and transmission to-) a wide variety of warm-blooded animals, reptiles, and birds, making *Salmonella* a most successful parasite.

III. Pathogenesis of systemic infection

Because humans are the only known reservoir for *S. typhi*, a good animal model to study this host specific pathogen does not exist (16, 85, 147). However, mice infected with *S. typhimurium* develop a typhoid-like disease (186). Consequently, the murine model of infection is an excellent experimental system to study systemic disease caused by *Salmonella*.

S. typhimurium infection in mice can be divided into an intestinal phase and a systemic phase. During the intestinal phase of infection, *Salmonella* adheres to and colonizes the intestinal mucosa prior to penetration of this epithelial barrier. During the systemic phase of infection, *Salmonella* primarily, but not exclusively, resides and proliferates within macrophages. In fact, professional phagocytes within the liver and spleen are the major sites for bacterial replication within the mouse. In this respect, this dissertation focuses on two key aspects of *Salmonella* virulence, (i) bacterial adhesion and colonization during early stages of infection, and (ii) interactions between *Salmonella* and the macrophage.

A. Intestinal phase of infection

The oral infectious dose for *Salmonella* is much higher than for *Shigella*, the causative agent of bacillary dysentery, because unlike *Shigella*, less than one percent of the ingested bacteria survive the acidic environment of the stomach (37, 222). Upon arrival in the intestinal lumen, *Salmonella* faces new barriers, including bile salts, peristaltic bowel movements and resident gut flora. Here, *Salmonella* must compete with commensals for attachment sites and nutrients (182, 196), as bacterial adhesion and colonization is a prerequisite for efficient penetration of the intestinal mucosa.

Several studies indicate that *Salmonella* adheres most tightly to murine tissue derived from the terminal ileum (37, 105), a process that depends on various filamentous cell-surface organelles that are collectively referred to as fimbriae (18, 46). Within the distal ileum, *Salmonella* resides primarily within specialized lymphoid follicles (Peyer's patches), despite the fact that they account for only a small fraction of the small intestine (37, 43, 79, 105, 113, 213, 221). Here, *Salmonella* efficiently invades and destroys M cells in the follicle associated epithelium (FAE) overlaying Peyer's patches (42, 113). M cells play an important role in mucosal immunity and are structurally and functionally distinct from other cells found in the FAE (including Goblet cells, Paneth cells and enterocytes). M cells efficiently sample and subsequently present lumenal antigens to immune cells following transcytosis (112, 117, 119, 161, 237). These

characteristics suggest that *Salmonella* may have exploited M cells to reach its preferred niche within a host, the macrophage.

Invasion and destruction of M cells is accompanied by uncontrolled bacterial replication, polymorphonuclear (PMN) cell infiltration, intestinal ulceration, perforation, and hemorrhage (26, 42, 78, 112, 113, 173). Penetration of the intestinal mucosa depends on a highly specialized type III protein secretion system encoded within SPI1 (55, 72, 74), although a SPI1 independent strategy to cross the intestinal epithelial barrier has recently been described (21, 233).

1. Bacterial adhesion to- and colonization of the intestinal mucosa

The importance of bacterial adhesion during infection was first recognized in the late 1940s (121). However, fimbriae, non-conjugative pili that mediate bacterial attachment, were not described until later (60). These thin, filamentous proteinaceous cell-surface organelles are structurally distinct from flagellar filaments, which mediate bacterial motility. Fimbriae are 1-2 μm in length and range from 2-8 nm in diameter, whereas flagella are 5-10 μm long with a diameter of circa 20 nm (139, 141).

2. *Salmonella* fimbriae

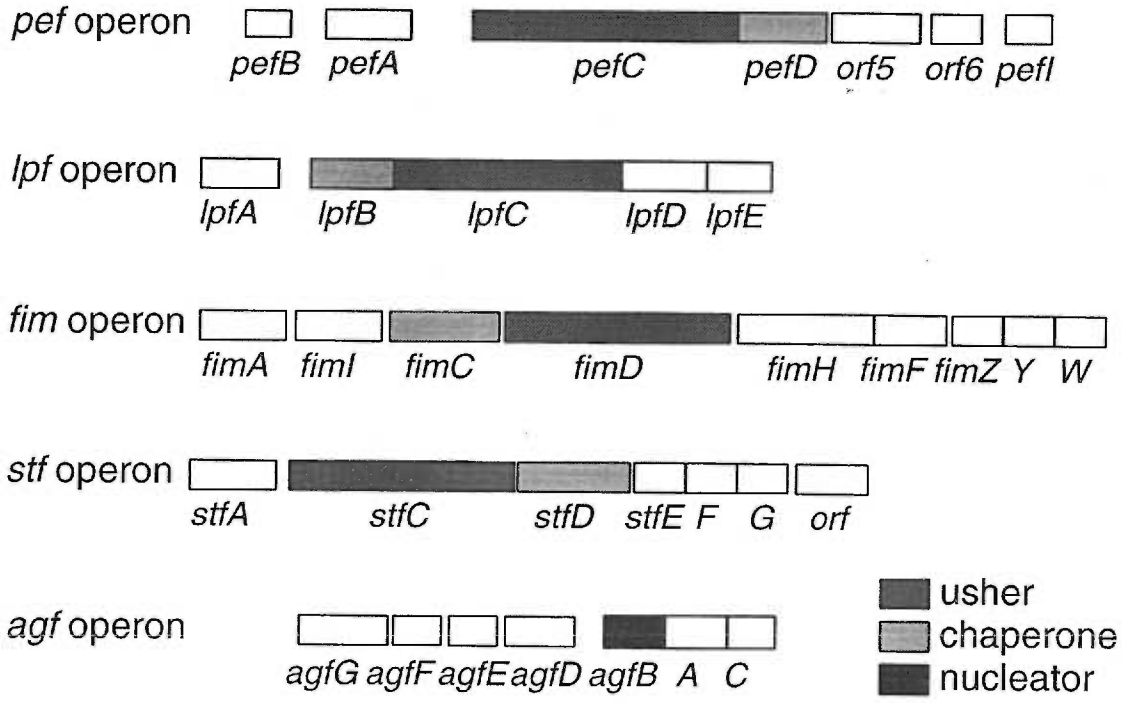
S. typhimurium produces at least four distinct fimbrial structures, designated type I, long polar (LP), plasmid encoded (PE), and thin aggregative (curli) fimbriae, which are encoded by the *fim*, *lpf*, *pef*, and *agf* operons,

respectively (13, 44, 48, 49, 69) (Fig. 1-2). Recently, a fifth fimbrial operon (*stf*) was identified in *S. typhimurium* by subtractive hybridization between *S. typhimurium* and *S. typhi* (157). However, it is not known whether this operon is functional.

Salmonella fimbriae can be grouped according to their assembly mechanism. Type I, LP, and PE fimbriae require outermembrane ushers and periplasmic chaperones for successful pilus assembly on the cell-surface, whereas thin aggregative fimbriae are assembled via a novel extracellular nucleation and precipitation pathway. Sequence homologies indicate that the *stf* operon encodes both a chaperone and usher homologue (157). The periplasmic chaperone is necessary for both release of newly synthesized pilus subunits into the periplasmic space and subsequent docking of the chaperone-subunit complex to the usher. The usher forms an outermembrane pore that facilitates ordered translocation- and incorporation of fimbrial subunits into a growing pilus structure (57, 108, 110, 114, 128, 171, 200, 211, 224). Assembly of thin aggregative fimbriae requires extracellular nucleation and subsequent precipitation of secreted fimbrial subunits onto the bacterial cell surface. This process depends on a membrane associated nucleator protein and an outermembrane lipoprotein that may either function as a chaperone during the secretion of pilus subunits or form a pore that facilitates translocation of subunits (25, 91, 92, 138, 170, 188).

Figure 1-1. Structural organization of fimbrial operons in *S. typhimurium*.

S. typhimurium encodes at least four distinct fimbrial operons. PE, LP, and type I fimbriae (encoded by the *pef*, *lpf* and *fim* operon, respectively) are assembled via a chaperone and usher dependent pathway. Based on sequence homology, the *stf* fimbrial operon also encodes a putative outer membrane usher and putative chaperone. Thin aggregative (curli) fimbriae (encoded by the *agf* operon) are assembled via a novel extracellular nucleation and precipitation pathway.


pef operon
pefB pefA pefC pefD orf5 orf6 pefI
lpf operon
lpfA lpfB lpfC lpfD lpfE
fim operon
fimA fimI fimC fimD fimH fimF fimZ Y W
stf operon
stfA stfC stfD stfE F G orf
agf operon
agfG agfF agfE agfD agfB A C
usher
chaperone
nucleator

3. Role of *Salmonella* fimbriae in adhesion

Numerous studies indicate that fimbrial adhesins of *S. typhimurium* play a role in bacterial adhesion and invasion of the intestinal mucosa (21, 63, 106, 115, 116, 136, 137, 223). Mixed infection experiments using murine ileal loop assays show that LP fimbriae mediate adhesion to Peyer's patches (19), whereas PE fimbriae promote binding to the villous intestinal epithelium (15). Other *in vitro* studies indicate that thin aggregative fimbriae and type I fimbriae play a role in bacterial attachment to tissue culture cells. Thin aggregative fimbriae mediate attachment to murine derived H10 cells (small intestine), whereas type I fimbriae promote binding to human derived HeLa cells (cervix carcinoma) (15, 106, 219).

Despite the role of *Salmonella* fimbriae in mediating adhesion to epithelial cells *in vitro*, blockage of individual fimbrial adhesins does not strongly reduce mouse virulence (105). In addition, mutations in essential fimbrial biosynthetic genes only moderately decrease (15, 19), or even slightly increase (137) mouse virulence. Thus, the contribution of individual fimbrial adhesins to *Salmonella* virulence *in vivo* is not apparent from these studies. In order to gain new insights into the role of *Salmonella* fimbriae during infection *in vivo*, we have evaluated the possibility that fimbrial adhesins in *S. typhimurium* are functionally redundant.

B. Systemic phase of infection

Following successful penetration of the intestinal mucosa, *Salmonella* is released into the intestinal subepithelium, where bacteria are ingested by

resident phagocytes. *Salmonella* survives and replicates within macrophages and disseminates systemically via the mesenteric lymph nodes and reticuloendothelial system, eventually reaching the bloodstream. Transient bacteremia is usually controlled by resident macrophages in the liver and spleen. However, bacteria that resist phagocyte killing re-enter the bloodstream after extensive replication inside macrophages and cause a second round of bacteremia that kills the infected host (85, 147, 197). Collectively, these studies indicate that *Salmonella* interacts extensively with host macrophages and may have even exploited the longevity of these terminally differentiated cells to escape serum antibodies and complement attack.

1. *Salmonella* survives and replicates within macrophages

Intracellular *Salmonella* reside within altered membrane-bound vacuoles (4, 5, 34, 109, 135, 152, 169, 183, 184, 229). Numerous studies indicate that *Salmonella* survives and replicates within professional phagocytes, including (i) the direct observation of *Salmonella* within hepatic phagocytes (61, 187), (ii) persistance of infection in mice treated with gentamicin (an antibiotic that kills extracellular, but not intracellular bacteria) (29, 61), (iii) protection from bactericidal serum components after uptake by phagocytic cells (192), and (iv) comparative infection studies in strains of mice that produce macrophages with varying resistance to *Salmonella* (153, 165). Furthermore, genetic studies

demonstrate that *Salmonella* mutants that cannot survive or replicate inside macrophages are attenuated for systemic infection in mice (14, 64, 71).

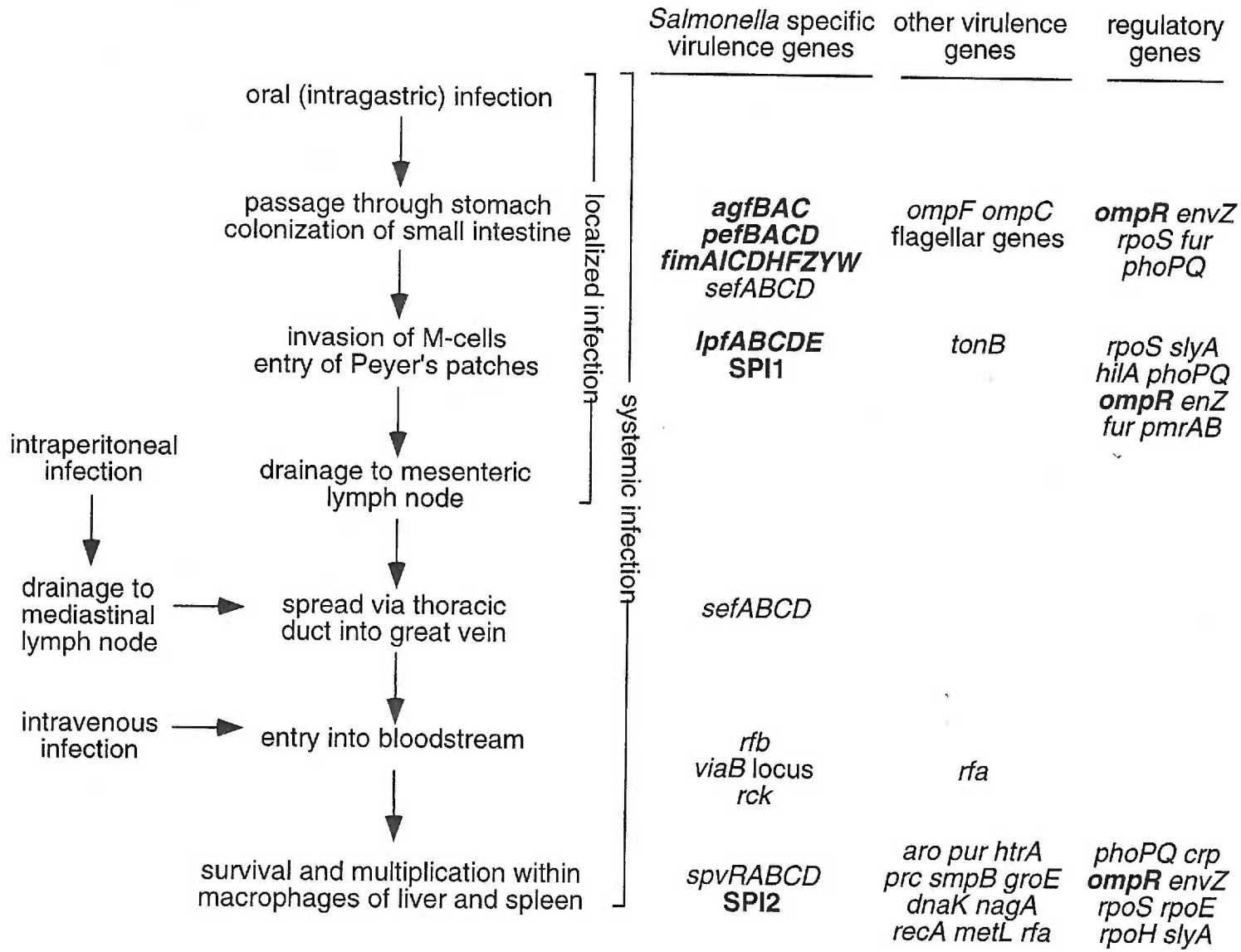
Long-term survival and growth inside macrophages depends on multiple genetic loci that are scattered throughout the *Salmonella* genome (6, 14, 31, 32, 35, 64, 65, 71, 82, 134, 148, 210). Moreover, numerous bacterial genes are specifically induced within these host cells (33, 36, 68, 97, 142, 180, 230, 231). In particular, the SPI2 encoded type III protein secretion system is highly induced inside macrophages and plays a role in intracellular proliferation at systemic sites of infection (41, 98-100, 167, 205, 229, 231, 234).

2. *Salmonella* kills infected macrophages

While all of these studies indicate that *Salmonella* survives and replicates within macrophages, several groups have recently demonstrated that *Salmonella* is also able to kill these host cells *in vitro* (7, 39, 135, 156). However, it is not clear how induction of macrophage cell death contributes to *Salmonella* virulence *in vivo* since intramacrophage survival is required for a persistent systemic infection. One report indicates that *Salmonella* kills macrophages as late as 18 hours post-infection (135), whereas other studies show that the SPI1 encoded type III protein secretion system is necessary for rapid induction of apoptosis (programmed cell death) (39, 140, 156). Similarly, *Shigella* and *Yersinia*, two other enteric pathogens, also use a type III protein secretion system to induce apoptosis in infected macrophages (40, 151, 155, 156, 246), suggesting that

Figure 1-2. Course of infection and *Salmonella* genes required during the development of murine typhoid fever.

Course of infection and *Salmonella* genes required during the development of murine typhoid fever. Bacterial genes required for infection may vary depending on the route of infection (intragastric, intraperitoneal, or intraveous). A number of virulence genes are studied in greater detail in this dissertation (bold). Adapted from Bäumler *et al.* (ref. 20) with permission.


Salmonella specific virulence genes
other virulence genes
regulatory genes
oral (intragastric) infection
passage through stomach colonization of small intestine
agfBAC pefBACD fimAICDHFZYW sefABCD
ompF ompC flagellar genes
ompR envZ rpoS fur phoPQ
invasion of M-cells entry of Peyer's patches
lpfABCDE SPI1
tonB
rpoS slyA hilA phoPQ ompR enZ fur pmrAB
drainage to mesenteric lymph node
localized infection
intraperitoneal infection
drainage to mediastinal lymph node
spread via thoracic duct into great vein
sefABCD
systemic infection
intravenous infection
entry into bloodstream
rfb viaB locus rck
rfa
survival and multiplication within macrophages of liver and spleen
spvRABCD SPI2
aro pur htrA prc smpB groE dnaK nagA recA metL rfa
phoPQ crp ompR envZ rpoS rpoE rpoH slyA

these pathogens may use similar strategies to activate programmed macrophage cell death.

IV. Bacterial induced apoptosis

A. Overview of apoptosis

Programmed cell death or apoptosis is an essential developmental process in multi-cellular eukaryotes. It is characterized by DNA fragmentation, chromatin condensation, membrane blebbing, production of reactive oxygen species (ROS), and exposure of phosphatidyl serine (PS) on the outer leaflet of the cell (122, 125, 143, 202, 226). Apoptosis is an important host defense mechanism against a variety of intracellular parasites as cell death prevents replication and spread of invading pathogenic microorganisms.

Cells that are instructed to die activate "effector" proteases that disassemble and degrade cellular components and structures before membrane integrity is lost, thereby minimizing the release of cytoplasmic and nuclear contents into the extracellular environment. These events account for ordered proteolysis in apoptotic cells, which prevents excessive localized inflammation typical of necrotic cell death. Thus, programmed cell death can be divided into three distinct phases: (i) an initiation phase, (ii) an effector phase, and (iii) a degradation phase (125, 226).

1. Initiation phase

Irreversible activation of the apoptotic machinery is initiated when a cell either (i) no longer receives necessary survival signals, (ii) receives conflicting signals during the cell cycle, or (iii) is specifically instructed to die (8, 235). This latter process depends on the transmission of pro-apoptotic signals via cell surface receptors, which include members of the tumor necrosis factor receptor (TNFR) superfamily (CD95 and TNFR1). The cytoplasmic tails of these receptors contain domains that interact with numerous pro-survival and pro-apoptotic adaptor proteins that ultimately lead to activation of either (i) NF-κB, a pro-survival transcription factor, or (ii) a family of death effectors (including caspase-8) (8, 160).

2. Effector phase

Many effector molecules are involved in determining cell fate, including Bcl-2 protein family members and a family of cysteine proteases collectively referred to as caspases (1, 124, 162, 185, 198, 227). Thus far, fifteen mammalian Bcl-2 family members have been identified. The Bcl-2 protein subfamily includes Bcl-2, Bcl-x_L, and Bcl-w, and promotes cell survival, whereas the Bax subfamily (including Bax, Bak and Bok) and BH3 subfamily (including Bik, Blk, and Bad) promote apoptosis (1, 185). Various Bcl-2 protein family members play a key role in determining cell fate as they are located in the mitochondrial outer membrane where they may form pores that monitor the

mitochondrial membrane potential. An apoptotic stimulus (such as ceramide) results in a drop in mitochondrial membrane potential, which triggers the release of pro-apoptotic molecules including cytochrome c, apoptosis inducing factor 1 (APF-1), and possibly procaspase-3, caspase-2 and caspase-9 (1, 80, 81, 93, 94, 111, 126, 179, 212, 220). Thus far, thirteen mammalian caspases have been identified. Initiator caspases (including caspase-8, 9) subsequently activate effector caspases (including caspase-3, 6, 7), which play a major role in disassembling and degrading cellular structures. Other caspases (including caspase-1, 11) provoke an inflammatory response (80, 227, 235) that may be required for subsequent removal of apoptotic bodies and cellular debris.

Two subdomains of a proteolytically processed pro-caspase heterodimerize, after which two heterodimers form a tetramer with two independent active sites (8, 227). Rapid autoproteolytic activation of initiator pro-caspase-8 depends on "death receptor" mediated signalling (TNFR1 and CD95), whereas activation of initiator pro-caspase-9 requires the release of mitochondrial cytochrome c and capase-8 (8, 227).

3. Degradation phase

During the degradative phase, effector caspases degrade and disassemble cellular structures and components through specific proteolysis. Proteins or protein complexes targeted for destruction include DNA repair proteins, structural components of the nuclear envelope, the actin cytoskeleton,

and cell signaling molecules (160, 162, 198). During later stages of apoptosis, membranes become porous and the remaining intracellular contents are released into the extracellular environment. This results in a local inflammatory response characterized by ingestion and subsequent degradation of apoptotic bodies by neighboring phagocytes.

B. Bacterial type III protein secretion and induction of apoptosis

Activation of programmed cell death is an important host defense mechanism against a variety of pathogenic microorganisms. However, several viruses have devised strategies to inhibit apoptosis (191), while various bacterial pathogens actively induce apoptosis to enhance their chances of survival and spread within the host (236). Here, activation of programmed macrophage cell death through bacterial type III protein export systems is reviewed in more detail.

1. Type III protein secretion in bacterial pathogens

Type III protein export systems are conserved amongst a variety of Gram negative bacterial pathogens in which they promote virulence by translocating multiple effectors into the cytoplasm of an infected eukaryotic cell through what has been referred to as a "molecular syringe" (50-53, 72, 73, 107, 127, 131, 146). Type III secreted proteins share only limited homology and have distinct targets and roles within the host-cell cytosol (40, 70, 76, 87, 95, 101, 120, 151, 155, 163, 172, 189, 190, 243, 244). Interestingly, numerous reports indicate that

Salmonella, *Shigella*, *Pseudomonas*, and *Yersinia* employ their respective type III protein secretion systems to deliver cytotoxins into the cytoplasm of a target cell (40, 66, 101, 151, 155, 172, 189, 190, 244). Moreover, various studies indicate that delivery of a subset of type III secreted effectors into the host cell cytoplasm results in activation of the cellular apoptotic machinery (39, 40, 96, 151, 155, 156, 244, 246). However, the various mechanisms through which these pathogens induce apoptosis are not completely understood.

2. Intracellular *Shigella* induce apoptosis in macrophages

Shigella is unable to enter apical surfaces of colonic epithelial cells and depends on successful M cell invasion to gain access to basolateral epithelial cell surfaces in the large intestine (158, 199, 245). However, resident macrophages in the lamina propria engulf invading bacteria in response to infection (245). *Shigella* escapes from the macrophage phagosome and activates programmed cell death by secreting IpaB, a type III effector that binds to and activates caspase-1 (interleukin 1β converting enzyme, ICE). Caspase-1 in turn activates both the host apoptotic machinery and interleukin 1β (IL-1β), a potent pro-inflammatory cytokine (40, 88, 89, 102-104, 225, 244, 246, 247) that is secreted by the apoptotic macrophage. The massive influx of PMNs following the release of pro-inflammatory cytokines subsequently destabilizes the intestinal epithelial barrier, thereby permitting bacterial invasion of basolateral epithelial cell surfaces (22, 145, 177, 178). Rapid bacterial growth, followed by intercellular

dissemination, results in extensive tissue damage characteristic of Shigellosis (158, 199, 245). Together, these observations indicate that induction of macrophage apoptosis contributes to *Shigella* virulence by facilitating intestinal dissemination.

3. Extracellular *Yersinia* induce apoptosis in macrophages

In contrast to *Shigella*, *Yersinia* successfully resists phagocytosis by resident macrophages in the lamina propria after penetrating M cells in ileal Peyer's patches (51, 52). *Y. pseudotuberculosis* induces apoptosis in infected macrophages by translocating YopJ (YopP in *Y. enterolitica*), a type III effector, into the macrophage cytoplasm (151, 155, 195). Here, YopJ inhibits phosphorylation of various members of the mitogen activated protein kinase kinase (MAPKK) superfamily, as well as IκB-α and IκB-β, two NF-κB inhibitory proteins. These processes prevent activation of MAPKK superfamily members and inhibit prolonged degradation of IκB. Interference with these signalling pathways delay pro-survival responses that depend on NF-κB. Furthermore, these processes result in downregulation of IL-1α transcription and reduced secretion of other pro-inflammatory cytokines, including IL-8 and TNFα (172, 174, 175, 193, 194, 201). Thus, YopJ/P dependent induction of apoptosis in infected macrophages is an important immune evasion mechanism as macrophages and PMNs efficiently kill intracellular *Yersina* (152). In support of this view, it was recently shown that *yopJ* mutant bacteria are attenuated in their

ability to establish a systemic infection (154), directly demonstrating that induction of macrophage apoptosis contributes to *Yersinia* virulence *in vivo*.

4. Macrophages infected with *Salmonella* undergo apoptosis

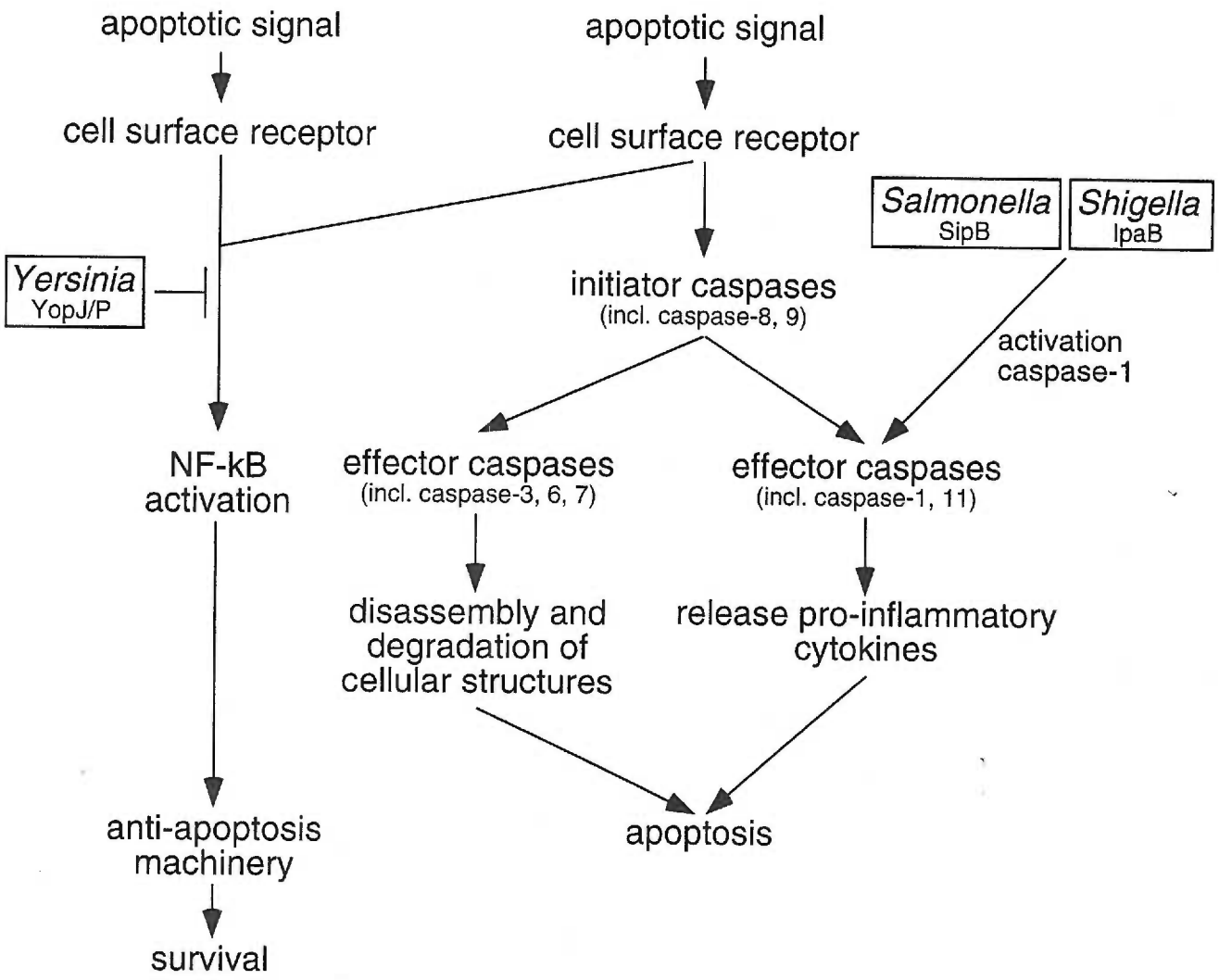
Several studies indicate that SipB, a SPI1 secreted type III effector molecule, and the SPI1 encoded type III protein export apparatus itself are required for the induction of apoptosis in infected macrophages (39, 101, 140, 156). The *sipB* gene is homologous to *ipaB* in *Shigella* and can functionally complement an *ipaB* defect with respect to epithelial cell invasion. Like IpaB, microinjection of SipB into the macrophage cytoplasm results in apoptosis (40, 101). SipB binds to and activates caspase-1, which results in production of IL1β, a potent pro-inflammatory cytokine. Additionally, SipB dependent induction of apoptosis is blocked in the presence of acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (Ac-YVAD-cmk), a chemical caspase-1 inhibitor. Lastly, peritoneal macrophages isolated from caspase-1 deficient mice are resistant to *Salmonella* induced programmed cell death (101). Cumulatively, these observations indicate that SipB is both necessary and sufficient for the induction of apoptosis in infected macrophages.

Unlike in *Shigella* and *Yersinia*, however, it is not clear how induction of apoptosis in infected macrophages contributes to *Salmonella* virulence *in vivo*, since long-term residence inside professional phagocytes is required for a persistent systemic infection (64). Interestingly, apoptotic phagocytes can be

detected in hepatic tissue derived from mice intravenously infected with *Salmonella* (187), suggesting that *Salmonella* induce apoptosis in host cells at systemic sites of infection. However, SPI1 mutant bacteria are attenuated only when administered to mice orally, indicating that the SPI1 encoded type III protein secretion system is dispensable during systemic infection (74). These observations suggest that *Salmonella* induced apoptosis in phagocytic cells at systemic sites of infection depend on genes other than those encoded within SPI1. Here, we have examined the possibility that *Salmonella* induces macrophage apoptosis independently of SPI1. We demonstrate that *Salmonella* can induce macrophage apoptosis via two independent pathways, both of which depend on a type III protein secretion system.

Figure 1-3. Various enteric pathogens interfere with the apoptotic machinery of the host by injecting type III secreted effectors into the macrophage cytoplasm.

Shigella, *Yersinia*, and *Salmonella* induce macrophage apoptosis using a type III protein secretion system. Translocation of the type III secreted effectors IpaB (*Shigella*), YopJ/P (*Yersinia*), or SipB (*Salmonella*) into the macrophage cytoplasm results in apoptosis. This diagram indicates at what step these various proteins interfere with the apoptotic machinery of the host. See text for further details.


apoptotic signal
apoptotic signal
cell surface receptor
cell surface receptor
Salmonella
SipB
Shigella
IpaB
Yersinia
YopJ/P
initiator caspases
(incl. caspase-8, 9)
activation
caspase-1
NF-kB
activation
effector caspases
(incl. caspase-3, 6, 7)
effector caspases
(incl. caspase-1, 11)
disassembly and
degradation of
cellular structures
release pro-inflammatory
cytokines
anti-apoptosis
machinery
apoptosis
survival

V. Research objectives

This dissertation focuses on two key aspects of *Salmonella* virulence. We will investigate the role of individual fimbrial adhesins *in vivo* by evaluating the possibility that *S. typhimurium* fimbriae are functionally redundant and coordinately facilitate bacterial adhesion and colonization during the development of murine typhoid. Furthermore, we will investigate the possibility that *Salmonella* activates programmed macrophage cell death independently of SPI1.

Chapter 2:

Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice

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Abstract

Adhesion is an important initial step during bacterial colonization of the intestinal mucosa. However, insertional inactivation or deletion of the *Salmonella typhimurium* fimbrial operons *lpf, pef,* or *fim* only moderately alter mouse virulence. The respective adhesins may thus play only a minor role during infection or *S. typhimurium* may encode alternative virulence factors that can functionally compensate for their loss. To address this question, we constructed mutations in all known fimbrial operons of *S. typhimurium*: *fim, lpf, pef,* and *agf.* A mutation in the *agfB* gene resulted in a three-fold increase in the oral 50% lethal dose (LD_{50}) of *S. typhimurium* for mice. In contrast, a *S. typhimurium* strain carrying mutations in all four fimbrial operons (quadruple mutant) had a 26-fold increased oral LD_{50}. The quadruple mutant, but not the *agfB* mutant, was recovered in reduced numbers from murine fecal pellets, suggesting that a reduced ability to colonize the intestinal lumen contributed to its attenuation. These data are evidence for a synergistic action of fimbrial operons during colonization of the mouse intestine and the development of murine typhoid fever.

Introduction

S. typhimurium causes murine typhoid fever. This systemic infection is initiated by colonization and penetration of the intestinal mucosa, which is commonly accepted as a necessary first step in the establishment of infection. Indeed, recent evidence suggests that fimbrial adhesins of *S. typhimurium* play a role during bacterial attachment to and invasion of the intestinal mucosa *in vitro* and *in vivo* (15, 19, 21, 137). For example, attachment mediated by fimbrial adhesins appears to be important for invasion of cultured epithelial cell lines *in vitro* (17, 59, 63). In addition, a mutation in *pefC*, encoding the putative outer membrane usher of plasmid encoded (PE) fimbriae, reduces the ability *of S. typhimurium* to attach to the murine villous small intestine (15). Furthermore, insertional inactivation of *lpfC*, encoding the putative outer membrane usher of long polar (LP) fimbriae, impairs colonization of murine Peyer's patches by *S. typhimurium* (19, 21). However, since mutations in fimbrial biosynthetic genes cause only a subtle decrease (19) or even a slight increase (137) in mouse virulence, it is not evident from these data that adhesion mediated by fimbriae is essential during the development of murine typhoid.

Neutralizing antibodies that block individual adhesins do not strongly reduce mouse virulence of *S. typhimurium* (105). It has therefore been speculated that attachment is not essential during murine typhoid (105). However, more recent evidence suggests an alternative interpretation of these

data, namely that *S. typhimurium* encodes alternate pathways for intestinal penetration (21, 137, 233). The presence of additional entry mechanisms may mask the effect of mutations in individual virulence genes of a single pathway. For example, a synergy of virulence factors involved in penetrating the intestinal mucosa is suggested by the fact that an *S. typhimurium lpfC invA* double mutant has a 150-fold increased oral 50% lethal dose (LD_{50}). In contrast, isogenic strains carrying a single insertion in either *lpfC or invA* are only 5-15-fold attenuated in mouse virulence, respectively (21). In addition, a similar synergistic effect has been observed for motility and type I fimbriation. Loss of motility has no effect on mouse virulence, and deletion of the *fim* operon, encoding type I fimbriae, results in a modest decrease in LD_{50}. However, a *S. typhimurium* strain that is both nonmotile and lacks type I fimbriae is 150-fold attenuated (137).

The presence of at least four distinct fimbrial operons in *S. typhimurium*, *fim* (45), *lpf* (13), *pef* (69), and *agf* (48), raises the possibility that *S. typhimurium* compensates for a functional defect of any individual fimbrial adhesin by producing alternate attachment elements. Redundancy in virulence determinants involved in intestinal colonization may explain why mutations that affect the expression of only one fimbrial structure have little to no effect on the ability *of S. typhimurium* to cause a lethal systemic infection in mice. Thus, a simultaneous loss of several fimbrial adhesins would be expected to reduce *S. typhimurium* virulence to a greater degree than mutations in individual fimbrial operons. To investigate whether inactivation of the genes essential to assembling distinct

fimbrial adhesins has a synergistic effect on the ability of *S. typhimurium* to cause murine typhoid, we determined the virulence properties of strains carrying mutations in one or more fimbrial operons.

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 incorporated into the media at the following concentrations: naladixic acid, 50 mg/l; kanamycin, 60 mg/l; chloramphenicol, 30 mg/l; carbenicillin, 100 mg/l. Analytical-grade chemicals were purchased from Sigma (St. Louis, Mo.) or Boehringer Mannheim (Indianapolis, Ind.). AJB3 is a fully mouse virulent naladixic acid resistant derivative of *S. typhimurium* strain SR-11 (15). SR-11 derivatives carrying a *pefC*::Tet^r allele (AJB9) or a deletion of the *fim* operon (AJB4) have been described previously (15, 17). Bacteriophage KB1*int* or P22HT*int* was used to transduce *pefC*::Tet^r and *lpfC*::Kan^r mutations from *S. typhimurium* strains AJB7 (15) and AJB1 (19), respectively, into the desired SR-11 background. Recombinant DNA techniques and Southern hybridizations were performed using standard protocols (9).

A 927-bp fragment internal to *agfB* was amplified from χ4252 (wild type SR-11 (137)) using primers *5'*-CTGACAGATGTTGCACTGCTGTG-*3'* and *5'*-TTCGCCCGATTATTTCCTCC-*3'*. This PCR product was cloned into the *Eco*RV site of pBluescript SK to yield plasmid pAV326. The *agfB* allele was inactivated upon insertion of a chloramphenicol resistance gene (a 1.2 kb *Sma*I fragment from pCMXX (21)) into a unique *Nru*I site (nucleotide 466). This plasmid was digested with *Sac*I and *Kpn*I, and a 2.2 kb fragment was cloned into suicide

vector pGP704 (149). The resulting plasmid (pAV328) was transformed into *Escherichia coli* S17λ*pir* (123) and conjugated into *S. typhimurium* AJB3 (wild type) and AJB12 (Δ*fim lpfC pefC*). A double cross-over was obtained by homologous recombination. Chloramphenicol-resistant, carbenicillin-sensitive (loss of vector pGP704) exconjugants were screened for and named AWM394 (*agfB*) and AWM401 (Δ*fim lpfC pefC agfB*).

DNA fragments specific for *fim*, *lpf*, *pef*, and *agf* were used as probes for Southern hybridization. In brief, a *Sph*I fragment of pISF101 (45) and a *Sac*I-*Kpn*I fragment of pMS1054 (13) served as probes to detect *fim*- and *lpf*- specific loci, respectively. A 520-bp fragment internal to *pefA* was amplified by PCR, with primers *5'*- GGGAATTCTTGCTTCCATTATTGCACTGGG-*3'* and *5'*-TCTGTCGACGGGGGATTATTTGTAAGCCACT-*3'* and cloned into the *Eco*RV site of pBluescript (206) to give rise to plasmid pAV323. The *Eco*RI- and *Cla*I-restricted insert of pAV323 was labeled and used as a *pef*- specific probe. A *Sac*I-*Kpn*I fragment of pAV326 was used to generate an *agf*- specific DNA probe. Restriction enzyme-digested chromosomal DNA was separated on an agarose gel and transferred onto a positively charged membrane (Boehringer Mannheim). The predicted sizes of hybridizing fragments were as follows. A *fim*- specific probe detected a 13.7-kb fragment in *Sph*I-restricted chromosomal DNA of *fim*$^+$ strains (AJB3, AJB5, AJB9, AJB11, AWM394, and AWM400) and 10.5- and 3.1-kb fragments in *Sph*I-restricted chromosomal DNA of *fim* mutants (AJB4, AJB6, AJB12, and AWM401). A *lpf*-specific probe detected a 3.7-kb fragment in *Pst*I-

restricted chromosomal DNA of *lpfC*$^+$ strains (AJB3, AJB4, AJB9, and AWM394) and 2.8- and 1.7-kb fragments in *Pst*I-restricted chromosomal DNA of *lpfC* mutants (AJB5, AJB6, AJB11, AJB12, AWM400, and AWM401). A *pef*-specific probe detected a 3.6-kb fragment in *Eco*RI- and *Hin*dIII-restricted chromosomal DNA of *pefC*$^+$ strains (AJB3, AJB4, AJB5, AJB6, and AWM394) and a 2.8-kb fragment in *Eco*RI- and *Hin*dIII-restricted chromosomal DNA of *pefC* mutants (AJB9, AJB11, AJB12, AWM400, and AWM401). An *agf*-specific probe detected a 1.8-kb fragment in *Eco*RI- and *Sal*I-restricted chromosomal DNA of *agfB*$^+$ strains (AJB3, AJB4, AJB5, AJB6, AJB9, AJB11, and AJB12) and a 3.0-kb fragment in *Eco*RI- and *Sal*I-restricted chromosomal DNA of *agfB* mutants (AWM394, AWM400, and AWM401). Detection was performed using the Renaissance random primer fluorescein dUTP labeling and detection system from DuPont NEN (Boston, Mass.).

Mouse experiments. Six- to eight-week-old female BALB/c mice (Jackson laboratories, Bar Harbor, Maine) were used throughout this study. To determine the (two-step) LD_{50}, a series of 10-fold dilutions of overnight cultures in a 0.2-ml volume were injected intragastrically into groups of four mice. The LD_{50}s were calculated 28 days postinfection by the method of Reed and Muench (186). For course of infection studies, approximately 10^8 bacteria were administered to groups of four mice by intragastric injection. Five days postinfection, the animals were sacrificed, after which internal organs (Peyer's patch, villous small intestine,

mesenteric lymph node, spleen, and liver) were collected and homogenized in 5 ml phosphate-buffered-saline (PBS) by using a stomacher (Tekmar, Cincinnati, Ohio). To test the ability of bacterial strains to colonize the intestinal lumen, fecal pellets were collected at days 1, 3 and 5 post infection and homogenized in 5 ml PBS. Ten-fold dilution series were plated on LB-agar plates containing the appropriate antibiotics to determine the number of colony forming units (CFU). Results are reported in CFU per organ or per gram of feces (single strain infections), or as percentages of total number of bacteria recovered (mixed infections). A paired *t* test was used to calculate statistical differences between arithmetic means.

Electron microscopy. Bacterial strains were grown as 3-ml static broth cultures to promote expression of fimbrial structures. Subsequently, 15 µl of bacterial suspension was pipetted onto a Formvar-coated grid (Ted Pella Inc., Redding, Calif.). Bacteria were allowed to adhere for 2 min and then fixed for 1 min using 1.5% glutaraldehyde in sodium cacodylate buffer (100 mM, pH 7.4). The grids were rinsed twice with water and negatively stained with 0.75% (wt/vol) uranyl acetate (pH 6.4) for 1 min. The grids were drained, and subjected to microscopic studies.

Table 2-1. Bacterial strains used in this study.

Strain	Genotype	Reference
E. coli		
DH5α	*endA* 1 *hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ0 d*lac* Δ(*lacZ*)*M15*]	Laboratory collection
S17λ*pir*	*pro thi recA hsdR*; chromosomal RP4-2 (Tn*1*::ISR1 *tet*::Mu Kan^r::Tn*7*); λ*pir*	(123)
S. typhimurium		
AJB3	wild type (SR11χ4252 Nal^r)	(15)
AJB4	Δ[*fim-aph*-11::Tn*10*]-391 Nal^r	(15)
AJB5	Δ[*aph*-11::Tn*10*]-251 Nal^r *lpfC*::Kan^r	This study
AJB6	Δ[*fim-aph*-11::Tn*10*]-391 Nal^r *lpfC*::Kan^r	This study
AJB9	Δ[*aph*-11::Tn*10*]-251 Nal^r *pefC*::Tet^r	(15)
AJB11	Δ[*aph*-11::Tn*10*]-251 Nal^r *lpfC*::Kan^r *pefC*::Tet^r	This study
AJB12	Δ[*fim-aph*-11::Tn*10*]-391 Nal^r *lpfC*::Kan^r *pefC*::Tet^r	This study
AWM394	Δ[*aph*-11::Tn*10*]-251 Nal^r *agfB*::Cam^r	This study
AWM400	Δ[*aph*-11::Tn*10*]-251 Nal^r *lpfC*::Kan^r *pefC*::Tet^r *agfB*::Cam^r	This study
AWM401	Δ[*fim-aph*-11::Tn*10*]-391 Nal^r *lpfC*::Kan^r *pefC*::Tet^r *agfB*::Cam^r	This study
IR715	ATCC14028 Nal^r	(215)
AJB1	IR715 *lpfC*::Kan^r	(19)
AJB7	IR715 *pefC*::Tet^r	(15)
SR-11χ4252	wild type Δ[*aph*-11::Tn*10*]-251	(137)

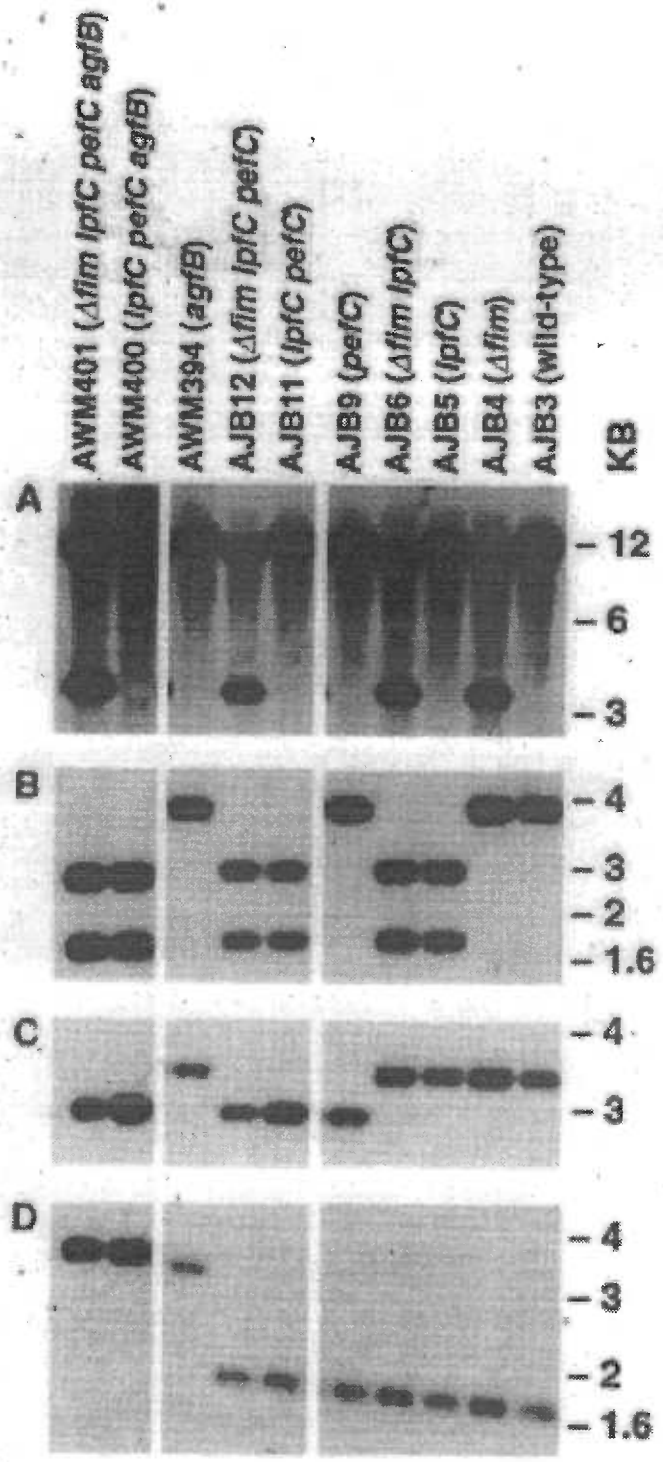
Results

Construction of *S. typhimurium* fimbrial mutants. Mutations in three fimbrial operons, including *fim*, *lpf* and *pef* have been reported previously (15, 19, 137), and were used to construct a set of isogenic *S. typhimurium* mutants that carried deletions of and/or insertions in essential fimbrial biosynthesis genes (Table 2-1). The *lpfC*::Kan^r allele of *S. typhimurium* ATCC14028 derivative AJB1 (19) was transduced into SR-11 derivatives AJB3 (wild type) and AJB4 (Δ*fim*), yielding strains AJB5 (*lpfC*) and AJB6 (Δ*fim lpfC*), respectively. The *pefC*::Tet^r allele of strain AJB7 (15) was transduced into AJB3 and AJB6 to give rise to strains AJB9 (*pefC*) and AJB12 (Δ*fim lpfC pefC*), respectively (Table 2-1). The *lpfC*::Kan^r allele of AJB1 was then transduced into AJB9 to give rise to AJB11 (*lpfC pefC*) (Table 2-1). All mutants were confirmed by Southern blot analysis with the appropriate DNA probes (Fig. 2-1).

Thin aggregative fimbriae, which are encoded by the *S. typhimurium agf* operon, are assembled by an export machinery that is distinct from the chaperone and usher dependent transport systems of type 1 fimbriae, PE fimbriae or LP fimbriae. Curli, encoded by the *csg* operon in *E. coli* is the prototypic member of this novel pilus assembly class. Recent evidence by Hammar *et al.* suggests that CsgB, a membrane associated nucleator protein, is required for the assembly of curli fimbriae on the bacterial cell surface (92). It was therefore decided to inactivate *agfB*, the *csgB* homologue in *S. typhimurium* (48). An *agfB* allele (carried on plasmid pAV328) was inactivated by insertion of a

Figure 2-1. Southern blot analysis of fimbrial mutants.

Southern blot analysis of chromosomal DNA digested with *Sph*I using a *fim*-specific probe (A), of chromosomal DNA digested with *Pst*I using an *lpf*-specific probe (B), of chromosomal DNA digested with *Eco*RI and *Hin*dIII using a *pef*-specific probe (C), and of chromosomal DNA digested with *Eco*RI and *Sal*I using an *agf*-specific probe (D). For further details, see Materials and Methods. Molecular sizes in kilobases (kb) are shown at right.


AWM401 (Δflm lpfC pefC agfB)
AWM400 (lpfC pefC agfB)
AWM394 (agfB)
AJB12 (Δflm lpfC pefC)
AJB11 (lpfC pefC)
AJB9 (pefC)
AJB6 (Δflm lpfC)
AJB5 (lpfC)
AJB4 (Δflm)
AJB3 (wild-type)
KB
A
12
6
3
B
4
3
2
1.6
C
4
3
D
4
3
2
1.6

1.2 kb chloramphenicol resistance cassette and introduced into strains AJB3 (wild type), AJB11 (*lpfC pefC*), and AJB12 (Δ*fim lpfC pefC*). A double cross-over events were obtained by homologous recombination, and the resulting strains were designated AWM394 (*agfB*), AWM400 (*lpfC pefC agfB*) and AWM401 (Δ*fim lpfC pefC agfB*), respectively (Table 2-1). All three mutants were confirmed by Southern blot analysis with an *agfB* specific DNA probe (Fig. 2-1).

Synergistic effect of mutations in fimbrial operons on mouse virulence. LD_{50} studies were conducted to investigate the effect of mutations in fimbrial operons on mouse virulence (Table 2-2) (186). Strains carrying mutations in a single fimbrial operon were either more virulent (AJB4, Δ*fim*) or less than five-fold attenuated (AJB5, *lpfC*; AJB9, *pefC*; and AWM394, *agfB*) in comparison with the wild type (AJB3). Strain AJB12 (Δ*fim lpfC pefC*) also exhibited slightly increased virulence, suggesting that the phenotype of a *fim* deletion mutant is dominant over the attenuating effect of mutations in *lpf* and *pef*. Interestingly, AWM400 (*lpfC pefC agfB*), is more strongly attenuated (> 29-fold) than the AWM401 (Δ*fim lpfC pefC agfB*) (below and Table 2-2). We and others observed that all *fim* mutants tested had a slight increase in virulence when compared to the wild-type (137) (Table 2-2 and our unpublished results) These data suggest a dominant phenotype for mutant *fim* alleles.

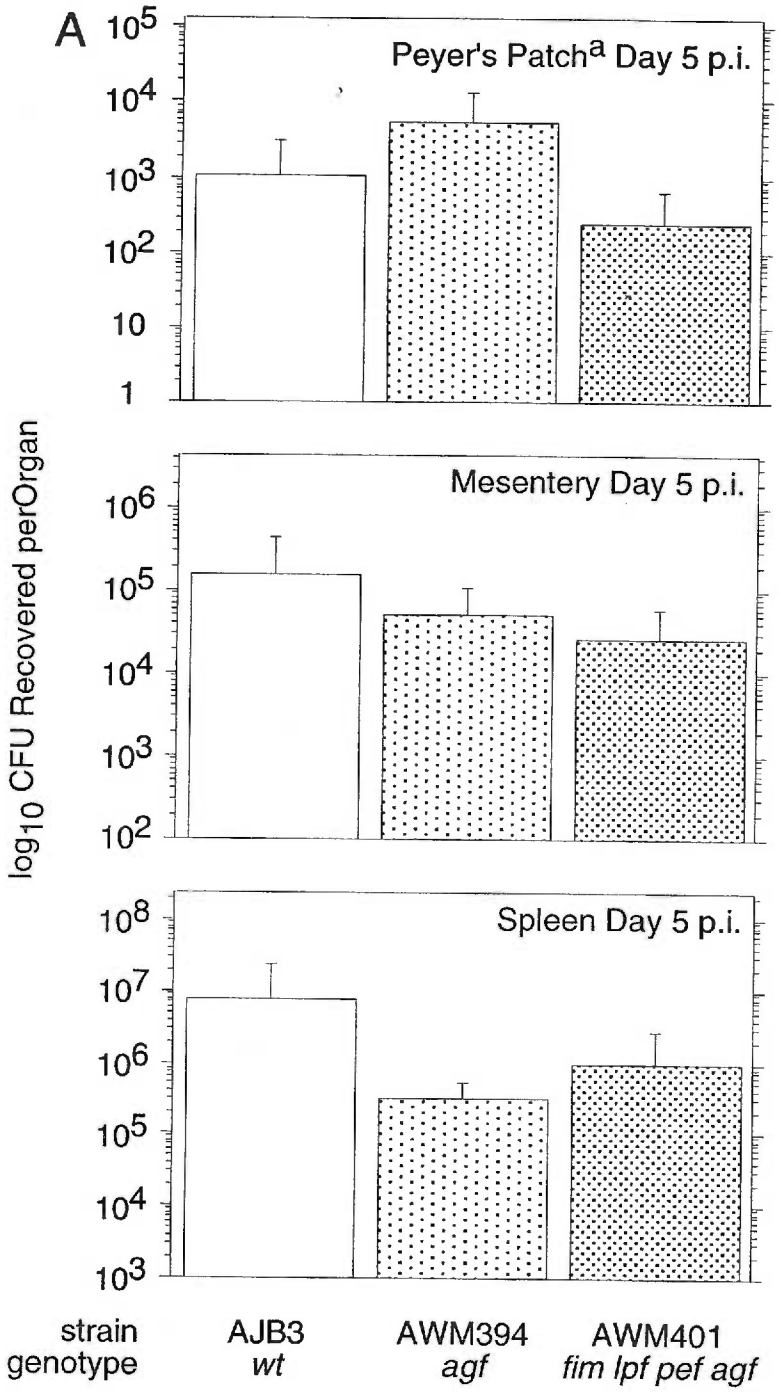
Table 2-2. Virulence properties of fimbrial mutants of *S. typhimurium* when administered orally to BALB/c mice.

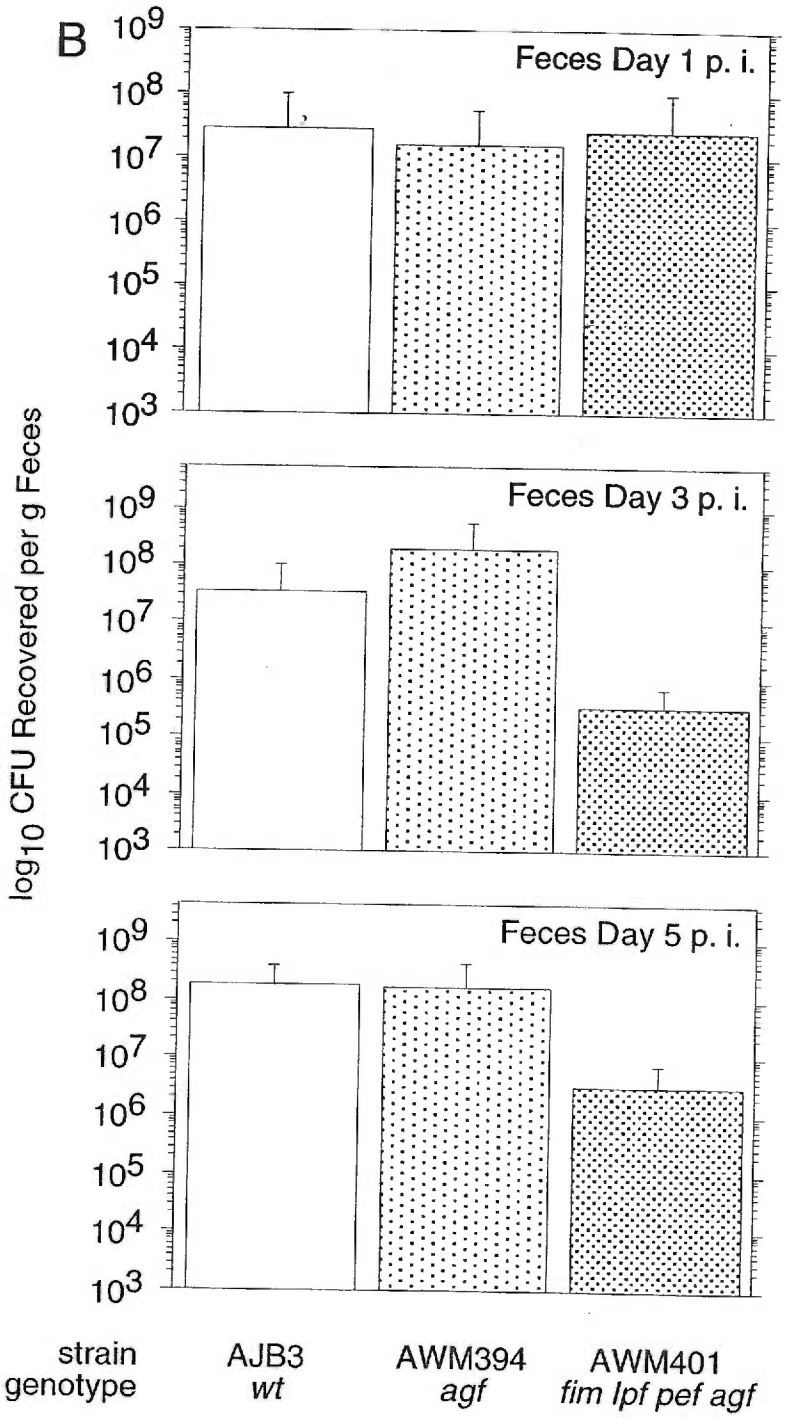
Strain	Relevant genotype	i.g.[a] LD_{50}	Fold attenuation	Reference
AJB3	wild type	5.8×10^5	1.0	(17)
AJB4	Δ*fim*	1.5×10^5	0.3	(17)
AJB5	*lpfC*	2.8×10^6	4.8	This study
AJB9	*pefC*	1.4×10^6	2.4	(15)
AWM394	*agfB*	1.9×10^6	3.3	This study
AJB12	Δ*fim lpfC pefC*	1.7×10^5	0.3	This study
AWM400	*lpfC pefC agfB*	1.7×10^7	>29	This study
AWM401	Δ*fim lpfC pefC agfB*	1.5×10^7	26.4	This study

[a] i.g., intragastric

Figure 2-2. Bacterial recovery from internal organs and feces after infection with AJB3 (*wt*), AWM394 (*agfB*), or AWM401 (Δ*fim lpfC pefC agfB*).

Bacterial recovery from internal organs 5 days postinfection (p.i.) (A) and feces 1, 3, and 5 days postinfection (B) reported in CFU per organ (three Peyer's patches in the distal ileum, close to the cecum, were collected and pooled for each mouse) or CFU per gram of feces. Three groups of four mice were each orally infected with 10^8 CFU of AJB3 (wild type [*wt*]), AWM394 (*agfB*), or AWM401 (Δ*fim lpfC pefC agfB*). Data are arithmetic means. Error bars indicate standard deviations.


A
Peyer's Patch[a] Day 5 p.i.
Mesentery Day 5 p.i.
Spleen Day 5 p.i.
log10 CFU Recovered perOrgan
strain
genotype
AJB3
wt
AWM394
agf
AWM401
fim lpf pef agf


B
Feces Day 1 p. i.
Feces Day 3 p. i.
Feces Day 5 p. i.
log10 CFU Recovered per g Feces
10^9
10^8
10^7
10^6
10^5
10^4
10^3
strain
genotype
AJB3
wt
AWM394
agf
AWM401
fim lpf pef agf

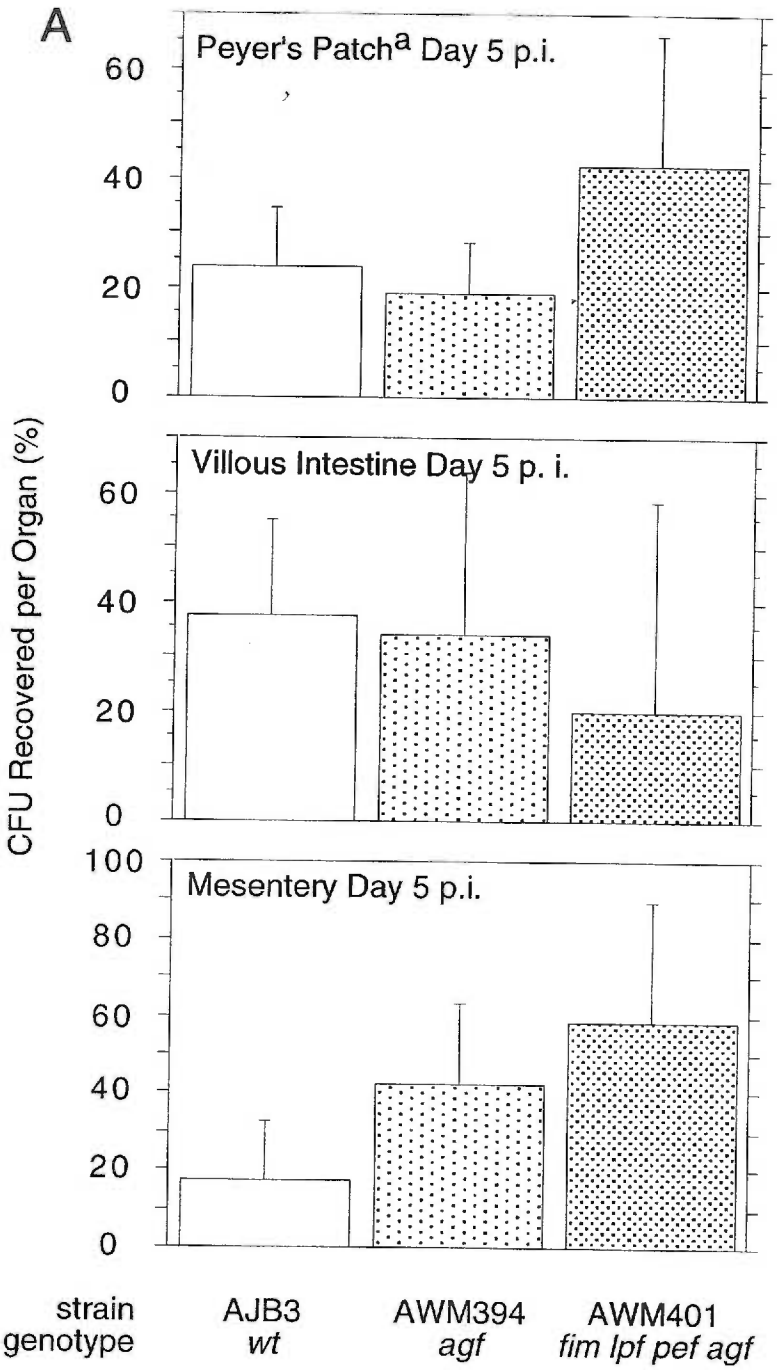
AWM401 (Δ*fim lpfC pefC agfB*), a quadruple fimbrial mutant, was more strongly attenuated (26-fold) than AJB12 (Δ*fim lpfC pefC*) or any of the strains carrying a single fimbrial mutation. This result suggested an additive attenuating effect of these mutations on the ability of *S. typhimurium* to cause murine typhoid (Table 2-2). Furthermore, the increased virulence of strain AJB12 (Δ*fim lpfC pefC*) compared to AWM401 (Δ*fim lpfC pefC agfB*) supports the idea that the insertional inactivation of *agfB* is one of the mutations responsible for the strong attenuation of the quadruple mutant.

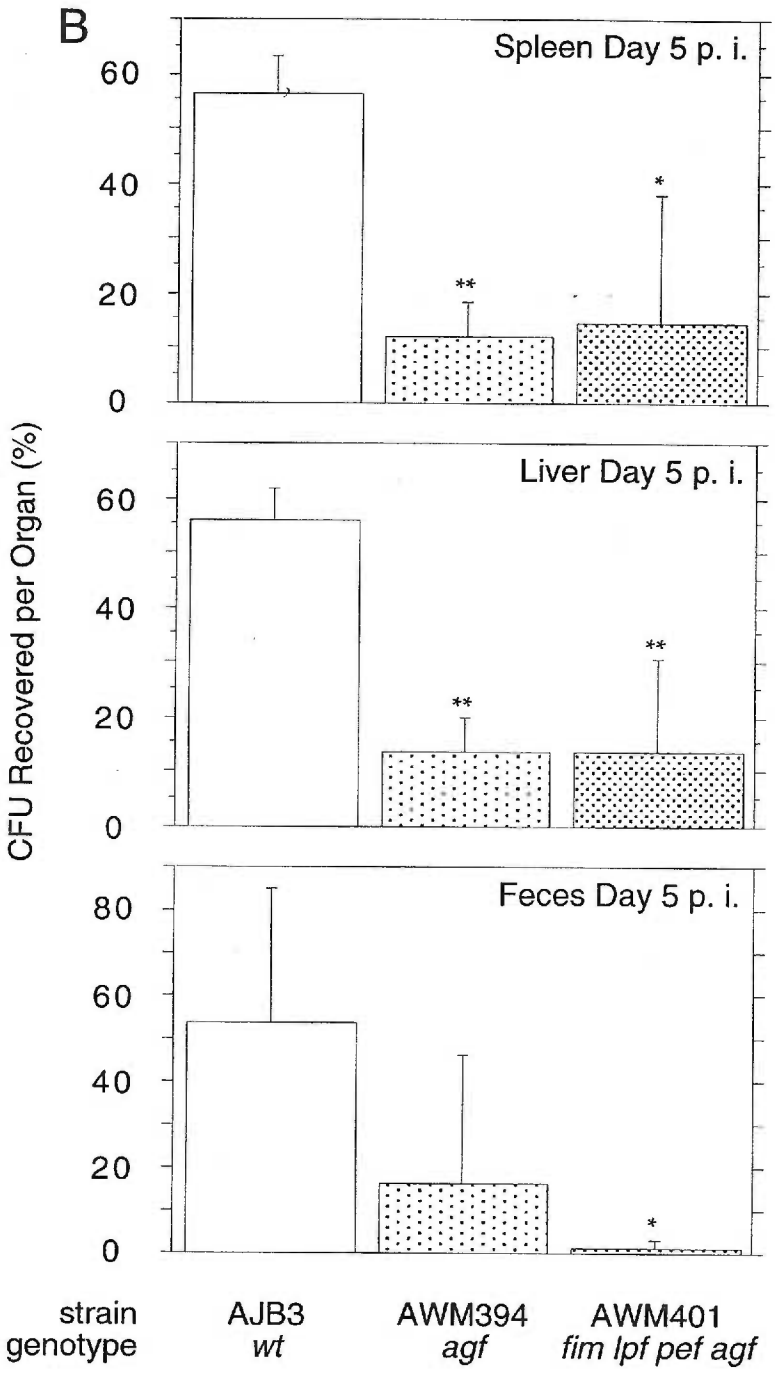
The quadruple mutant has a reduced ability to colonize liver, spleen and intestine. To investigate at which step during the infection process the AWM401 (Δ*fim lpfC pefC agfB*) is impaired, course of infection studies were conducted. Since our mouse virulence data (Table 2-2) suggested that a mutation in *agfB* in combination with a mutation in at least one other fimbrial operon is responsible for the attenuation of AWM401 (Δ*fim lpfC pefC agfB*), strain AWM394 (*agfB*) was included in these studies. Groups of four mice were orally infected with 10^8 colony forming units (CFU), upon which bacteria were recovered from the Peyer's patch, mesenteric lymph node and spleen on days 3 (data not shown) and 5 postinfection (Fig. 2-2A). In addition, bacteria were recovered from the feces on days 1, 3 and 5 postinfection to monitor intestinal colonization (Fig. 2-2B). Compared to the wild type (AJB3), reduced numbers of both AWM401 (Δ*fim lpfC pefC agfB*) and AWM394 (*agfB*) were recovered from

internal organs and fecal pellets. However, these differences proved not to be statistically significant ($P > 0.05$). As bacterial numbers recovered from individual animals may vary greatly during infection, small differences between wild type and mutant may go undetected. In order to control for the variability between experimental animals, mixed infections with AJB3 (wild type), AWM394 (*agfB*) and AWM401 (Δ*fim lpfC pefC agfB*) were performed, permitting a direct comparison between wild type and mutants. A group of four mice was orally infected with 10^8 CFU of a mixture containing approximately equal amounts of AJB3 (wild type), AWM394 (*agfB*), and AWM401 (Δ*fim lpfC pefC agfB*). On day 5 postinfection, CFU in internal organs (Peyer's patch, villous intestine, mesenteric lymph node spleen and liver) were determined. In addition, bacteria were recovered from the feces up to 5 days postinfection to monitor intestinal colonization (Fig. 2-3). Both AWM394 (*agfB*) and AWM401 (Δ*fim lpfC pefC agfB*) were able to compete with the wild type (AJB3) for colonization of Peyer's patches and villous intestinal tissue in the terminal ileum. Interestingly, increased numbers of both quadruple mutant (AWM401) and *agfB* mutant (AWM394) were recovered from the mesenteric lymph node compared to that of the wild type (AJB3). These differences were not statistically significant (Fig. 2-3, $P > 0.05$). However, both AWM394 (*agfB*) and AWM401 (Δ*fim lpfC pefC agfB*) were outcompeted by the wild type (AJB3) for colonization of liver and spleen ($P < 0.05$ and $P < 0.01$, respectively). In addition, AWM401 (Δ*fim lpfC pefC agfB*) failed to compete with the wild type for colonization of the intestine as suggested

Figure 2-3. Bacterial recovery from internal organs and feces 5 days postinfection after 1:1:1 mixed infection with AJB3 (*wt*), AWM394 (*agfB*), and AWM401 (Δ*fim lpfC pefC agfB*).

Bacterial recovery from internal organs and feces 5 days postinfection (p.i.) reported as percentages of the total number of bacteria recovered. Three groups of four mice each were orally infected with a 1:1:1 mixture of three strains, AJB3 (wild type [wt]), AWM394 (*agfB*), or AW M401 (Δ*fim lpfC pefC agfB*), respectively, for a total of 10^8 CFU per mouse. Three Peyer's patches in the terminal ileum, close to the cecum, were collected and pooled for each mouse. Data are arithmetic means. Error bars indicate standard deviations. *, $P < 0.05$ (paired *t* test); **, $P < 0.01$ (paired *t* test).


A
Peyer's Patch[a] Day 5 p.i.
Villous Intestine Day 5 p. i.
Mesentery Day 5 p.i.
CFU Recovered per Organ (%)
0
20
40
60
80
100
strain
genotype
AJB3
wt
AWM394
agf
AWM401
fim lpf pef agf

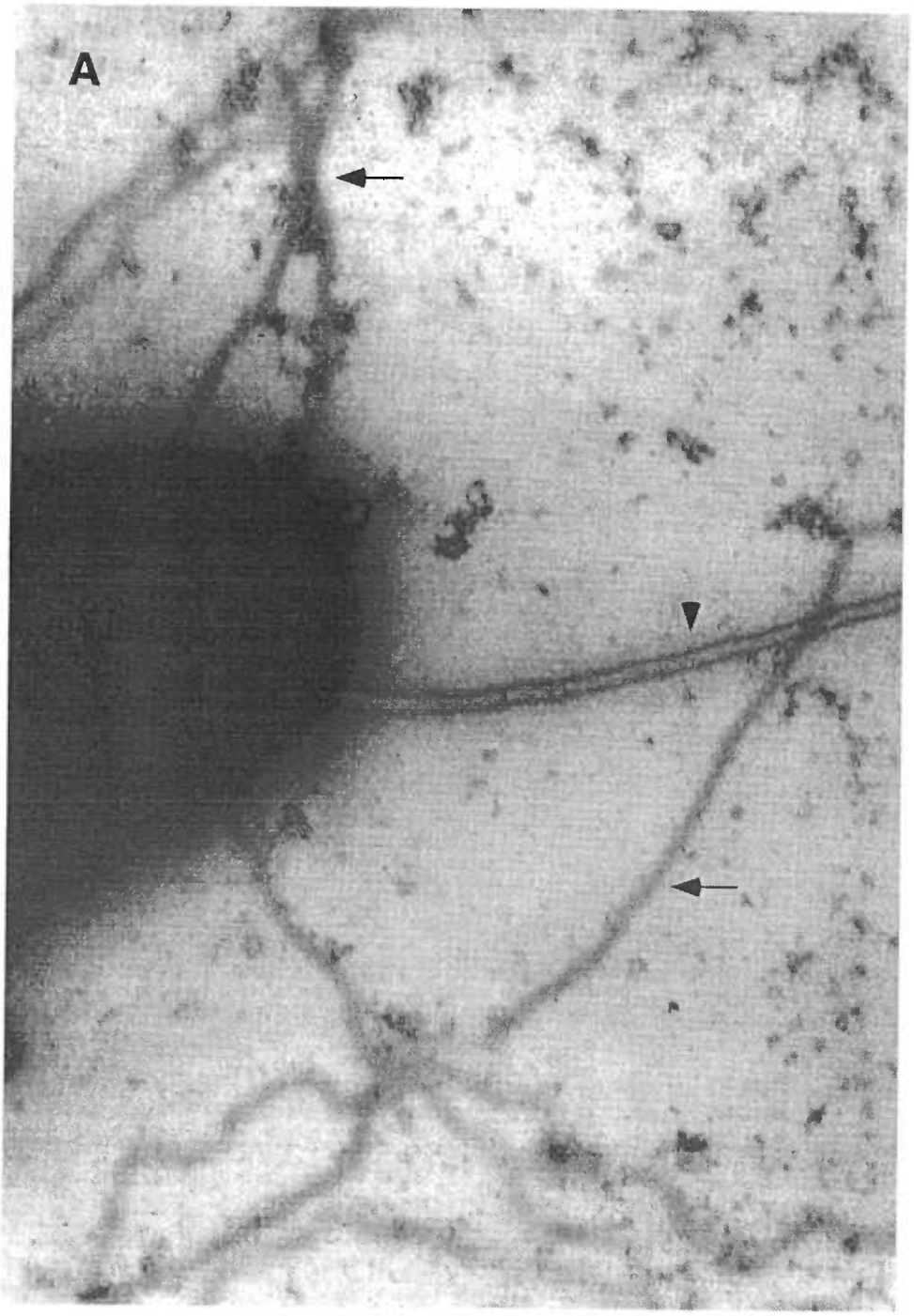

B
Spleen Day 5 p. i.
Liver Day 5 p. i.
Feces Day 5 p. i.
CFU Recovered per Organ (%)
0
20
40
60
80
**
*
strain
genotype
AJB3
wt
AWM394
agf
AWM401
fim lpf pef agf

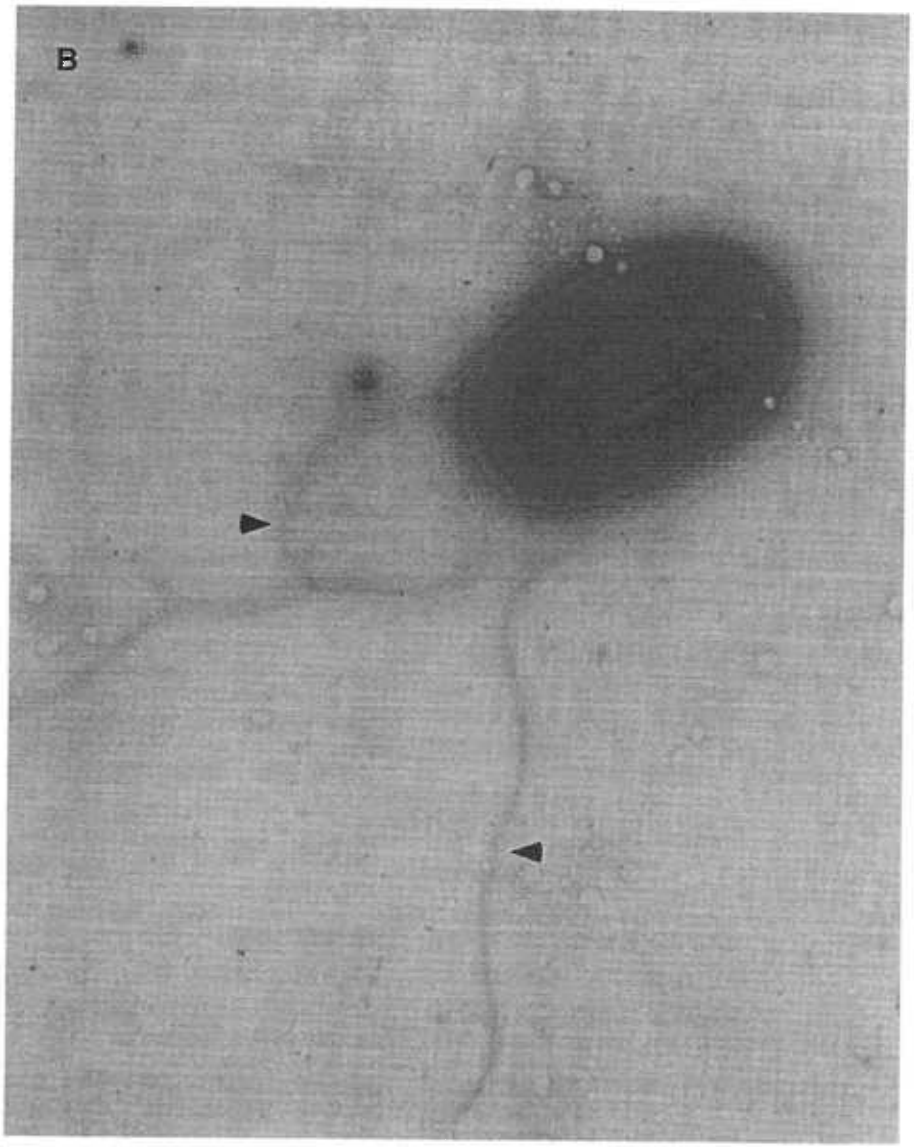
by recovery of significantly reduced numbers from fecal pellets ($P < 0.05$). These results provide evidence that fimbrial adhesins act synergistically during colonization of the mouse intestinal tract.

Identification of new fimbrial structures. Although we have demonstrated that fimbrial adhesins of *S. typhimurium* play an important role during infection (Table 2-2), AWM401 (Δ*fim lpfC pefC agfB*) was still able to cause a lethal systemic illness in mice when administered at higher doses. These data suggest that AWM401 (Δ*fim lpfC pefC agfB*) may express yet other factors for intestinal attachment. To investigate this possibility, we examined strain AWM401 (Δ*fim lpfC pefC agfB*) for the presence of fimbriae by electron microscopy. Interestingly, this mutant (AWM401) expressed thus far uncharacterized fimbrial structures (Fig. 4-4A), which could easily be distinguished from flagellar filaments required for cell motility (Fig. 4-4). Flagellar filaments varied in length from 5 to 10 μm, with a diameter of approximately 20 nm (141). Fimbriae could be distinguished from flagella by means of morphology and diameter (typically between 2 to 8 nm (139)). These data provide direct evidence for the expression of at least one other, yet uncharacterized fimbrial structure in *S. typhimurium* which may contribute to the redundancy of virulence factors involved in colonization of the intestinal mucosa.

Figure 2-4. Identification of a novel fimbrial structure.

Electron micrographs of AWM401, which harbors mutations in the *fim, lpf, pef,* and *agf* fimbrial operons. This quadruple mutant expresses a thus far uncharacterized fimbrial structure (A, arrows) that can be distinguished from flagellar filaments (A and B, arrowheads). Magnification, x35,000 (A) and x8,000 (B).


A


B

Discussion

Our results demonstrate that despite the moderate effect on mouse virulence of individual mutations in fimbrial operons, the simultaneous inactivation of genes involved in the biosynthesis of four distinct fimbrial adhesins markedly attenuates *S. typhimurium* virulence. To our knowledge, this is the first study to provide direct evidence for a synergistic effect of fimbrial adhesins during infection. Previous studies have shown that inactivation of biosynthetic genes for type-1 fimbriae, LP fimbriae, or PE fimbriae attenuate *S. typhimurium* mouse virulence only fivefold or less (15, 19, 137). Here, we report that a mutation in a fourth *S. typhimurium* fimbrial operon, *agf*, resulted in a threefold reduction in mouse virulence. A recent study indicated that thin aggregative fimbriae mediate adhesion to murine small intestinal epithelial cells *in vitro* (219). We have observed that strains carrying the *agfB* mutation have an altered colony morphology (data not shown). A pleiotropic effect for *agf* mutants regarding colony morphology has also been reported by others (219). However, our virulence data strongly suggests that this pleiotropic effect does not reduce the ability to cause murine typhoid (Table 2-2, Fig. 2-2, AWM394 [*agfB*]). Furthermore, from these data it is evident that inactivation of individual adhesins does not strongly reduce the ability of *S. typhimurium* to cause a lethal systemic infection in mice. However, strain AWM401, in which all four known fimbrial operons have been inactivated, was 26-fold attenuated when orally administered

to mice. These results are consistent with the idea that mutations in individual *S. typhimurium* fimbrial operons have only moderate effects on mouse virulence because lack of a single attachment factor can be compensated for by the presence of other adhesins.

Because a strain carrying mutations in *fim*, *lpf* and *pef* (AJB12) was not attenuated, insertional inactivation of *agfB* must, be partly, responsible for the strong attenuation of AWM401 (Δ*fim lpfC pefC agfB*). Neither the *agfB* mutant (AWM394) nor the quadruple mutant (AWM401) were able to compete with the wild type (AJB3) for colonization of the liver and spleen ($P < 0.05$ and $P < 0.01$, respectively). However, during mixed infection experiments, only AWM401 (Δ*fim lpfC pefC agfB*) was recovered in reduced numbers from fecal pellets ($P < 0.05$), suggesting that the decreased virulence of AWM401, compared to AWM394 (*agfB*), is caused by a defect in intestinal colonization. From these results, we conclude that the absence of at least two fimbrial structures (encoded by the *agf* and *fim*, *lpf*, or *pef* operons) may significantly decrease adherence to murine intestinal tissue and further reduce mouse virulence. Additional virulence studies and course of infection studies are needed to identify precisely which combination of mutations in fimbrial operons reduces virulence.

The ability of AWM401 (Δ*fim lpfC pefC agfB*) to cause a lethal systemic infection in mice upon intragastric injection of large inocula suggested that a quadruple mutant might express additional means of adhesion and colonization. Electron microscopic studies demonstrated that, in addition to flagellar filaments,

AWM401 (Δ*fim lpfC pefC agfB*) expresses at least one additional, yet uncharacterized, fimbrial structure. Thus, this fimbrial structure, and possibly others, may be the adhesive organelle(s) that allow residual colonization of the mouse intestine by *S. typhimurium* in the absence of type-1, LP, PE, and thin aggregative fimbriae.

Acknowledgements

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Chapter 3:

Salmonella enteritidis lpf and *pef* fimbrial operons contribute to mouse virulence

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Abstract

Salmonella enteritidis expresses SEF14, SEF17, and SEF21 fimbriae. Although *lpf* and *pef* fimbrial operons are present, their role in *S. enteritidis* pathogenesis is unclear. Here, we show that these fimbrial operons are functional and contribute to *S. enteritidis* colonization of the murine intestinal mucosa and development of murine typhoid fever.

Results and Discussion

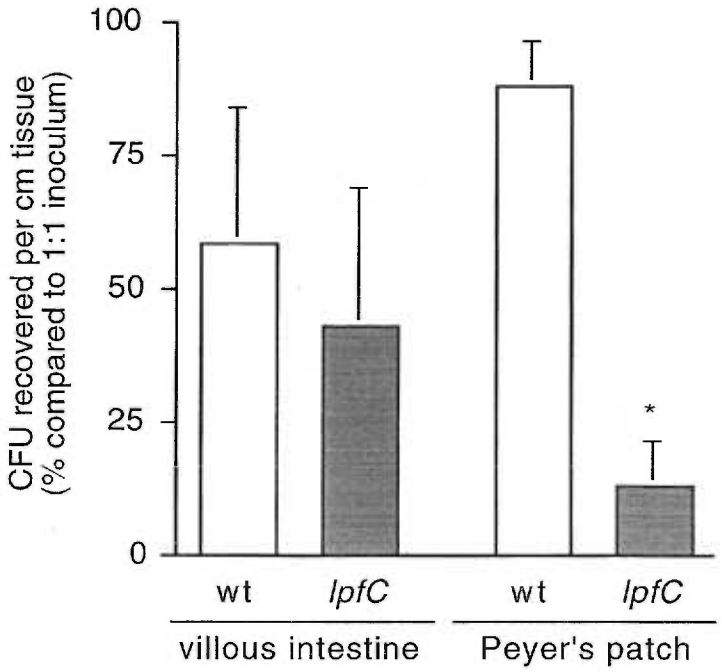
Salmonella enterica serotype Enteritidis (*S. enteritidis*), a common cause of food poisoning, causes acute gastroenteritis and is a major health problem worldwide. Necessary first steps in the establishment of a *Salmonella* infection include colonization and penetration of the intestinal mucosa. Indeed, recent evidence indicates that fimbrial adhesins of *Salmonella* play an important role during bacterial adhesion to and invasion of the intestinal barrier (15, 19, 21, 137, 168, 219, 232). *S. enteritidis* expresses at least three fimbrial structures, designated SEF14, SEF17 (curli), and SEF21 (type I) fimbriae, encoded by the *sef*, *agf*, and *fim* operons, respectively (47, 48, 159). Furthermore, *S. enteritidis* encodes genes homologous to the *lpf* and *pef* fimbrial operons in *S. typhimurium* (12-14, 69). In *S. typhimurium*, these operons encode long polar fimbriae and plasmid encoded fimbriae, respectively, while *S. typhimurium* also encodes homologs of both *fim* and *agf* operons. *S. typhimurium* long polar (LP) fimbriae mediate attachment to murine Peyer's patches (19), whereas plasmid encoded (PE) fimbriae permit binding to murine small intestine (15). However, the role of the *lpf* and *pef* operons in *S. enteritidis* pathogenesis is not known. Since the *S. enteritidis* and *S. typhimurium pefA* genes share only 82% amino acid identity (240), it is unclear whether these fimbrial operons function similarly because most genes from these two organisms share > 90% identity and > 95% homology on the amino acid level. Mutations in either the *lpf* or *pef* operon do not reduce

the ability of *S. enteritidis* to adhere to chick gut explant (3), even though PefA specific antibodies can be detected in chicks infected with wild type *S. enteritidis* (240). Thus, these fimbrial operons may either be non-essential, non-functional, or play only a minor role in *S. enteritidis* adhesion to chicken intestinal tissue. Alternatively, the cognate fimbrial receptors may not be present on these epithelial cell surfaces. Here, we have inactivated essential fimbrial biosynthetic genes *lpfC* and *pefC* to study the effect of these mutations on the ability of *S. enteritidis* to adhere to murine intestinal tissue.

Bacteria were grown as described previously (232). Defined mutations in either *lpfC* (19) or *pefC* (15) were introduced into a spontaneous naladixic acid resistant-, mouse-virulent strain of *S. enteritidis* (134). Insertional inactivation was confirmed by Southern hybridization (data not shown). The oral 50% lethal dose (LD_{50}) was determined in BALB/c mice. The oral LD_{50} was similar for all strains tested. The oral LD50 for the wild type (AJB72) was 2.1×10^5 colony forming units (CFU), whereas 3.9×10^5 and 1.4×10^5 CFU were required to kill 50% of mice infected orally with either AJB73 (*lpfC*) or AWM255 (*pefC*), respectively. Although these data indicate that the *lpf* and *pef* operons are not essential during the development of murine typhoid fever, these results do not exclude the possibility that *S. enteritidis* compensates for a defect in the *lpf* or *pef* fimbrial operons by producing alternate attachment factors, possibly including SEF 14, SEF17, and SEF21 fimbriae. This view is supported by a recent study in which it was demonstrated that, in *S. typhimurium*, fimbrial adherence factors are

Figure 3-1. Bacterial association with the murine small intestine after 1:1 mixed infection with AJB72 (*wt*) and AJB73 (*lpf*).

Bacterial association with the murine small intestine after 1:1 mixed infection with AJB72 (*wt*) and AJB73 (*lpf*). Three Peyer's patches in the terminal ileum were collected and pooled. Data are arithmetic means of three independent experiments. Error bars indicate standard devations. *, $P < 0.001$ (paired *t* test).

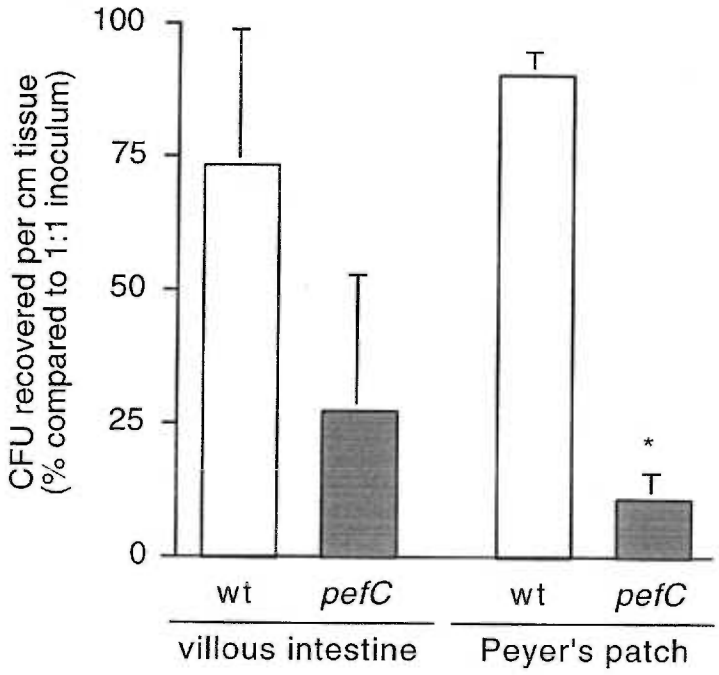

CFU recovered per cm tissue
(% compared to 1:1 inoculum)
100
75
50
25
0
*
wt
lpfC
wt
lpfC
villous intestine
Peyer's patch

functionally redundant and act in synergy to facilitate adhesion and colonization of the murine intestinal mucosa (232).

Next, we tested the ability of AJB73 (*lpfC*) and AWM255 (*pefC*) to compete with the wild type (AJB72) for adherence to murine intestinal tissue *in vitro* using the intestinal organ culture model (IOC) developed in our laboratory (15, 19). In short, bacteria were grown as standing overnight cultures in 3 ml of Luria-Bertani (LB) broth at 37 °C and 5% CO_2 to promote fimbrial expression. Murine ileal loops were infected with 1 ml of a 1:1 mixture (6-8 x 10^8 CFU total) of either wild type (Nal^r) and *lpfC* mutant (Kan^r), or wild type (Nal^r) and *pefC* mutant (Tet^r). After incubating for 30 min at 37 °C and 5% CO_2, non-adherent bacteria were removed by washing with PBS. A 10-fold dilution series was plated on LB agar plates containing the appropriate antibiotics to determine the number of CFU for each strain. Compared to the wild type, similar numbers of *lpfC* mutant bacteria were recovered from the villous intestinal tissue. However, reduced numbers of *lpfC* mutant bacteria were recovered from the Peyer's patch (Fig. 3-1). These results indicate that the *S. enteritidis lpf* operon is important during bacterial attachment to murine ileal Peyer's patches. Compared to the wild type, reduced numbers of *pefC* mutant bacteria were recovered from both murine Peyer's patches and small intestine (Fig. 3-2).

Figure 3-2. Bacterial association with the murine small intestine after 1:1 mixed infection with AJB72 (*wt*) and AWM255 (*pef*).

Bacterial association with the murine small intestine after 1:1 mixed infection with AJB72 (*wt*) and AWM255 (*pef*). Three Peyer's patches in the terminal ileum were collected and pooled. Data are arithmetic means of three independent experiments. Error bars indicate standard devations. *, $P < 0.001$ (paired *t* test).


CFU recovered per cm tissue
(% compared to 1:1 inoculum)
100
75
50
25
0
*
wt
pefC
wt
pefC
villous intestine
Peyer's patch

These results indicate that the *S. enteritidis pef* operon plays a role in mediating attachment to murine ileal tissue.

In conclusion, *S. enteritidis* encodes at least five functional fimbrial operons. In addition to *sef*, *agf*, and *fim*, evidence presented here indicates that *S. enteritidis* encodes two additional fimbrial operons, *lpf* and *pef*, that play a role in pathogenesis. Mutations of *lpfC* and *pefC* reduce the ability of *S. enteritidis* to adhere to murine ileal Peyer's patches and villous intestine, respectively. Reduced adherence of an *lpfC* mutant to murine Peyer's patches, but not to villous intestinal tissue, suggests that the *S. enteritidis lpf* operon mediates attachment to cells unique to Peyer's patch tissue. In contrast, reduced adherence of the *pefC* mutant to both villous intestine and Peyer's patches suggests that the *S. enteritidis pef* operon mediates attachment to enterocytes abundantly present in both Peyer's patches and villous intestinal tissue. *S. enteritidis* strains mutated in *lpfC* and *pefC* are not significantly attenuated in virulence when administered to mice orally. We propose that alternate attachment factors, including SEF14, SEF17, and SEF21 fimbriae, functionally complement the lack of either LP fimbriae or PE fimbriae *in vivo*. This hypothesis is supported by a recent study in which it was demonstrated that multiple fimbrial adhesins in *S. typhimurium* coordinately facilitate adhesion and colonization of murine intestinal mucosa (232). The presence of yet additional fimbrial structures is suggested by the fact that *S. enteritidis* encodes genes homologous *bfp* genes in *Escherichia coli* (209). However, evidence for the expression of

bundle forming pili in *S. enteritidis* has not yet been presented.

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Chapter 4:

SPI1 independent induction of apoptosis in infected macrophages by *Salmonella typhimurium*

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Key words: *Salmonella*, macrophage, apoptosis

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Abstract

The enteric pathogen *Salmonella enterica* serotype Typhimurium (*S. 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 *S. typhimurium* can activate programmed macrophage cell death independently of SPI1. SPI1 independent induction of apoptosis in infected macrophages is observed as early as 12-13 hours post-infection, even in the absence of intracellular bacterial replication. Delayed activation of programmed macrophage cell death is not observed with *S. 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*, as *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 non-cytotoxic phenotype, whereas simultaneous inactivation of genes required for both rapid and delayed induction of apoptosis renders *S. typhimurium* non-cytotoxic under all conditions tested. We hypothesize that differential activation of programmed macrophage cell death by *S. typhimurium* occurs under discrete physiological conditions at distinct locations within an infected host.

Introduction

Salmonella enterica serotype Typhimurium (*S. 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 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 (187), comparative infection studies in genetic strains of mice that produce macrophages with varying resistance to *Salmonella* (153, 165), and the persistence of infection in mice treated with gentamicin, an antibiotic that primarily kills extracellular bacteria (29, 61). Finally, genetic studies indicate that *Salmonella* mutants that are attenuated for intramacrophage survival are also attenuated for systemic infection in mice (64). 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 (7, 39, 135, 156).

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 *S. typhimurium* kills macrophages as late as 18 hours post-

infection (135). 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 SPI1 encoded type III export apparatus, whereas SipB is a SPI1 secreted effector molecule (72, 118). Null mutations in either *invA* or *sipB*, two genes within *Salmonella* pathogenicity island I (SPI1), had no effect on the ability of *S. typhimurium* to kill infected macrophages in this study (135). However, other studies appear to contradict these observations and demonstrate that within a few hours upon contact, *S. typhimurium* induces apoptosis in infected macrophages in an *invA* (thus SPI1) dependent process (39, 140, 156). SipB is both necessary and sufficient for the rapid activation of this apoptotic pathway (101).

Here, we resolve this apparent controversy by demonstrating that *S. typhimurium* kills macrophages via two independent processes. We demonstrate that SPI1 gene expression accounts for rapid induction of apoptosis, whereas delayed induction of apoptosis depends on *ompR* and SPI2. Implications of these results for understanding *Salmonella* pathogenesis are discussed.

Materials and Methods

Bacterial strains, bacteriophages, and recombinant DNA techniques. Bacteria were grown overnight in Luria-Bertani broth at 37 °C. Antibiotics, when required, were used at the following concentrations: naladixic acid (nal), 50 μg/ml; chloramphenicol (cam), 30 μg/ml; kanamycin (kan), 60 μg/ml; ampicillin (amp), 100 μg/ml. Recombinant DNA techniques and Southern hybridizations were performed using standard protocols (9, 144). Analytical grade chemicals were purchased from Sigma (St. Louis, MO, USA) or Roche Biochemicals/Boehringer Mannheim (Indianapolis, IN, USA).

Mutations in the *ompR*, *invA*, *spiB,* and *prc* genes have been described previously (64, 74, 135, 228) and were used to construct a set of isogenic *S. typhimurium* mutants (Table 1). Bacteriophage KB1*int* was used to transduce the *ompR*::Mud*J* allele of SWL350 (135) into SR-11 χ3041 (*wt*), yielding strain AWM405 (*ompR*). Bacteriophage P22HT*int* was used to transduce the *invA*::Tn*phoA* allele of AJB75 (21) into AWM501 (*sipB*, see below), and AWM527 (*ssrB*, see below), yielding AWM544 (*invA sipB*), and AWM545 (*ssrB invA*), respectively. Bacteriophage P22HT*int* was used to transduce the *spiB*::mTn*5* allele of STN119 (228) into SR-11 χ3041 (*wt*), yielding strain AWM568 (*spiB*). Bacteriophage P22HT*int* was also used to transduce the *prc*::Tn*10* allele of MS4290 (64) into SR-11 χ3041 (*wt*), yielding strain AWM664 (*prc*).

Allelic exchange was performed to disrupt the *S. typhimurium invA* gene. An internal fragment of the *invA* gene was amplified from *S. typhimurium* ATCC14028 (*wt*) using primers *5'*- GCATGAATTCGCAGAACAGCGTCG –*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 (21)) into a unique internal *Sna*BI site, and cloned into suicide plasmid pKAS32 (208). The resulting plasmid was electroporated into *E. coli* SM10λ*pir* and conjugated to *S. typhimurium* ATCC14028 derivative BA715 (*rpsL*) (2). A double cross-over 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 *S. typhimurium sipB* gene. A fragment of the *sipB* gene was amplified from *S. 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 (21)) 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 (176). The resulting plasmid was electroporated into *E. coli* S17λ*pir* (123) and conjugated to AJB3, a naladixic acid resistant derivative of *S. typhimurium* SR-11 (232). A chloramphenicol- and naladixic 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 *S. typhimurium ssrB* gene. An 853-bp fragment of the *ssrB* allele was amplified from *S. typhimurium* ATCC14028 (*wt*) using primers *5'*- CTTAATTTTCGCGAGGGCAGC –*3'* and *5'*-TAGAATACGACATGGTAAAGCCCG –*3'*. This PCR product was cloned into pCR-Blunt (Invitrogen, Carlsbad, California, USA). The *ssrB* allele was inactivated upon insertion of a chloramphenicol resistance gene (a 1.2 kb *Sma*I fragment from pCMXX (21)) into a unique *Ssp*I site. This plasmid was digested with *Eco*RI and the disrupted *ssrB* allele was ligated into suicide vector pKAS32 (208). The resulting plasmid (pMJW99) was transformed into *E. coli* SM10λ*pir* and conjugated to *S. typhimurium* ATCC14028 derivative BA715 (*rpsL*) (2). A double cross-over 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, Manassas, VA, USA) and RAW264.7 (ATCC) were cultured (37°C/5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL, Rockville, MD, USA), supplemented with 10% Fetal Bovine Serum (FBS,Gibco-BRL), glutamine (Gibco-BRL), sodium pyruvate (Gibco-BRL), and non-essential amino acids (Gibco-BRL). Bone marrow derived macrophages (BMDM) were isolated from C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) 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, OR, USA), glutamine and sodium pyruvate (Gibco-BRL).

Macrophage survival assays (gentamicin protection assays) were performed as described by Fields et al. (64). In brief, 1 x 10^5 J774 macrophages were infected with stationary phase cultures (below) at a number of input bacteria (IB) ≤1. Use of the term "multiplicity of infection (MOI)" as it relates to these assays is confusing as the actual rate at which macrophages are infected depends on many factors, including the number of input bacteria (IB) and the rate at which they infect macrophages (usually about 1 percent). We will therefore

use the term IB and leave MOI as the term to describe the actual number of internalized bacteria. At 18 hours post-infection, monolayers were washed three times with phosphate-buffered saline (PBS) and lysed with Triton X-100 (Sigma). Bacterial viability was determined by plating for intracellular colony forming units (CFU) at various times post-infection. 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 IB~60. At one hour 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 one percent of input bacteria). At 6 h and 18 h post-infection, the release of LDH was quantified colorimetrically using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). The absorbance (A_{490nm}) was determined on a microplate reader (Dynatech Laboratories, Inc., Chantilly, VA, USA), after which the percentage of cytotoxicity was calculated using the following formula: 100% x ((experimental release – spontaneous release)/(maximum release – spontaneous release)). 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.* (135) (data not shown). In brief, to determine the number of input bacteria (IB) at which 50% of the infected macrophages are killed (IB_{CD50}), 1 x 10^5 J774 macrophages were infected with 2-fold serial dilutions of bacterial cultures ($31 \leq IB \leq 1000$, the limits of detection) as verified by plating for Colony Forming Units (CFU). At 6 h and 18 h post-infection, the remaining viable macrophages were fixed in a 10% formalin solution (10-15 min.) and stained in a 0.13% crystal violet solution (> 2 h). The absorbtion (A_{595nm}) was determined on a microplate reader (Dynatech Laboratories, Inc., Chantilly, VA, USA); the MOI for the well that gave 50% of the absorbtion recorded for uninfected wells was considered the IB_{CD50} (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., USA) was used to determine whether *S. typhimurium* infected macrophages were undergoing apoptosis. This assay has been used successfully to study *Pseudomonas aeruginosa* induced apoptosis in eukaryotic cells (96). 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 (MOI). The amount of cytoplasmically located histones bound to

fragmented DNA was quantified colorimetrically at 18 h post-infection, after which the absorbance (A_{410nm}) was determined on a microtiterplate reader. An enrichment factor indicative of apoptosis was calculated using the following formula: (A_{410nm} [experimental]) / (A_{410nm} [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 as under these culture conditions, high levels of β-galactosidase were produced (data not shown).

Table 4-1. Bacterial strains used in this study.

Strain	Genotype	Reference
E. coli		
DH5α	*endA1 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*	(123)
S. typhimurium		
AJB3	SR-11 χ4252 (Nal^r)	(232)
AJB75	ATCC14028 *invA*::Tn*phoA* (Kan^r)	(21)
ATCC14028	wild type	ATCC
BA715	ATCC14028 *rpsL* (Str^r)	(2)
MJW129	ATCC14028 *ssrB*::*cat* (Cam^r)	This study
MS4290	ATCC14028 *prc*::Tn*10* (Tet^r)	(64)
SR11χ3041	wild type	R. Curtiss III
STN119	IR715 *spiB*::mTn*5* (Kan^r)	(228)
SWL350	SR-11 *ompR*::Mud*J* (Kan^r)	(135)
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

Table 4-1. (Continued)

Strain	Genotype	Reference
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

Results

***S. typhimurium* kills macrophages independently of SPI1.** Conflicting reports on macrophage killing (39, 135, 156) prompted us to investigate the effect of bacterial growth phase on the ability of *S. 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 dehydrogenase (LDH), macrophage killing was calculated using a quantified macrophage cytotoxicity assay (data not shown)(135). 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 multiplicities of infection were also tested with identical results (data not shown).

Under SPI1 inducing conditions (late-log phase, see materials and methods) (39), 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 12-13 hours post-infection (Fig. 1C). These results suggest that *S. typhimurium* induces delayed macrophage cell death independently of SPI1.

Figure 4-1. *S. typhimurium* kills macrophages independently of SPI1.

J774 macrophages were infected with late-log phase (A) or stationary phase (B) cultures of wild-type *S. typhimurium*, or strains carrying null mutations in *invA* or *sipB*. Bacterial growth was monitored by measuring optical density at OD_{600} (experimental procedures, data not shown). Macrophage cell death was quantitated at 6 h (A) and 18 h (B) p. i. by measuring the release of LDH. Using stationary phase cultures of either wild-type *S. typhimurium* or an *invA* deficient strain, macrophage cytotoxicity was monitored for 20 h (C) and quantitated at 2 h intervals by measuring the release of LDH. Data from graphs (A) and (B) are arithmetic means of at least three independent experiments. Error bars indicate standard deviations of the mean. Data from graph (C) are representative of two independent experiments.

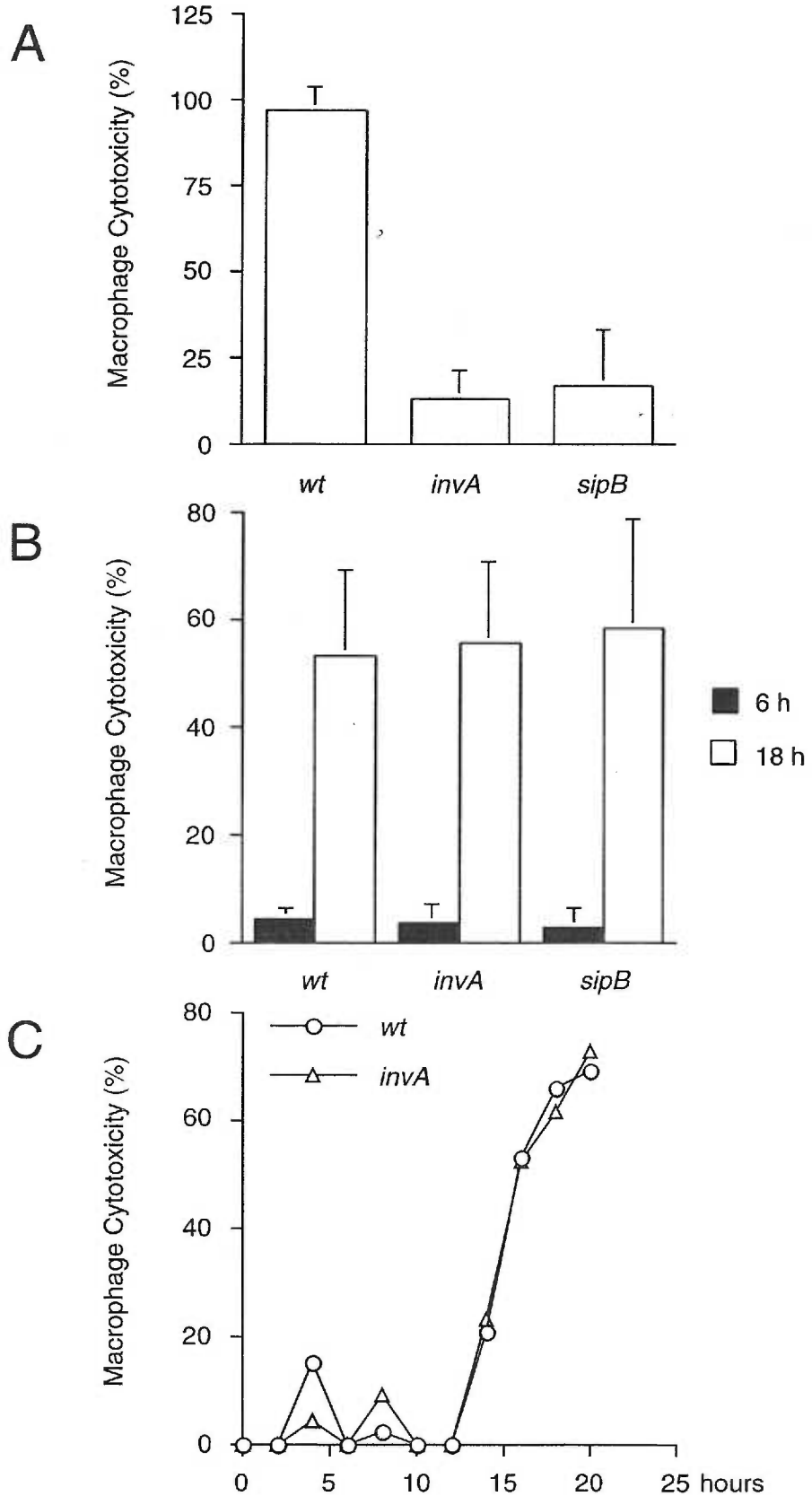
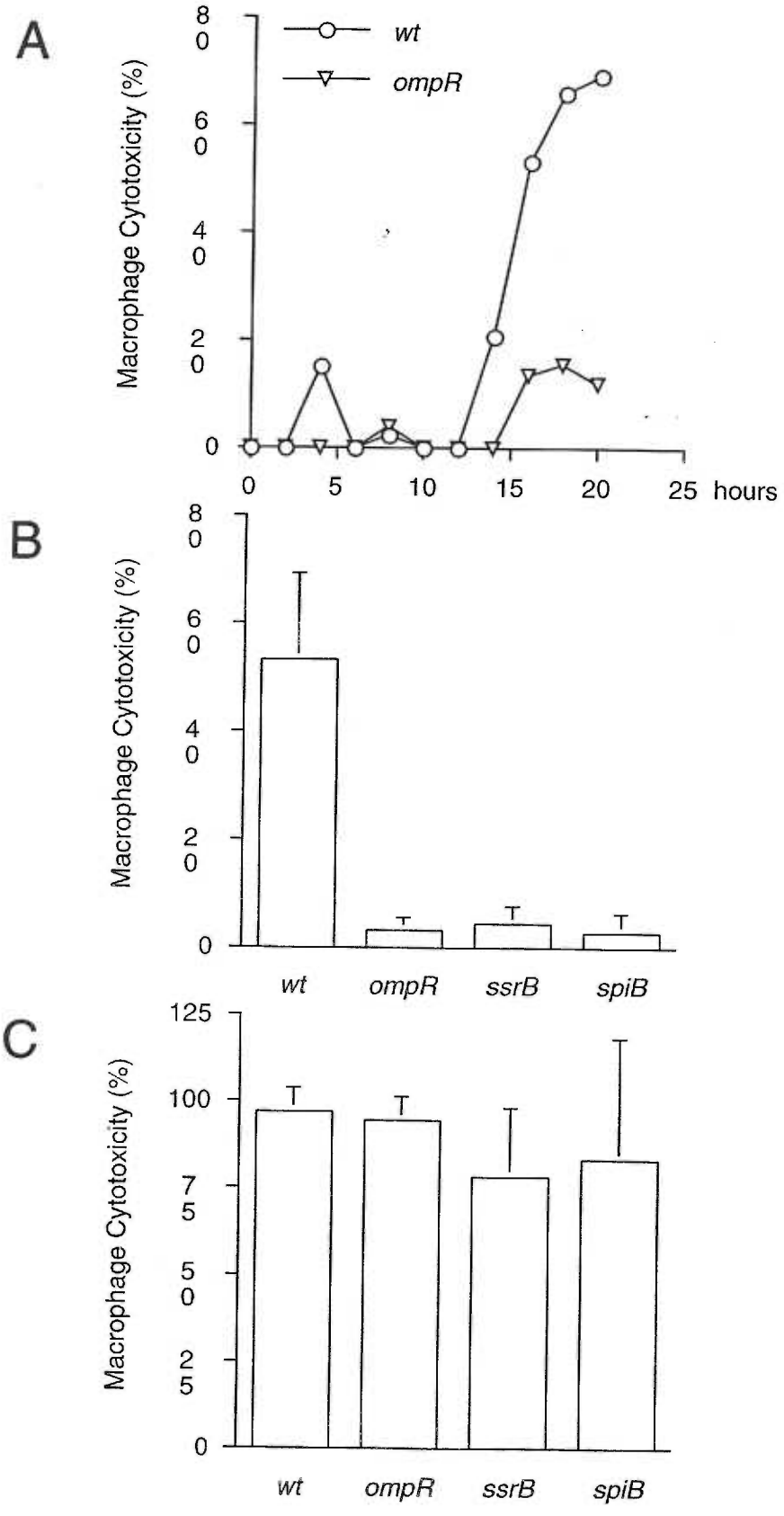

A
Macrophage Cytotoxicity (%)
125
100
75
50
25
0
wt
invA
sipB
B
Macrophage Cytotoxicity (%)
80
60
40
20
0
6 h
18 h
wt
invA
sipB
C
Macrophage Cytotoxicity (%)
80
60
40
20
0
wt
invA
0
5
10
15
20
25 hours

Figure 4-2. SPI2 and *ompR* are required for delayed macrophage killing.

J774 macrophages were infected with stationary phase cultures of either wild-type *S. 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 (A). In addition, J774 macrophages were infected with stationary phase (B) or late-log phase (C) cultures of wild-type *S. typhimurium*, or strains carrying null mutations in *ompR*, *ssrB*, or *spiB*. Macrophage cell death was quantitated at 18 h (B) and 6 h (C) p. i. by measuring the release of LDH. Data from graph (A) are representative of two independent experiments. Data from graphs (B) and (C) are arithmetic means of at least three independent experiments. Error bars indicate standard deviations of the mean.


A
Macrophage Cytotoxicity (%)
wt
ompR
0
20
40
60
80
0
5
10
15
20
25 hours
B
Macrophage Cytotoxicity (%)
0
20
40
60
80
wt
ompR
ssrB
spiB
C
Macrophage Cytotoxicity (%)
0
25
50
75
100
125
wt
ompR
ssrB
spiB

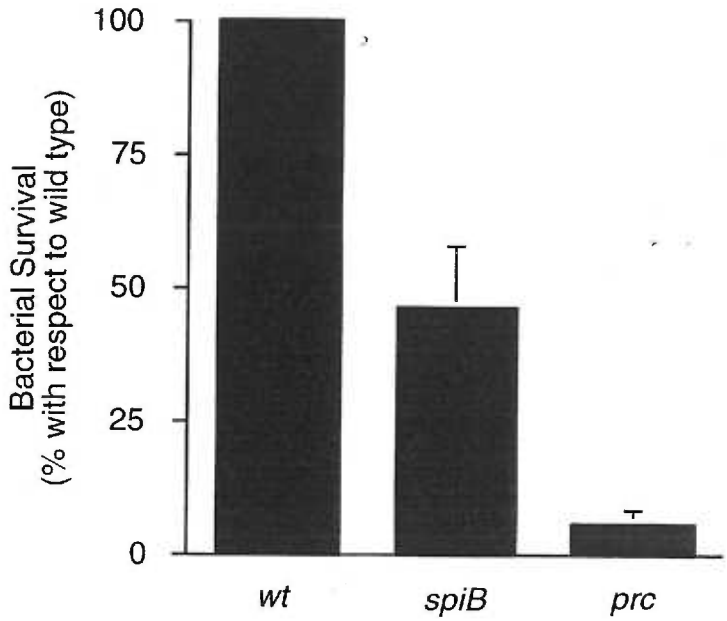
SPI2 and *ompR* are required for delayed macrophage killing. Delayed cytotoxicity was dependent on a functional *ompR* locus, as *ompR* mutant bacteria have a reduced ability to kill infected macrophages (Fig. 2A). Recent evidence suggests that OmpR activates transcription of the SPI2 encoded regulon *ssrA/B* (130). This operon is essential for the transcription of SPI2 genes (41), which are highly induced inside macrophages (56, 230). To test whether, in addition to *ompR*, SPI2 is required for delayed induction of macrophage cell death, *S. 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 (167). As shown in Fig. 2B, *S. typhimurium* strains mutated in *ompR*, *ssrB*, or *spiB* had a strongly reduced ability 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 independent cytotoxic effects are masked under conditions that turn on SPI1 gene expression.

In agreement with the literature, we observe a defect (2-10 fold) in intracellular proliferation for SPI2 mutant strains at 15 hours post-infection (41, 99, 100, 167, 204). However, long-term intracellular survival and proliferation are not required for delayed macrophage killing *per se*, as a *prc* mutant, encoding a periplasmic protease (14, 64) required for intracellular survival and growth (Fig.

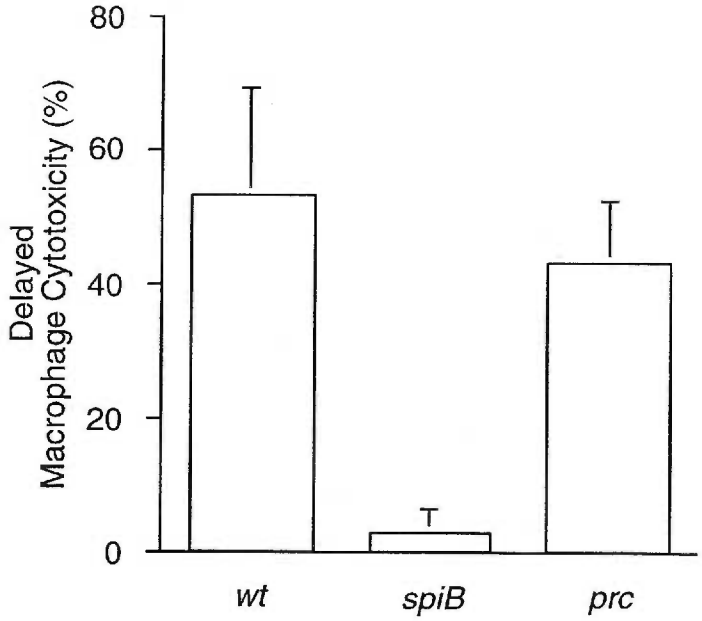
Figure 4-3. Long term intracellular survival and growth is not required for delayed macrophage killing.

J774 macrophages were infected with stationary phase cultures (conditions shown to turn off SPI1 dependent rapid induction of macrophage cell death) of wild-type *S. typhimurium*, a *spiB* mutant strain, or a macrophage sensitive *prc* deficient strain, after which macrophage survival was determined at 15 and 18 hours p. i. (three times each) by measuring viable intracellular CFU (A). Macrophage cytotoxicity was quantitated at these times by measuring the release of cytoplasmic LDH (B). Data are arithmetic means of at least three independent experiments from 15 h time points. Error bars indicate standard deviations of the mean.

A


Bacterial Survival
(% with respect to wild type)
100
75
50
25
0
wt
spiB
prc

B


Delayed
Macrophage Cytotoxicity (%)
80
60
40
20
0
wt
spiB
prc

3A)(31, 71), 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 *S. typhimurium* mutants that are defective in intramacrophage survival, 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. We are currently in the process of identifying SPI2 secreted effector(s) that mediate this delayed cytotoxic effect.

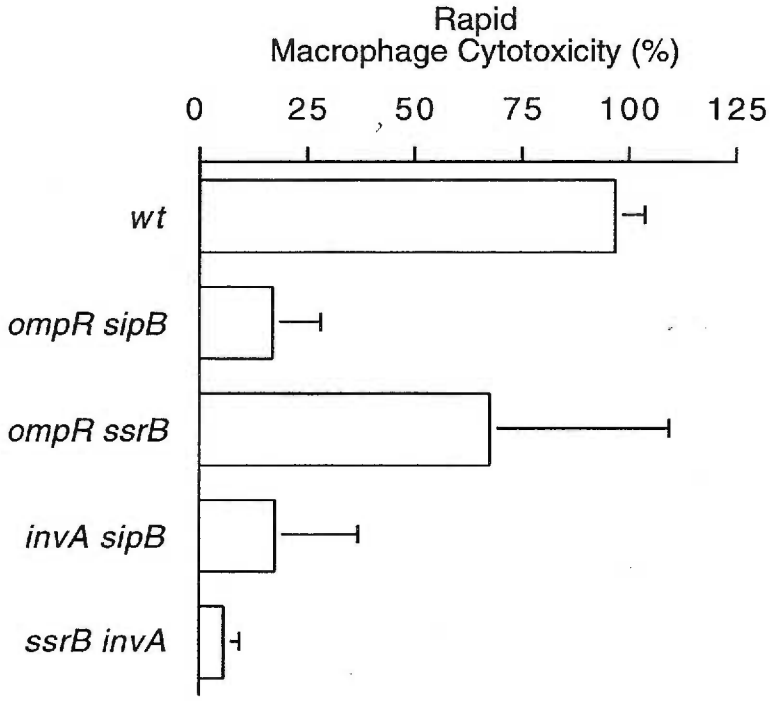
Rapid and delayed macrophage killing processes are independent.

To determine whether rapid and delayed macrophage killing were independent of one another, double mutant strains were constructed. Strains were constructed that carried 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* double mutants were non-cytotoxic, whereas an *ompR ssrB* double mutant was as cytotoxic as the wild-type (Fig. 4A). Under conditions that favored delayed macrophage killing, an *invA sipB* double 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

Figure 4-4. Rapid and delayed macrophage killing processes are independent.

J774 macrophages were infected with wild-type *S. typhimurium*, or double mutants, inactivated in *ompR sipB*, *ompR ssrB*, *invA sipB*, or *ssrB invA*, respectively. 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) p. i. by measuring the release of LDH. Data from each graph are arithmetic means of at least three independent experiments. Error bars indicate standard deviations of the mean.

A


Rapid
Macrophage Cytotoxicity (%)
0
25
50
75
100
125
wt
ompR sipB
ompR ssrB
invA sipB
ssrB invA

B

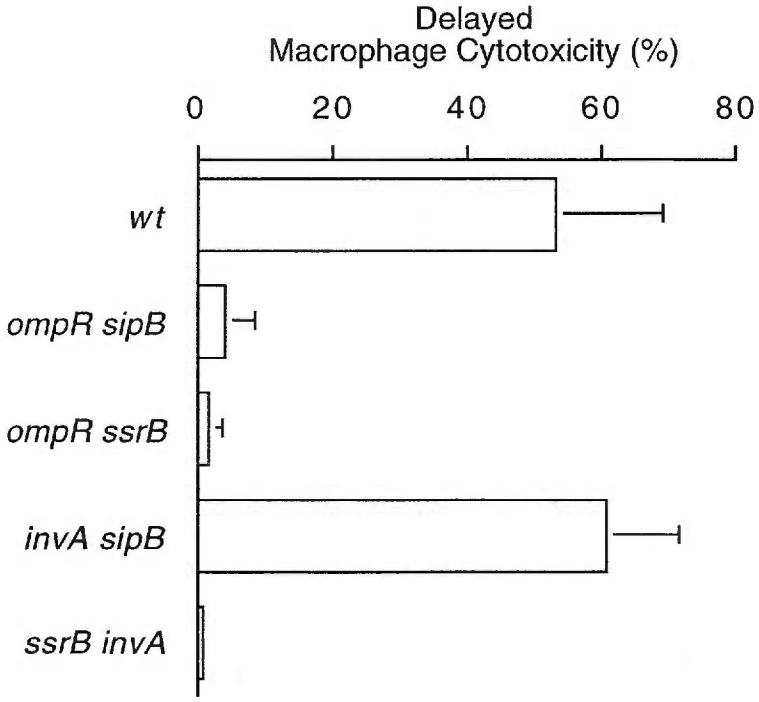
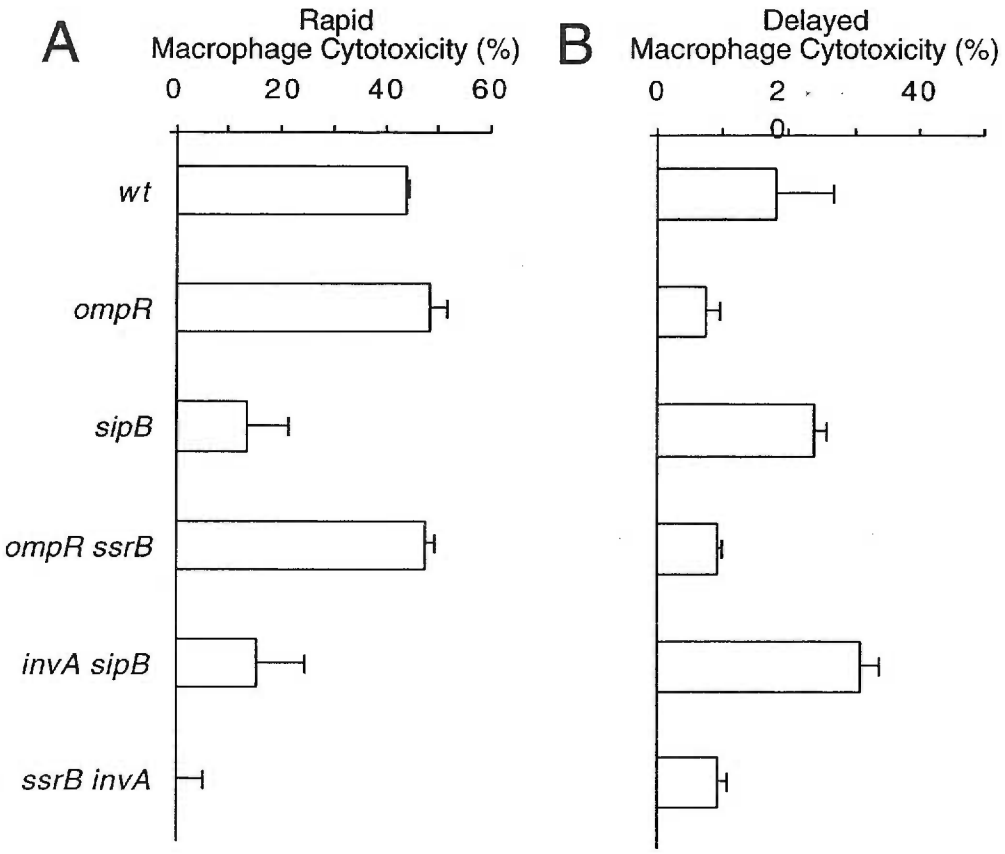

Delayed
Macrophage Cytotoxicity (%)
0
20
40
60
80
wt
ompR sipB
ompR ssrB
invA sipB
ssrB invA

Figure 4-5. *S. typhimurium* induces rapid and delayed macrophage cell death in bone marrow derived macrophages.

To demonstrate that *S. typhimurium* induced rapid and 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*), delayed macrophage cell death (*ompR, ompR ssrB*), or a mutant strain defective in both rapid and delayed macrophage killing (*ssrB invA*). Bacterial strains were grown to either late-log phase (A) or stationary phase prior to infection. Macrophage cell death was quantitated at 6 h (A) and 30 h (B) post-infection by measuring the release of LDH. Data from each graph are arithmetic means of three independent experiments. Error bars indicate standard deviations of the mean.


A
Rapid
Macrophage Cytotoxicity (%)
0
20
40
60
wt
ompR
sipB
ompR ssrB
invA sipB
ssrB invA
B
Delayed
Macrophage Cytotoxicity (%)
0
2
40

RAW264.7 macrophages (data not shown) and bone marrow derived macrophages (Fig. 5).

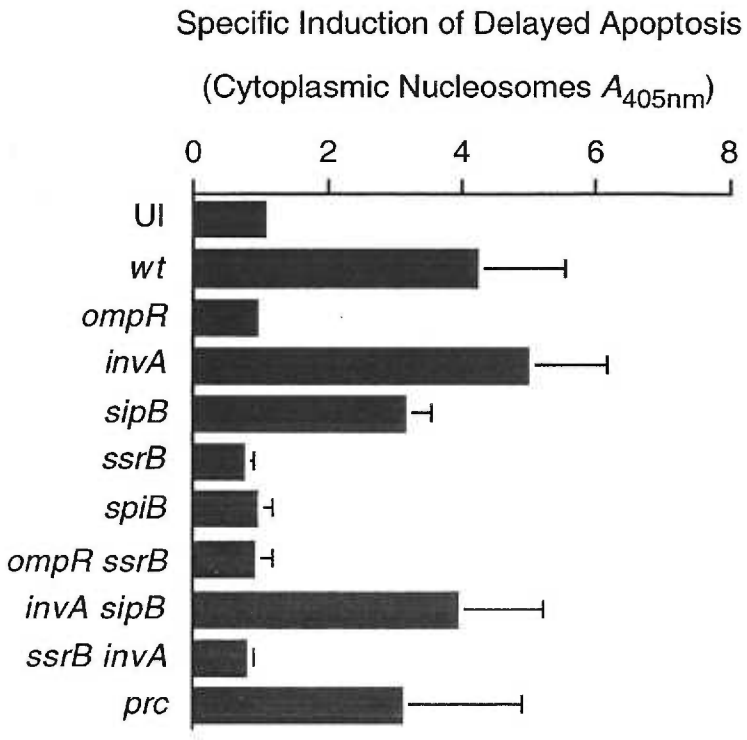
Collectively, these results indicate that bacterial strains mutated in genes required for either rapid or delayed induction of macrophage cell death are non-cytotoxic only under specific growth conditions. However, bacterial strains mutated in loci that affect both rapid and delayed macrophage killing are non-cytotoxic 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 investigated the nature of *S. typhimurium* induced rapid and delayed macrophage cell death. Thus far, a non-specific method, measuring the release of host cytoplasmic lactate dehydrogenase (LDH), was used as a readout for macrophage cytotoxicity. To determine whether macrophages were undergoing apoptosis upon infection with *S. typhimurium*, the amount of cytoplasmically located histones bound to fragmented DNA was quantified. Under SPI1 inducing conditions, *S. typhimurium* rapidly induced apoptosis via a SPI1 dependent process (data not shown). Under conditions that favored delayed macrophage cytotoxicity, induction of apoptosis was independent of SPI1 (Fig. 6). Delayed induction of apoptosis in infected macrophages was abrogated in strains defective in either *ompR* or SPI2 (Fig. 6). These results indicate that *S. typhimurium* induces either rapid or delayed

macrophage apoptosis. As shown previously, rapid activation of programmed cell death depends on SPI1. Here, we demonstrate that delayed activation of programmed macrophage cell death depends on *ompR* and SPI2.

Figure 4-6. *ompR* and SPI2, but not SPI1, are required for delayed induction of apoptosis in infected macrophages.

J774 macrophages were infected with either wild-type *S. typhimurium* or mutant strains defective in either rapid killing (*sipB, invA, invA sipB*), delayed killing (*ompR, ssrB, spiB, ompR ssrB*), a mutant strain defective in both rapid and delayed macrophage killing (*ssrB invA*), or a strain defective in macrophage survival (*prc*). The ability of these strains to induce apoptosis was determined at 18 h p. i. by measuring the amount of cytoplasmically located histones bound to fragmented DNA. Data from this graph are arithmetic means of three independent experiments. Error bars indicate standard deviations of the mean.


Specific Induction of Delayed Apoptosis
(Cytoplasmic Nucleosomes A_{405nm})
0
2
4
6
8
UI
wt
ompR
invA
sipB
ssrB
spiB
ompR ssrB
invA sipB
ssrB invA
prc

Discussion

In this study, we demonstrate that macrophages undergo either rapid or delayed apoptosis upon infection with *S. 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 non-cytotoxic 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 multiplicity of infection (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 non-specific effect such as uncontrolled intracellular proliferation can not be excluded until we have identified a SPI2 effector(s) that is both necessary and sufficient for the activation of delayed programmed macrophage cell death.

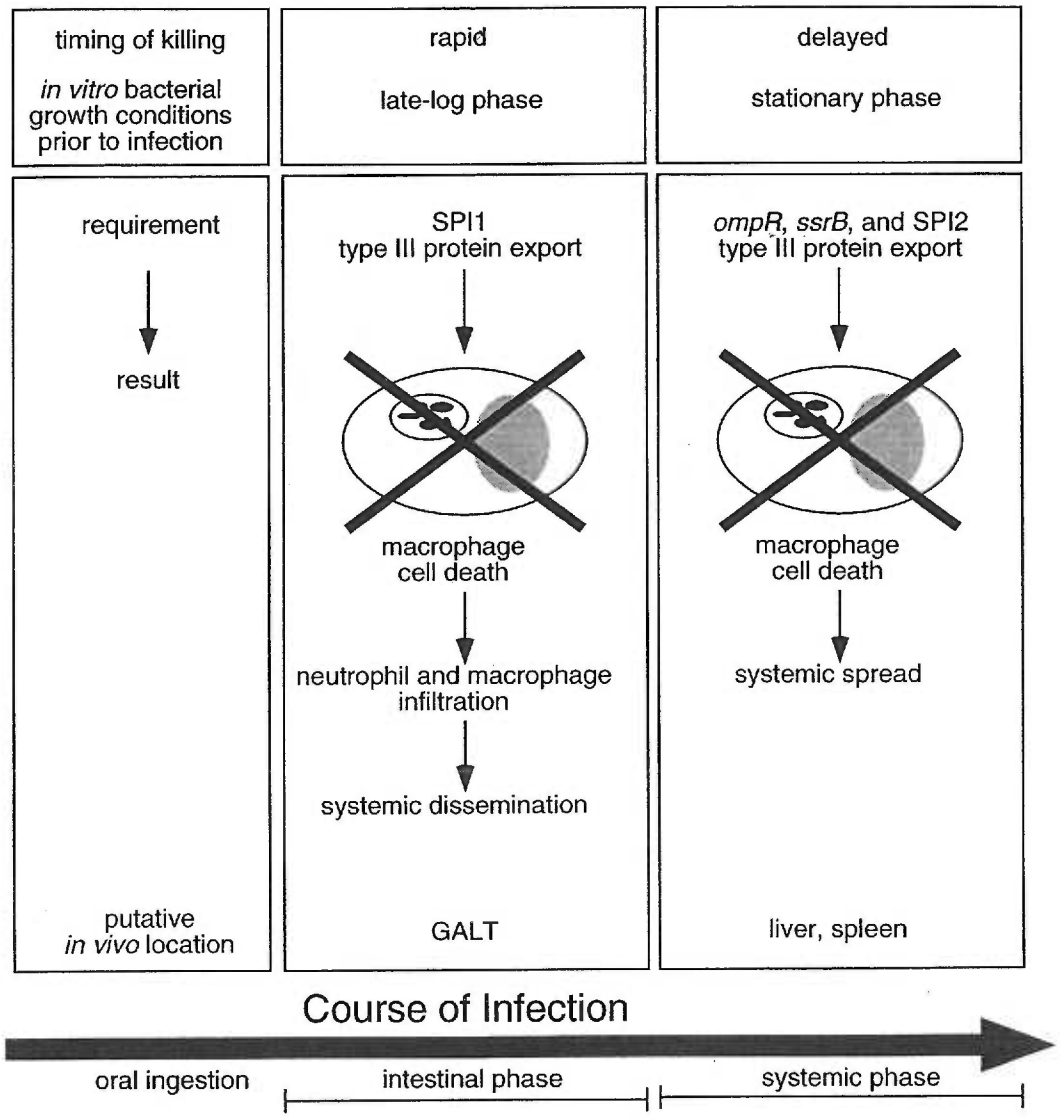
In agreement with the literature, we observe a defect (2-10 fold) in intracellular proliferation for SPI2 mutants at 15 and 18 hours postinfection (41, 99, 100, 167, 204). However, *prc, htrA,* and eleven other macrophage sensitive mutants tested are fully cytotoxic, yet more severely defective in their ability to

survive and grow inside phagocytic cells (Fig. 3A) (31, 64). In fact, MS4290 (*prc*) was the most sensitive mutant isolated in an extensive search for *Salmonella* mutants that can not survive inside macrophages (31, 64). Despite this substantial defect, *prc* mutant bacteria, as well as a large panel of other macrophage sensitive *S. 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 distinct regulatory circuits control these two specialized protein secretions systems. For example, substrates for the SPI1 encoded type III protein export apparatus are secreted under mildly alkaline conditions (54), whereas SseB, a substrate for the type III protein export system encoded within SPI2, is secreted at pH 5.0 (24). Furthermore, numerous studies suggest that, once inside a phagocytic host, *S. typhimurium* represses SPI1 gene expression and turns on genes that are important for long-term residence, growth, and survival inside these host cells (6, 10, 23, 41, 56, 63, 75, 77, 132, 133, 180, 181, 230). It is therefore unlikely that substrates for SPI1 and SPI2 encoded type III

Figure 4-7. Model of *S. typhimurium* induced apoptosis *in vivo*.

We propose that *S. 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 (74), 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 (38, 56, 58, 167, 205, 230). This model predicts that *Salmonella* induces delayed apoptosis in infected macrophages to spread intercellularly within apoptotic bodies.


timing of killing
in vitro bacterial growth conditions prior to infection
rapid
late-log phase
delayed
stationary phase
requirement
result
SPI1 type III protein export
ompR, ssrB, and SPI2 type III protein export
macrophage cell death
neutrophil and macrophage infiltration
systemic dissemination
macrophage cell death
systemic spread
putative in vivo location
GALT
liver, spleen
Course of Infection
oral ingestion
intestinal phase
systemic phase

protein export systems are secreted simultaneously.

We hypothesize that *S. typhimurium* induces rapid and delayed apoptosis in infected macrophages under distinct 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, as SPI1 mutants are significantly attenuated only when administered to mice orally (72, and references therein, 74). In contrast, *ompR* and SPI2 are absolutely required during the systemic phase of infection (38, 56, 58, 167, 205, 230). In fact, ample evidence suggests that plays a role in growth inside phagocytic cells at systemic sites of infection (38, 56, 58, 167, 205, 230). 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 neighboring phagocytes ingests apoptotic cells, we propose that delayed induction of apoptosis in infected macrophages may allow *Salmonella* to spread intercellularly within apoptotic bodies. This model is supported by (i) a recent study demonstrating that *S. typhimurium* is transported from the intestine, via the bloodstream, to the liver and spleen by

CD18-expressing monocytes in a SPI1 independent process (233) and (ii) studies demonstrating that *Salmonella* virulence was unaffected by treatment with antibiotics that kill extracellular bacteria (29, 61).

Acknowledgements

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Chapter 5: Discussion

Salmonella virulence is a complex, multi-factorial process that requires numerous genes that are scattered throughout the genome. This dissertation focuses on two important aspects of *Salmonella typhimurium* virulence, (i) the role of fimbrial adhesins during infection *in vivo*, and (ii) the ability of this enteric pathogen to induce apoptosis in infected macrophages.

Initiation of *S. typhimurium* infection in mice depends on successful attachment to- and colonization of the intestinal mucosa in the distal ileum. Although *Salmonella* fimbriae play a role in mediating attachment to epithelial cells *in vitro*, the role of individual fimbrial adhesins during infection is not clear as blockage of individual adhesins does not greatly affect mouse virulence. Furthermore, inactivation of essential fimbrial biosynthetic genes only moderately affects *Salmonella* virulence in the mouse model of infection. Here, we present evidence that *S. typhimurium* fimbriae are functionally redundant and act in synergy during colonization of the intestinal mucosa and subsequent development of a typhoid-like systemic infection in mice. A *S. typhimurium* mutant that lacks all four known fimbrial adhesins (quadruple mutant) is significantly attenuated for mouse virulence. Despite its attenuation, however, this strain still causes a lethal systemic infection in mice, suggesting that *Salmonella* produce alternate attachment factors. Indeed, our electron

microscopic studies demonstrate that the quadruple mutant expresses at least one additional, yet uncharacterized fimbriae-like structure.

This structure, and possibly others, may contribute to residual adhesion and colonization of the murine intestinal mucosa by *S. typhimurium* in the absence of type-1, LP, PE, and thin aggregative fimbriae. It remains to be determined whether the novel fimbrial structure observed in our studies is encoded by the recently identified *stf* operon (157). Several studies suggest that *Salmonella* may produce bundle forming (type IV) pili (67, 209). We have attempted to identify *Salmonella* homologues of type IV pilus assembly genes using a genetic screen that relies on functional complementation of a *Pseudomonas aeruginosa pilD* mutation. The *pilD* gene encodes a highly conserved pillin-specific leader peptidase (217) that is required for proteolytic processing and subsequent secretion of type IV pilus subunits (164, 216, 218). Although a similar screen was used successfully to identify *bfpP*, a *pilD* homologue in enteropathogenic *E. coli* (242), we were unable to functionally complement the *pilD* defect in *P. aeruginosa* using a *S. typhimurium* cosmid library. It is possible that this library is not representative of the entire genome, however, other interpretations of this result include (i) *S. typhimurium* does not encoded a functional *pilD* homolog, or (ii) the putative *pilD* homologue in *S. typhimurium* cannot complement a *pilD* defect in *P. aeruginosa*.

Following adhesion to- and colonization of epithelial cells in the distal ileum, *S. typhimurium* penetrates the intestinal mucosa and enters the lamina

propria, where bacteria are taken up by resident macrophages and eventually reach the bloodstream. Although long-term survival and growth within macrophages is required for a persistent systemic infection in mice, recent studies indicate that *S. typhimurium* can also kill these host cells *in vitro*. One study shows that *S. typhimurium* induces macrophage cell death as late as 18 hours postinfection in a process that depends on *ompR*. Other reports indicate that *S. typhimurium* induces macrophage apoptosis within a few hours after infection in a process that depends on SipB, a SPI1 secreted type III effector. Finally, one study reports that hepatic phagocytes undergo apoptosis upon intravenous infection with *S. typhimurium*. Because SPI1 is not required for systemic infection in mice, these findings suggest that *S. typhimurium* has devised an alternate strategy to induce apoptosis in phagocytes at systemic sites of infection. Here we evaluate this possibility using a genetic approach to study the kinetics of *Salmonella* induced macrophage cell death. We demonstrate that macrophages undergo either rapid- or delayed apoptosis upon infection with *S. typhimurium*. Rapid activation of programmed macrophage cell death requires SPI1, whereas delayed induction of apoptosis is SPI1-independent and is abrogated in *S. typhimurium* strains mutated in either *ompR* or SPI2. Rapid- and delayed induction of apoptosis are two independent processes, as only mutants that are defective in both rapid and delayed macrophage killing are unable to induce apoptosis under all conditions tested. Even though SPI2 mutants have a defect in intracellular growth, our results indicate that long-term intracellular

survival and proliferation are not required for delayed macrophage killing *per se*, as *Salmonella* mutants that are severely defective in intracellular growth still induce delayed apoptosis.

These findings have important implications for understanding *Salmonella* pathogenesis and suggest that *S. typhimurium* activates rapid- and delayed programmed macrophage cell death under discrete physiological conditions at distinct times and locations within an infected host. Because the SPI1 encoded type III protein secretion system is important primarily during the intestinal phase of infection, we propose that rapid induction of apoptosis in macrophages of the GALT results in increased inflammation and recruitment of phagocytes that may be required for systemic dissemination. Subsequent repression of rapid killing upon internalization is necessary for systemic dissemination, allowing continued proliferation and systemic spread prior to *ompR* and SPI2 dependent induction of delayed apoptosis at systemic sites of infection. In support of this view, *ompR* and SPI2, unlike SPI1, are absolutely required during the systemic phase of infection. Finally, this model predicts that *Salmonella* induces delayed apoptosis in infected macrophages to spread intercellularly without being exposed to the extracellular environment since apoptotic cells are taken up by neighboring phagocytes.

We are currently further characterizing delayed induction of apoptosis in infected macrophages and have preliminary evidence for two novel *Salmonella* specific genes that may play a role in this process (van der Velden and Heffron,

data not shown; Chai and Heffron, personal communication). Delayed induction of apoptosis depends on a functional SPI2 encoded type III protein export apparatus, suggesting that *Salmonella* translocates a type III secreted toxin into the macrophage cytoplasm. However, a non-specific effect cannot be excluded until we have identified a SPI2 secreted effector(s) that is both necessary and sufficient for the delayed activation of programmed cell death. SsrB, a transcriptional activator, may regulate the expression of such a toxin because a *S. typhimurium* strain mutated in *ssrB* cannot induce delayed programmed cell death in infected macrophages. It was recently demonstrated that SsrB controls a global regulon of putative intracellular virulence genes in *S. typhimurium* (241). Interestingly, SsrB activates a gene (*srfJ*) that is remarkably homologous to human glucosyl ceramidase (241), an enzyme that is necessary for the production of ceramide, a potent activator of the cellular apoptotic machinery (93, 94, 111, 179, 212). We used a genetic selection to identify mutations in SsrB regulated genes that affected the ability of these strains to induce delayed macrophage cell death. Unfortunately, this selection was not successful (van der Velden, Worley, and Heffron, data not shown). We interpret these observations to mean that either (i) the SPI2 export apparatus, but not the SPI2 secreted toxin, is regulated by SsrB, or (ii) *Salmonella* may translocate multiple SPI2 secreted toxins into the macrophage cytoplasm. Alternatively, the SsrB regulated gene screen may have failed to represent the entire *S. typhimurium* genome. This

latter interpretation is supported by the fact that not all known SsrB regulated genes were recovered (Worley and Heffron, unpublished).

Besides increasing the size of our SsrB regulated gene screen, we are currently testing two additional strategies to identify *Salmonella* genes that play a role in delayed activation of programmed macrophage cell death. The first strategy is based on the assumption that a toxin translocated into the macrophage cytoplasm is subject to proteolytic degradation and subsequent presentation onto the macrophage cell surface in the context of a class I major histocompatibility complex (MHC) molecule. Ellefson et al. developed a resolvable Tn*5*-based transposon that randomly distributes a class I MHC epitope tag throughout the bacterial genome (62). *S. typhimurium* mutants that secrete fusion proteins into the macrophage cytoplasm are isolated by fluoresence activated cell sorting (FACS) using an antibody that specifically recognizes cell surface located class I MHC epitopes in context of Class I MHC receptors. We are currently using this strategy to identify secreted proteins of *S. typhimurium* that play a role in delayed induction of apoptosis in infected macrophages. The second strategy also uses a Tn*5*-based transposon that contains a constitutive neomycin promoter (133, 207, 214). Upon random insertion throughout the genome, the transposon creates promoter fusions that result in constitutive expression of the target gene. We are currently using this strategy to select for mutants that have an increased ability to induce delayed macrophage cell death.

Cumulatively, these strategies should provide new means to identify the putative SPI2 secreted macrophage toxin(s). Information gained from these experiments will contribute important new insights into the molecular mechanism *Salmonella* induced apoptosis in infected macrophages. These studies will allow us to identify cellular targets of the SPI2 encoded type III protein secretion system during delayed activation of programmed macrophage cell death.

Collectively, this dissertation describes the characterization of two key virulence mechanisms of the enteric pathogen *Salmonella.* It is demonstrated that *S. typhimurium* fimbriae act synergistically during the colonization of mouse intestinal tissue and subsequent development of murine typhoid *in vivo.* Furthermore, evidence is presented that two fimbrial operons in *S. enteritidis*, *lpf* and *pef*, mediate attachment to murine intestinal tissue *in vitro* and contribute to mouse virulence *in vivo.* Finally, it is demonstrated that *S. typhimurium* has devised two independent strategies to induce apoptosis in infected macrophages. Our findings have important implications for understanding *Salmonella* pathogenesis and provide new insights into the complexity of *Salmonella* virulence.

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