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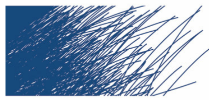

Salmonella Typhimurium infections in pigs: a closer look at the pathogenesis

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LIST OF ABBREVIATIONS

BGA	Brilliant Green Agar
BHI	Brain Heart Infusion Broth
BPW	Buffered Peptone Water
CFU	Colony Forming Units
GALT	Gut-Associated Lymphoid Tissue
GFP	Green Fluorescent Protein
HBSS	Hank’s Balanced Salt Solution
IL-8	Interleukin 8
iNOS	Inducible Nitrogen Oxyde Synthetase
LB	Luria-Bertani Broth
LPS	Lipopolysaccharide
NO	Nitrogen Oxyde
PAI	Pathogenicity Island
PAM	Porcine Alveolar Macrophages
PAMP	Pathogen-Associated Molecular Pattern
PBM	Peripheral Blood Monocytes
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PEEC	Pathogen Elicited Epithelial Chemo-attractant
PI	Post Inoculation
PMN	Polymorphonuclear Leukocytes
RLU	Relative Light Units
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
SCV	*Salmonella* Containing Vacuole
SD	Standard Deviation
SEM	Standard Error of the Mean
SIF	*Salmonella* Induced Filaments
SP	Spacious Phagosome
SPI	*Salmonella* Pathogenicity Island
STM	Signature-Tagged Mutagenesis
T3SS	Type 3 Secretion System
TLR	Toll-Like Receptor
VAP	Vacuole-associated Actin Polymerizations
WT	Wild Type

GENERAL INTRODUCTION

1. The genus *Salmonella*

Salmonella Choleraesuis was the first *Salmonella* serotype to be isolated from pigs (Salmon and Smith, 1886), only 2 years after the first isolation ever of *Salmonella*, performed by Gaffky in 1884 (Le Minor, 1994). In the course of time, more than 2400 different serotypes were isolated from different animal species, including pigs. These serotypes are designated based on their variation in somatic (O) and 2 phases of flagellar (H_1 and H_2) antigens. Based on the variations in these antigens, Kauffmann (1941) and White (1926) started the first taxonomical efforts, resulting in the classification scheme named after them. This classification formed the basis for the nomenclature proposed by Le Minor and Popoff (1987), which was adjusted by Reeves et al. (1989) to the classification generally accepted nowadays.

Until recently, the bacterial genus *Salmonella* contained 2 species: *enterica* and *bongori*. Even though *Salmonella choleraesuis* originally was the official type species, *Salmonella enterica* has always been widely accepted and has recently been designated the type species (Judicial Commission, 2005). In 2004, a new species was discovered: *Salmonella subterranea* (Shelobolina *et al*. 2004). *Salmonella enterica* is divided in 6 subspecies: *enterica* (I), *salamae* (II), *arizonae* (III), *diarizonae* (IV), *houtenae* (V) and *indica* (VI). Recently the *Salmonella* nomenclature has been reviewed (Heyndrickx et al., 2005; Tindall et al., 2005). The biochemical properties of some pig-associated *Salmonella* serotypes are summarized in Table 1.

On the basis of host specificity and pathogenesis, *Salmonella* serotypes can be classified in three distinct types of infection (Wallis and Barrow, EcoSal website). A small number of serotypes are capable of producing severe systemic disease in healthy adult individuals of a narrow range of animal species (host restricted serotypes). In general, bacterial multiplication is considered to take place primarily in the macrophages and monocytes. The alimentary tract becomes involved pathologically only in the later stages of the disease, and thus, in the absence of disease little intestinal colonization takes place. Examples of these serotypes are *Salmonella* Typhi and Paratyphi A, which produce typhoid in humans, and *Salmonella* Gallinarum causing systemic infections in poultry. A second group of host adapted serotypes is primarily associated with one or two closely related host species, but may also infrequently cause disease in other hosts. For example, *Salmonella* Dublin and *Salmonella* Choleraesuis are generally

Table 1: Biochemical properties of pig-associated serotypes. + = positive; (+) = most strains positive; v = variable reactions; - = negative

	***Salmonella* Typhisuis**	***Salmonella* Choleraesuis**	***Salmonella* Choleraesuis var Kunzendorf**	**most serotypes of *Salmonella* (*Salmonella* Typhimurium,…)**
Gram staining	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod
Motility at 37°C	+	+	+	+
Indol production	-	-	-	-
Glucose fermentation	+	+	+	+
Lactose fermentation	-	-	-	-
Lysine decorboxylase	-	+	+	+
Urease	-	-	-	-
Hydrogen sulphide	v	-	+	+
Citrate fermentation	-	+	+	+
Mannitol fermentation	-	+	+	+
Inositol fermentation	+	-	-	v
Sorbitol fermentation	-	(+)	(+)	+
Trehalose fermentation	-	-	-	+
Maltose fermentation	-	+	+	+

associated with severe systemic disease in ruminants and pigs, respectively, but may also cause disease in humans or other animal species. The vast majority of the remaining serotypes (containing for example *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Derby,…) seldom produce systemic infections in healthy adult animals. They are, however, able to colonize the alimentary tract of a broad range of animals and may cause acute enteritis or subclinical infections, also in pigs (Loynachan et al., 2004). As a consequence of intestinal colonization and high levels of fecal shedding, particularly in food producing animals, these serotypes can enter the human food chain and cause human salmonellosis.

In the next few chapters and where possible, *Salmonella* Typhimurium will be discussed as the serotype of interest and the pig as host of interest. The importance, epidemiology and control of *Salmonella* infections in pigs, both at the level of the primary production and the slaughterhouse has been described extensively (Schwartz, 1999). In addition, some interesting PhD theses handling these topics, have been produced at the UGent the last few years (i.e. Nathalie Nollet, 2005; Nadine Botteldoorn, 2006). In order to avoid repetition, these matters will not be handled in this introduction. Instead, special emphasis has been put on the pathogenesis of *Salmonella* infections in pigs and associated *Salmonella* virulence factors.

2. The pathogenesis of *Salmonella* Typhimurium infections in pigs

2.1. Intestinal phase of infection

Transmission of *Salmonella* between pigs is thought to occur mainly via the faecal-oral route. Oral experimental infection of pigs with *Salmonella* Typhimurium can result in clinical signs and excretion of high numbers of bacteria (Wood and Rose, 1992; Loynachan et al., 2005). Some studies showed that the upper respiratory tract and lungs may be an important portal of entry as well (Fedorka-Cray et al., 1995; Proux et al., 2001). Indeed, in a recent report, Oliveira and coworkers (2006) found that airborne *Salmonella* transmission in weaned pigs over short distances is possible. However, the pathogenesis of *Salmonella*-infections caused by infection of the respiratory tract has not been studied in detail. For the remaining of the text, the pathogenesis of *Salmonella* infections will be discussed using the oral route of infection.

During ingestion, *Salmonella* enters the tonsils in the soft palate and persists in the tonsillar crypts (Wilcock and Olander, 1978; Wood et al., 1989; Gray et al., 1995; Horter et al., 2003). Until now, no detailed information has been gathered on how *Salmonella* interacts with and persists in the porcine tonsillar tissue, although some observations mention persistence of *Salmonella* on the superficial epithelium of the tonsillar crypts (Fedorka-Cray et al., 1995; Horter et al., 2003).

Porcine epithelial beta-defensin 1 is expressed in the dorsal tongue at antimicrobial concentrations and may contribute to the antimicrobial barrier properties of the dorsal tongue and oral epithelium (Shi et al., 1999). After ingestion, the bacteria encounter a second antimicrobial barrier: the stomach. Although it has been shown that salmonellae can survive acidic environments by producing acid shock proteins (Smith, 2003), the stomach can function as a barrier against *Salmonella*, especially when the pigs are fed a coarsely ground meal (Mikkelsen et al., 2004). Bearson and coworkers (2006) determined that the lethal effects of the porcine stomach contents are pH-dependent but that low pH is not the sole killing mechanism and identified *Salmonella* genes important in survival of the gastric environment. Bacteria that have survived the stomach travel to the small intestines. Even though *Salmonella* Typhimurium can be highly resistant against bile salts (van Velkinburgh and Gunn, 1999), these salts can still repress the invasion of *Salmonella* in epithelial cells, possibly by decreasing virulence gene expression (Prouty and Gunn, 2000). In the gut, adhesion to the intestinal mucosa is generally accepted as the first step in the pathogenesis of *Salmonella* infections in pigs. Although multiple putative adhesines have been described in *Salmonella* Typhimurium, the type 1 fimbriae are the only ones which have been shown to contribute to the attachment to porcine enterocytes and the colonization of swine (Isaacson and Kinsel, 1992; Althouse et al., 2003).

Following adhesion, *Salmonella* invades the intestinal epithelium. It has been shown that *Salmonella* can invade porcine absorptive enterocytes, M-cells and even goblet cells (Pospischil et al., 1990; Schauser et al., 2004). Also Meyerholz et al. (2002) suggested that early cellular invasion by *Salmonella* Typhimurium is nonspecific and rapid in swine. Furthermore, they suggest that *Salmonella* Typhimurium may use sites of cell exfoliation as an additional mechanism for early invasion.

After invasion, *Salmonella* Typhimurium and *Salmonella* Choleraesuis are found within the porcine enterocytes (Reed et al., 1986). Both serotypes penetrate the intestinal mucosa and can be isolated from mesenteric lymph nodes at 2 hours after inoculation. Intracellular bacteria are morphologically intact, free in the cytoplasm or membrane bound, and cause no detectable cytotoxic effect to the cell. Schauser and coworkers (2005), however, observed increased cell loss during *Salmonella* infection, as a result of caspase 3 dependent and independent apoptosis of epithelial cells in the proximal region of the jejunum.

As a result of epithelial invasion, the porcine gut will react with the production of several cytokines, of which IL-8 is the most studied one and for *Salmonella* pathogenesis probably the most important one (Trebichavsky et al., 1997; Grondahl et al., 1998; Splichal et al., 2002; Cho and Chae, 2003; Trebichavsky et al., 2003; Cho and Chae, 2004; Hyland et al., 2006a; Hyland et al., 2006b).

Infection of polarized porcine intestinal epithelial cells with *Salmonella* Typhimurium or *Salmonella* Choleraesuis increases polarized IL-8 secretion i*n vitro*, but also *in vivo* at least 24 hours after inoculation of weaned piglets (Skjolaas et al., 2006). The secretion of IL-8 results in the attraction of neutrophils to the lamina propria and eventually the lumen of the gut. These cells are the first line of defense against a *Salmonella* infection. Inefficient uptake of *Salmonella* by PMN may provide an opportunity for the pathogen to colonize and/or replicate to levels that facilitate establishment of a carrier state or clinical infection in swine (Stabel et al., 2002). The presence of large amounts of neutrophils in the porcine gut is able to prevent successful salmonellosis (Foster et al., 2003). The damage induced by these activated neutrophils, however, is considered the most important cause of the gut pathology distinctive for *Salmonella* infections. Nevertheless, Foster and coworkers (2005) demonstrated that PMN influx in the gut in itself is not necessarily associated with clinical symptoms and/or intestinal pathology.

Luminal *Salmonella* endotoxin affects epithelial and mast cell function in the proximal colon of pigs (Aschenbach et al., 2003). In addition, gilts at 30 days of gestation that were experimentally injected with LPS of *Salmonella* Typhimurium showed increased PGF2 alpha metabolite concentrations accompanied by abortion (Cort and Kindahl, 1990). Although not very

likely, it is not known yet if resorption of LPS may be a common phenomenon in pigs naturally (subclinically) infected with *Salmonella* Typhimurium.

At least two types of defensins are described in the porcine small intestine. They are differentially expressed along the gut, and expression of both defensins is not altered by *Salmonella* Typhimurium infection (Veldhuizen et al., 2007).

2.2. Systemic phase of infection

The systemic part of a *Salmonella* infection in pigs is not well documented. Although not yet investigated in pigs, it is generally accepted that *Salmonella* can spread throughout an animal using the blood stream or the lymphatic fluids and infect internal organs. By hiding inside macrophages or other cells, the bacterium is able to conceal itself from the hostile extracellular environment. Being a facultative intracellular organism, *Salmonella* is able to survive and even replicate inside professional phagocytic cells. Most of the research concerning this stage of infection was done *in vitro* using isolated primary cell suspensions.

Porcine monocytes and polymorphonuclear cells (PMN; neutrophils) respond *in vitro* to *Salmonella* Typhimurium with phagocytosis, oxidative burst and to some extent intracellular killing (Riber and Lind, 1999; Donné et al., 2005). Monocytes obtained from different pigs differed markedly in their reactive oxygen species (ROS) production and in their ability to kill the bacteria. Interestingly, high ROS production did not coincide with increased intracellular killing. No reactive nitrogen intermediates (RNI) production was detected by porcine PBM after stimulation with *Salmonella* Typhimurium or LPS (Donné et al., 2005). This is in agreement with other studies (Pampusch et al., 1998; Akunda et al., 2001) that suggested that NO synthase (iNOS) is not inducible in porcine immune cells with little or no upregulation following stimulation. Therefore, RNI production by iNOS does not appear to be an important component of the innate immune response to control intracellular *Salmonella* populations in pigs. However, a significant increase of nitrite and/or nitrate plasma levels, 3-nitro-tyrosine expression and pathological changes in mesenteric lymph nodes have been observed in gnotobiotic piglets orally infected for 1 day with a virulent strain of *Salmonella* Typhimurium (Trebichavsky et al., 2001). The colonization of the mesenteric lymph nodes, spleen and liver can result in prominent systemic and local immune responses (Dlabac et al., 1997).

2.3. Interactions between *Salmonella* and other microbial agents

Secondary immunodeficiency is a well known consequence of some bacterial (*Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*) and viral infections (hog cholera virus, porcine reproductive and respiratory syndrome (PRRSV), Aujeszky's disease virus) in pigs. These infections are capable of suppressing immune function sufficiently to make the animal more susceptible to secondary infections. Furthermore, these and also other swine pathogens (porcine parvovirus, swine influenza virus, African swine fever virus) are able to replicate in a variety of immune system cells and impair their function (Segales et al., 2004). These infections may lead to easier colonization by *Salmonella*, increased shedding or even higher mortality rates. For example, in utero infection with PRRSV inhibits phagocytosis of *Salmonella* in blood monocytes as well as the oxidative burst capacity of alveolar macrophages (Riber et al., 2004) and both pathogens may work synergistically to produce disease or persistence (Wills et al., 2000).

Exposure to rumen protozoa led to enhancement of pathogenicity of *Salmonella* in a bovine infection model (Rasmussen et al., 2005). There are no reports describing similar effects in a porcine infection model. Interactions with intestinal nematodes may alter the excretion of *Salmonella* Typhimurium (Steenhard et al., 2002) or the development of enteritis symptoms (Arechavaleta et al., 1998). A dose dependency of the interaction was suggested. The relatively high number of parasites necessary to influence the *Salmonella* infection suggests that these common pig helminths generally do not influence the course of concurrent *Salmonella* Typhimurium infections under natural conditions (Steenhard et al., 2006). In contrast, the counts of *Salmonella* Typhimurium in the feces and in the cecal contents of piglets infected with both *Salmonella* Typhimurium and *Isospora suis* were significantly lower than in those infected with *Salmonella* Typhimurium alone (Baba and Gafaar, 1985). No mechanistic model was proposed by the authors to explain this phenomenon.

2.4. Genetic resistance to *Salmonella*

Although genetic-linked variance in immune responses is well known to occur in large domestic mammals such as pigs, specific resistance to *Salmonella* is less characterized (Wigley, 2004). The influence of miniature swine major histocompatibility complex genes (SLA) upon phagocytic and bactericidal activities of peripheral blood monocytes against *Salmonella* Typhimurium was measured *in vitro* using cultured cells. Uptake and killing of *Salmonella* Typhimurium was highest in homozygous aa and cc haplotypes at 4 weeks and pigs with the c x d

recombinant haplotype had highest uptake and killing of *Salmonella* Typhimurium at 8 weeks (Lacey et al., 1989).

The natural resistance-associated macrophage protein (SLC11a1; also called NRAMP1) has been identified and cloned in a number of domestic mammals including pigs (Sun et al., 1998; Zhang et al., 2000). In pigs NRAMP1 is strongly expressed on macrophages and neutrophils following stimulation with LPS, but any role in *Salmonella* infection is yet to be demonstrated (Zhang et al., 2000). A reference population of pigs bred to study resistance to *Salmonella* Choleraesuis infection indicated that a number of inherited immunological traits influence resistance to salmonellosis (Van Diemen et al., 2002). Neutrophils from the resistant animals showed increased phagocytic and antimicrobial activity and T-lymphocytes increased mitogen-induced proliferation, though no genes associated with this resistance were described.

3. Virulence factors of *Salmonella* situated on *Salmonella* Pathogenicity Islands

Pathogenicity Islands (PAI) are genetic elements on the chromosome of pathogenic bacteria encoding virulence factors (for an overview in different bacterial species see Schmidt and Hensel, 2004). These Islands are considered "quantum leaps" in bacterial evolution (Groisman and Ochman, 1996; Hensel, 2004) and were probably acquired by horizontal gene transfer. As a result, the GC content of PAI often differs from that of the core genome. Typically, PAI are present in the genome of pathogenic bacteria but absent in nonpathogenic strains of the same or related species. PAI are often inserted at specific sites of the genome, frequently tRNA or tRNA-like genes. Mobility genes, such as integrases, are regularly located at the beginning of the island, close to the attachment site. PAI are also often interspersed with other mobility elements, such as insertion elements or remnants of insertion elements (Schmidt and Hensel, 2004; Figure 1).

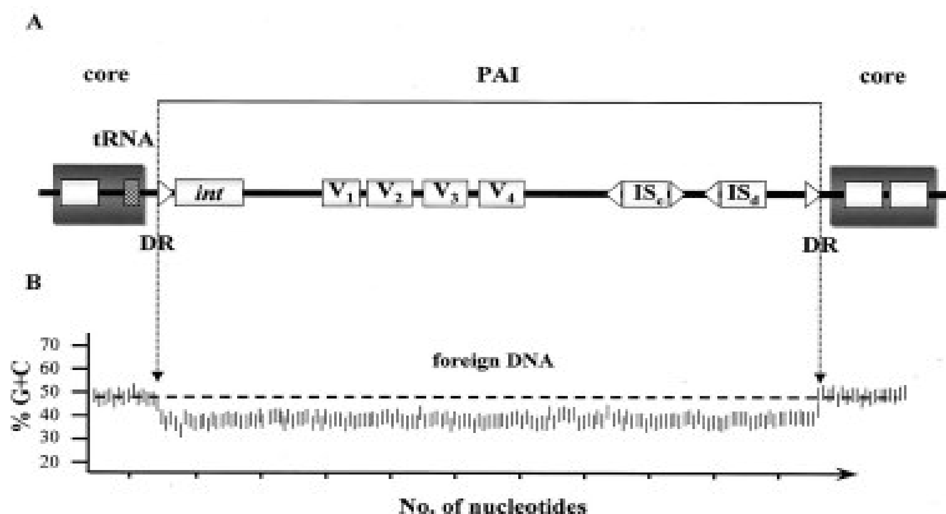

Figure 1

General structure of Pathogenicity Islands (PAI). (A) PAI are mostly inserted in the backbone genome of the host strain (dark grey bars) in specific sites that are frequently tRNA or tRNA-like genes (hached grey bar). Mobility genes, such as integrases (*int*), are frequently located at the beginning of the island, close to the tRNA locus or the respective attachment site. PAI harbor one or more genes that are linked to virulence (V1 to V4) and are frequently interspersed with other mobility elements, such as IS elements (Is_c, complete insertion element) or remnants of IS elements (IS_d, defective insertion element). The PAI boundaries are frequently determined by Direct Repeats (triangle), which are used for insertion and deletion processes. (B) A characteristic feature of PAI is a G+C content different from that of the core genome. This feature is often used to identify new PAI (Schmidt and Hensel, 2004).

Many of the virulence factors of *Salmonella* are encoded by genes situated on so-called *Salmonella* Pathogenicity Islands or "SPI". To date, 17 different SPI have been described in *Salmonella*. These 17 SPI all comply with at least some of the above mentioned criteria, although not all SPI can yet be linked to virulence. The first SPI was discovered in 1995, but since then, several hundreds of scientific articles have been written addressing additional functions to "old" SPI and describing new SPI. The most important and best characterized SPI are SPI-1 and SPI-2. Although until recently the role of SPI-1 was considered limited to the intestinal phase of infection and SPI-2 to the systemical phase of infection, the last few years, more and more researchers are pointing to evidence suggesting that these 2 SPI may act in concert on different stages of the pathogenesis (Jiang et al., 2004; Coombs et al., 2005; Drektrah et al., 2005; Hapfelmeier et al., 2005; Mangan et al., 2006). Most of the SPI-related researches were conducted with *Salmonella* Typhimurium in BALB/c mice as the *in vivo* model of choice for the systemic phase of infection and the intestinal loop model in calves as the model of choice for the intestinal phase of infection.
An overview of all known SPI and their virulence properties is given below; a summary is presented in Table 2.

Table 2: The characteristics of all currently known Pathogenicity Islands in *Salmonella*.

Designation (alternative)	Distribution	Stability	Associated virulence treats
SPI-1	*Salmonella* spp.	Conserved	T3SS, epithelial invasion, diarrhoea and macrophage cytotoxicity
SPI-2	*Salmonella enterica*	Conserved	T3SS, intracellular survival and macrophage cytotoxicity
SPI-3	*Salmonella* spp.	Variable	Mg^{2+} uptake, intestinal colonization factors
SPI-4	*Salmonella* spp.	Conserved	T1SS, intestinal colonization factors
SPI-5	*Salmonella* spp.	Variable	T3SS effectors for SPI-1 and SPI-2
SPI-6 (SCI)	*Salmonella enterica* subsp. I, parts in IIIb, IV, VII	?	Invasion in epithelial cells, fimbriae
SPI-7 (MPI)	*Salmonella enterica* subsp. I	Instable	Capsular antigen, type IV pilus assembly, T3SS effector SPI-1
SPI-8	*Salmonella* Typhi	?	Unknown
SPI-9	*Salmonella enterica* subsp. I	Conserved	T1SS, Putative toxin
SPI-10	*Salmonella enterica* subsp. I	?	Sef fimbriae
SPI-11	*Salmonella enterica* subsp. I	Conserved	Survival in macrophages
SPI-12	*Salmonella enterica* subsp. I	Conserved	Unknown
SPI-13	*Salmonella* Pullorum biotype Gallinarum	?	Virulence factors in chicken infection model
SPI-14	*Salmonella* Pullorum biotype Gallinarum	?	Virulence factors in chicken infection model
SPI-15, 16, 17	*Salmonella* Typhi	?	Unknown
SGI-1	*Salmonella enterica* subsp. I	Variable	Antibiotic resistance genes
HPI	*Salmonella enterica* subsp. IIIa, IIIb, IV	?	Iron uptake

3.1. *Salmonella* Pathogenicity Island 1

SPI-1 was the first genomic region ever called a *Salmonella* pathogenicity island (Mills et al., 1995). It forms a 40 kb insertion between 2 genes that are consecutive in *Escherichia coli*, a bacterium presumed to have an ancestor in common with *Salmonella* (Mills et al., 1995; Groisman and Ochman, 2000). SPI-1 encodes a type three secretion system (T3SS; reviewed by Galan, 2001). T3SS are complex assemblies that require the function of more than 20 genes for their activity. Many of the subunits of T3SS involved in virulence show similarities to the flagellum assembly machinery system. Although termed "secretion systems," the main function of the T3SS is not the secretion of effector proteins into the medium but rather, the translocation across a third membrane: the membrane of a eukaryotic host cell (reviewed by Ehrbar et al., 2002; Schlumberger and Hardt, 2006). The T3SS consists of a hollow, needle-like structure (Figure 2) and a pore-forming ring (also called translocon) through which the effectors are transported into the cytoplasm of the infected host cell. Surprisingly, a substantial number of the SPI-1 effectors are not encoded in SPI-1 itself. The effectors may be situated on other parts of the bacterial chromosome, in other SPI or even be associated with prophages (Figure 3). The association of SPI-1 virulence genes with bacteriophages can result in an extensive exchange of virulence factors between *Salmonella* serovars and strains and create a mosaic of strains with potential presence or absence of different effector genes (Mirold et al., 2001; Ehrbar and Hardt, 2005). Not only the presence/absence of virulence genes, but also the *Salmonella*-specific regulatory systems of the different SPI-1 effectors may result in strains of different behaviour (Streckel et al., 2004; Ben-Barak et al., 2006).

Apart from their localization, the SPI-1 effectors can be subdivided in three major categories regarding their mode of action. They can modulate the host's actin skeleton with invasion as a consequence, alter the host's cellular responses with diarrhoea as a consequence and switch host cell death pathways on and off. Strikingly, a lot of the SPI-1 effectors have multiple tasks, either as a translocator and effector protein or as an effector protein exerting different functions. In Table 3, all known SPI-1 related effector genes and their respective functions are summarized.

Table 3: The characteristics of all currently described SPI-1 related effector genes.

Protein	Localization	Structural function	Function as effector protein	References
SspA/SipA	SPI-1	None	Binding and stabilization of actin and subsequently role in cell invasion Induction of PMN influx (PEEC)	Higashide et al., 2002; Raffatellu et al., 2005 Lee et al., 2000
SspB/SipB	SPI-1	Pore forming translocon	Induction of macrophage cell death	Hersch et al., 1999; Van der Velden et al., 2003
SspC/SipC	SPI-1	Pore forming translocon	Nucleation and bundling of actin and subsequently role in cell invasion	Hayward and Koronakis, 1999; Chang et al., 2005
SspD/SipD	SPI-1	Pore forming translocon	May repress expression of sip genes	Kaniga et al., 1995
SptP	SPI-1	None	Reverses cellular changes stimulated by the other effectors	Fu and Galan, 1998; Murli et al., 2001
AvrA	SPI-1	None	Inhibits the pro-inflammatory and anti-apoptotic NF-κB pathway	Hardt and Galan, 1997; Collier-Hyams et al., 2002
SopA	Chromosome	None	Targets to mitochondria Role in epithelial cell invasion Induction of enteritis	Layton et al., 2005 Raffatellu et al., 2005 Zhang et al., 2002
SigD/SopB	SPI-5	None	Modulates actin organization and subsequently cell invasion Enhances intestinal chloride secretion and induces diarrhoea Modulates lysosome/vesicular traficking Prevents apoptosis in epithelial cells Induces NO production	Zhou et al., 2001; Raffatellu et al., 2005 Zhang et al., 2002; Bertelsen et al., 2004 Hernandez et al., 2004; Dukes et al., 2006 Knodler et al., 2005 Drecktrah et al., 2005
SopD	chromosome	None	Role in epithelial cell invasion Induction of enteritis Role in systemic mouse virulence	Raffatellu et al., 2005 Zhang et al., 2002 Jiang et al., 2004
SopE	prophage	None	Modulates actin organization and subsequently cell invasion Enhances intestinal chloride secretion and induces diarrhoea	Hardt et al., 1998; Friebel et al., 2001 Zhou et al., 2001
SopE2	Chromosome	None	Modulates actin organization and subsequently cell invasion Induction of enteritis	Stender et al., 2000; Raffatellu et al., 2005 Zhang et al., 2002
SlrP	Chromosome	None	Role in mouse virulence	Tsolis et al., 1999
SspH1	Chromosome	None	Inhibits pro-inflammatory pathways	Haraga and Miller, 2003
SteA	Chromosome	None	Colonization of mouse spleens	Geddes et al., 2005
SteB	Chromosome	None	Putative dipicolinate reductase	Geddes et al., 2005

Salmonella invasion: modulation of the host cell's actin cytoskeleton

Invasion of *Salmonella* in non-phagocytic cells is considered a crucial step in its pathogenesis and was already observed almost 40 years ago (Takeuchi, 1967). Nowadays, the invasion of *Salmonella* in both phagocytic and non-phagocytic cells is considered completely SPI-1 dependent. Through modulation of the host cell's actin cytoskeleton, the bacterium forces the cell to engulf the attached *Salmonella* (reviewed by Guiney and Lesnick, 2005). The finger-like changes in the actin cytoskeleton and the plasma membrane are called ruffles and are accompanied by macropinocytosis (Garcia-del Portillo and Finlay, 1994). These changes are the result of 3 coordinated steps: stimulation of host signalling pathways to promote indirect actin cytoskeleton rearrangements, direct modulation of actin dynamics and a subsequent inversion of the cytoskeletal changes. As a result, the *Salmonella* bacterium has invaded the host cell, which has become normal again after invasion. The bacteria reside in the *Salmonella* containing vacuole (SCV; in epithelial cells) or spacious phagosome (SP; in phagocytic cells).

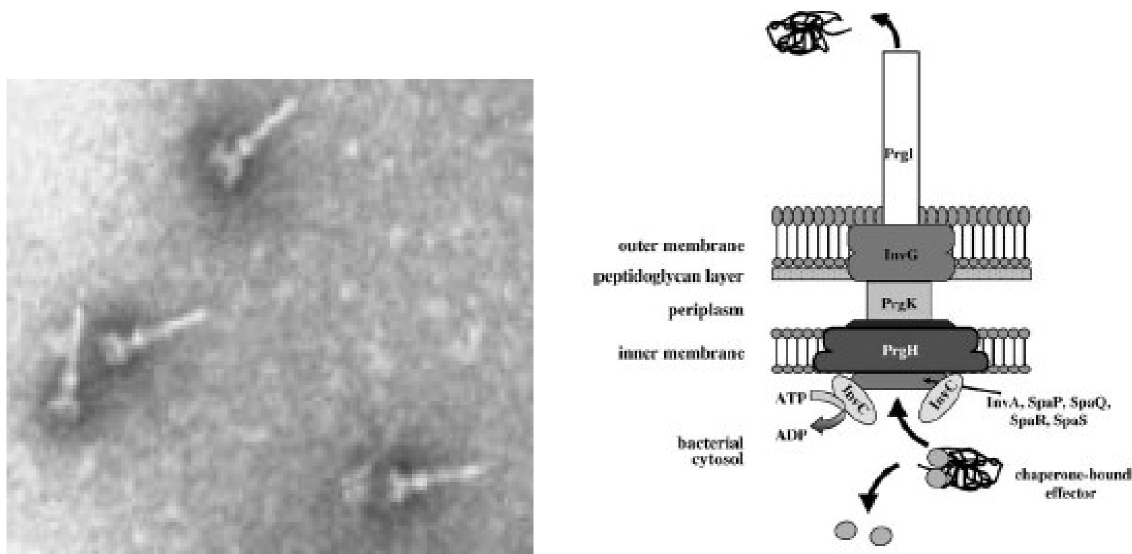

Figure 2: Electron microscopic picture and schematic representation of the SPI-1 related type 3 secretion system of *Salmonella* (Galan, 2001; Ehrbar and Hardt, 2005).

The Rho-family of small GTPases Rho, Cdc42 and Rac1, has a central role in the actin cytoskeleton rearrangements (Hall, 1998). The injection of SPI-1 effectors SopB, SopE and $SopE_2$ results in the recruitment and activation of Rac1 and/or Cdc42 to the apical cell membrane, but have no known effect on RhoA. SopB is an inositol phosphatase that increases cellular levels of $(1,4,5,6)P_4$, leading to Cdc42 activation (Norris et al., 1998; Zhou et al., 2001) and decreases the levels of $(4,5)P_2$ leading to the rapid fission of the invaginating

membranes (Terebiznik et al., 2002). SopE and $SopE_2$ are guanine nucleotide exchange factors for the GTPases (Hardt et al., 1998; Stender et al, 2000; reviewed in Schlumberger and Hardt, 2005). SopE can activate both Cdc42 and Rac1 and $SopE_2$ was efficiently activating Cdc42, but not Rac1 (Friebel et al., 2001). $SopE_2$ is commonly present in all *Salmonella enterica* strains, including in *Salmonella* Typhimurium. SopE, however, is encoded by a bacteriophage and is found predominantly in a small group of epidemic *Salmonella* Typhimurium strains (Mirold et al., 1999). Although SopE also has been implicated in the mechanism of increasing cellular levels of $(1,4,5,6)P_4$ (Zhou et al., 2001), it was recently shown that the SopE-dependent remodelling of the actin cytoskeleton occurs independently from the changes in the inositol phosphate turnover (Deleu et al., 2006).

The presence of multiple, partially redundant effectors may provide an efficient mechanism for fine-tuning the interaction of *Salmonella* with different cell types or host species.

In concert with the indirect effect on actin rearrangement, *Salmonella* Typhimurium also directly influences actin changes through translocation of two actin binding proteins, SipA and SipC. SipA binds actin subunits on opposite strands of polymers, acting as a molecular staple to stabilize the actin filaments (Lilic et al., 2003). SipC is a membrane bound protein and has separate actin bundling and actin nucleating domains. In this way it is presumed to target the actin filament formation to the site adjacent to the attached bacterium (Hayward and Koronakis, 1999). SipA and SipC are thought to cooperate in their actions and potentiate each others actitivities (McGhie et al., 2001). The exact role of SipC in invasion has not been established yet, since *sipC* mutants are defective for the transport of all SPI1 T3SS effectors, being also a translocator protein.

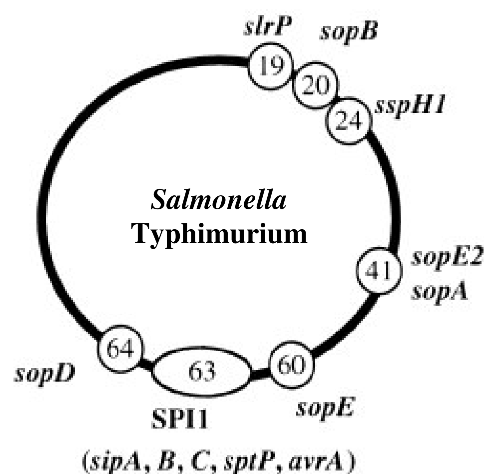

Figure 3: The location of important SPI-1 related effector genes in the *Salmonella* Typhimurium chromosome (Ehrbar et al., 2002).

After the invasion has occurred, *Salmonella* actively reverses the changes it has induced, returning the cytoskeleton to its original state (reviewed in Schlumberger and Hardt, 2005). To accomplish this, *Salmonella* injects another effector protein, SptP, into the cytosol of the infected cell. SptP has a GTPase activating domain in the N-terminal region and a tyrosine phosphatase activity in its C-terminal region (Fu and Galan, 1999; Murli et al., 2001). In order to be able to exert its functions after the phase of invasion, the SptP protein degradation by the proteasome pathway is delayed, compared to the degradation of the other SPI-1 effector proteins (Kubori and Galan, 2003).
Finally, the function of SopA and SopD is not fully understood, but both effectors also contribute to invasion (Raffatellu et al., 2005).

Salmonella *induced diarrhoea: taking over the host cell responses*
A characteristic feature of the *Salmonella*-associated pathology is the induction of an inflammatory response in the gut and the subsequent influx of neutrophils (reviewed by Tükel et al., 2006). SPI-1 plays a central role in both the stimulation and inhibition of the pro-inflammatory cytokine production. Like every other bacterium, *Salmonella* can activate the pro-inflammatory pathways of the innate immunity system by stimulation of Toll-like receptors. These receptors recognize so-called “Pathogen Associated Molecular Patterns” (PAMPs), conservated antigenic structures like LPS, flagella, etc. However, since the majority of the Toll-like receptors are situated at the basolateral side of the polarized intestinal epithelial cells, they can only be reached when the epithelial monolayer was disrupted before infection or after disruption, for example through the action of cytotoxins (Hershberg et al., 2002; Rhee et al., 2005). The SPI-1 dependent induction of an inflammatory response, however, possesses the power to initiate an inflammatory response, even when the intestinal epithelium is intact. After the integrity of the tight junctions is compromised through the *Salmonella* induced changes of the actin skeleton, the PAMPs and predominantly Toll-like receptor 5 are likely to contribute to the intensification of the host response (Jepson et al., 2000; Huang et al., 2004).
The activation of the Rho GTPases by SopB, SopE and $SopE_2$, as discussed in the mechanism of invasion, also stimulates the Mitogen Activated Protein kinase (MAP kinase) pathways. The unity in the mechanisms of stimulation, however, may lead to the incorrect conclusion that bacterial invasion per se stimulates the pro-inflammatory cytokine production (Gewirtz et al., 1999). The activation of the MAP kinase pathways result in their turn, via activation of the

transcription factor NF-κB, in the secretion of pro-inflammatory cytokines, for example IL-8 (Hobbie et al., 1997; Murli et al., 2001). IL-8, released from the basolateral side of infected epithelial cells, plays an important role in the initial movement of neutrophils from the circulation into the subepithelial region (McCormick et al., 1995).

The actual transepithelial migration of the PMN into the lumen of the gut is mediated by another cytokine, called the pathogen elicited epithelial chemo-attractant (PEEC), wich is secreted on the apical side of the epithelial cells, in response to the SPI-1 effector SipA (McCormick et al., 1998; Lee et al., 2000). Recently, PEEC has been identified as the key regulator of mucosal inflammation, hepoxilin A_3 (Mrsny et al., 2004). The overwhelming influx of PMN results in detachment of the epithelial cells from the basal membrane and the release of ROS, proteases and other PMN mediators induces extensive necrosis of the superficial mucosa.

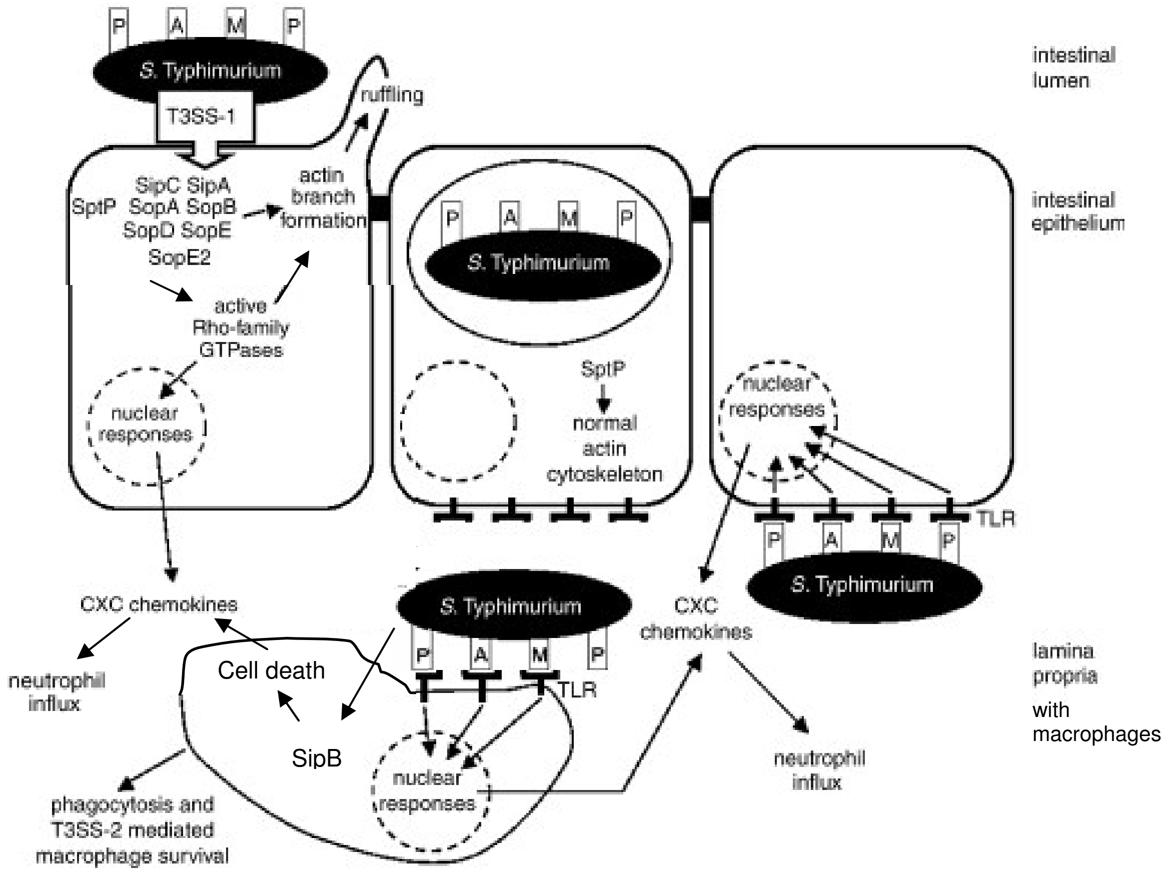

Figure 4: A schematic representation of the different mechanisms leading to invasion of the gut epithelium and underlying tissues by *Salmonella* and to the induction of an inflammatory response (Adapted from Tükel et al., 2006).

In addition to the mechanisms described above, *Salmonella* can modulate the chloride secretion, thereby contributing to the development of diarrhoea. As described above, SopB is an inositol 3-phosphate phosphatase that increases cellular levels of (1,4,5,6)P_4 (Norris et al., 1998). This increase not only results in the activation of cytoskeletal changes, but also antagonizes the closure of chloride channels on the intestinal eptithelial cells (Eckmann et al., 1997; Marcus et al., 2001). Also SopE has been implicated in this mechanism, although not having phosphatase properties (Zhou et al., 2001). Recently, it was shown that the Inositol-P_5 dephosphorylation is not promoted by SopE itself, but requires the interaction of SopE with other *Salmonella* virulence proteins (Deleu et al., 2006).

The SPI-1 effector SipB mediates macrophage death in the intestine (see further) by caspase-1 activation, which causes the release of IL-1beta and IL-18, contributing to the inflammatory response (Hersch et al., 1999).

Finally, the function of SopA and SopD is not fully understood, but both effectors also contribute to enteritis in a bovine intestinal loop model (Zhang et al., 2002).

The tools available for *Salmonella* to induce diarrhoea seem overwhelming. Keeping this in mind, it may seem rather peculiar that most of the *Salmonella* Typhimurium infections in pigs and humans are subclinical and asymptomatic. Apart from environmental factors such as the infection pressure, the age and immunological status of the host, again *Salmonella* SPI-1 effectors may play a role.

At least 3 SPI-1 effector proteins have been described, able to downregulate the transcription factor NF-κB and, subsequently, the host's inflammatory responses. AvrA was first identified as a homologue to a plant anti-virulence factor (Hardt and Galan, 1997). It is a cysteine protease which inhibits IL-8 production and in turn the inflammatory response (Collier-Hyams et al., 2002). Although it was first considered an anti-virulence protein, the lack of inflammatory response may help *Salmonella* to "sneak in" unnoticed. Most of the *Salmonella enterica* strains are *avrA* positive (Prager et al., 2000), but only epidemic strains of *Salmonella* Typhimurium and Enteritidis seem to express *avrA* constitutively (Streckel et al., 2004; Ben-Barak et al., 2006). SspH1 belongs to a family of T3SS effector proteins that share leucine-rich repeat motifs and are called *Salmonella* Translocated Effectors (STE's; see further SPI-2; Miao et al., 1999). Although not all STE's are linked to a certain virulence property, SspH1 is translocated through the SPI-1 T3SS and inhibits NF-κB-dependent gene expression (Haraga and Miller, 2003). SptP is described above as the protein reversing SPI-1 effects concerning invasion. It has also been shown to downregulate NF-κB (Haraga and

Miller, 2003). A schematic overview of the different players in the development of enteritis is shown in Figure 4.

Salmonella *induced host cell death: switching it on and off on demand?*

Salmonella Typhimurium is cytotoxic for several cell types. The most intensively studied cell types are murine monocytes/macrophages and dendritic cells. When these phagocytic cells are infected with *Salmonella* Typhimurium that has been grown under conditions that allow high SPI-1 expression, death occurs within the next hours (Chen et al., 1996; Van der Velden, 2003). Although some of the infected cells show signs of apoptosis (Monack et al., 1996), an important part do not (Watson et al., 2000). Recently, it has become clear that *Salmonella* induced cell death is very complicated and that more than 1 mechanism is triggered by multiple virulence genes (reviewed in Hueffer and Galan, 2004). For example, salmonellae that do not cause the SPI-1 dependent rapid cell death and reside in the phagocytic vacuole can trigger a SPI-2 or *spv* dependent cell death 12 to 24 hours after infection (see further). Finally, also the toll-like receptors are thought to play a role in *Salmonella* induced apoptosis (Hsu et al., 2004; Zeng et al., 2006). A schematic overview is presented in Figure 5.

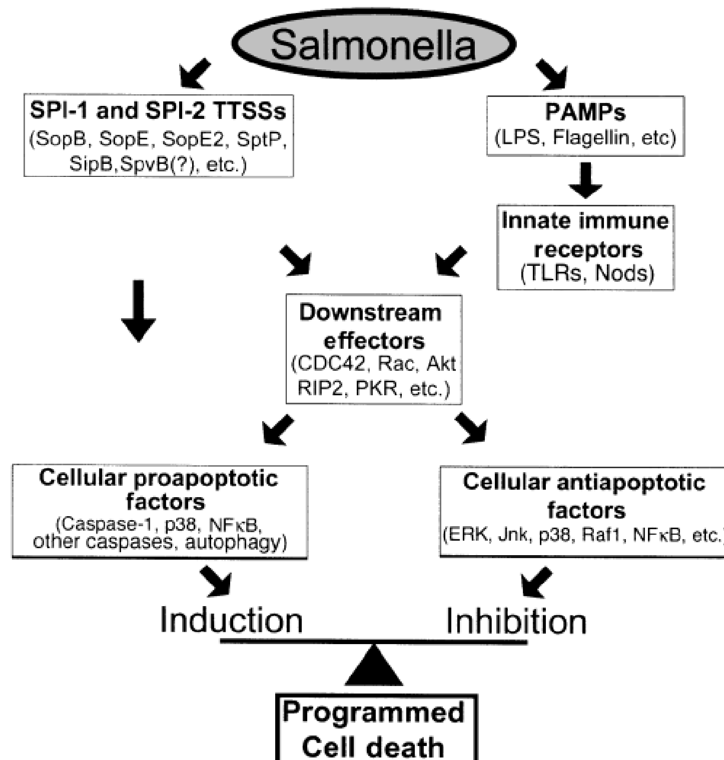

Figure 5: A schematic representation of the different mechanisms used by *Salmonella*, that lead to the induction or inhibition of cell death (Hueffer and Galan, 2004).

Hersch and colleagues (1999) first demonstrated that the SPI-1 translocator protein SipB is involved in triggering programmed cell death in macrophages in a caspase 1 dependent manner, although also caspase 2 has been implied (Jesenberger et al., 2000). SipB was found to disrupt mitochondria, thereby inducing autophagy and a type II programmed cell death (Hernandez et al., 2003). The exact mechanism of action, however, is yet to be elucidated. Recently, SPI-1 induced membrane permeabilization of the SCV was shown to induce a lysosomal repair response and autophagy (Roy et al., 2004; Birmingham et al., 2006).

Whilst in macrophages a rapid, SipB dependent cell death occurs, the opposite takes place in epithelial cells. An anti-apoptotic effect was shown for SopB in epithelial cells (Knodler et al., 2005). Comparing the anti-apoptotic versus pro-apoptotic activities of SPI-1 in epithelial cells and macrophages, respectively, it is apparent that *Salmonella* can selectively modulate host cell events depending on the type of cell infected, a further example of the remarkable ability of this pathogen to adapt to its host cell environment.

AvrA does induce a late form of apoptosis in epithelial cells through inhibition of the anti-apoptotic NF-κB pathway (Collier-Hyams et al., 2002). The presence of AvrA resulted in accelerated apoptosis, allowing elimination of the infected cells and prevention of systemic spread. As epithelial cells are rapidly replaced, cell death occurring only 24 hours after infection probably does not have harmful consequences. *Salmonella* Typhi and Paratyphi are strains that evade epithelial defenses and result in severe systemic disease (within macrophages). Strikingly, these strains invariably do not possess an *avrA* allele. Also in pigs, apoptotic intestinal epithelial cells have been noticed after *Salmonella* Typhimurium infection (Schauser et al, 2005), but no correlation was made with any virulence factor.

The biological significance in the pathogenesis of *Salmonella* infections of these phenomena in different cell types and host species is not yet clear at all. A model has been proposed by Guiney (2005).

SPI-1: does it stop after invasion?

SPI-1 has long been considered to play an exclusive role in the invasive phase of infection. The last few years, a few researchers found additional SPI-1 related functions, for example intracellular proliferation and vacuole biogenesis in epithelial cells and macrophages (Mukherjee et al., 2001; Steele-Mortimer et al., 2002), contribution to systemic infection and persistence in mice (Jiang et al., 2004; Lawley et al., 2006) and the induction of nitric oxide production long after invasion (Drecktrah et al., 2005). Coombes and coworkers (2005) recently found that SPI-1 does not play an important role in gut pathology appearing long (>5

days) after infection. Aguirre et al. (2006) saw that in contrast to the rest of the genes in the SPI-1 locus, orgBC is expressed during and after *Salmonella* entry into its host cell, and they suggest a role for the products of this operon after host cell internalization.
The most interesting observations, however, were made by Drecktrah and colleagues (2006). They showed that the initial interactions of *Salmonella* with the host macrophages (SPI-1 dependent invasion, complement mediated or Ig-mediated phagocytosis) determine the development of the intracellular niche and the bacterial response. For example, intracellular upregulation of SPI-1 was seen when the bacteria were taken up by macrophages by phagocytosis, but a downregulation occurred after active invasion of the cells.
Not all SPI-1 encoded genes are related to the T3SS. The *sit* gene cluster is also located at SPI-1, but it encodes an iron uptake system (Zhou et al., 1999). The sitABCD operon is preferentially expressed during the systemic stages of *Salmonella* Typhimurium infection in mice and a *sit* null mutation confers a virulence defect (Janakiraman and Slauch, 2000).

Regulation of SPI-1

The control of invasion involves a number of genetic regulators and environmental stimuli in complex relationships (reviewed by Altier, 2005 and Jones, 2005). Several environmental conditions are known to regulate the expression of SPI-1, including pH, osmolarity, oxygen tension, bile, Mg2+ concentration, and short chain fatty acids (Bajaj et al., 1996; Gantois et al., 2006). SPI-1 itself encodes several transcriptional regulators (HilA, HilD, HilC, and InvF) with overlapping sets of target genes. These regulators are, in turn, controlled by both positive and negative regulators outside SPI-1. A simplified schematic drawing of the SPI-1 regulation is presented in Figure 6.

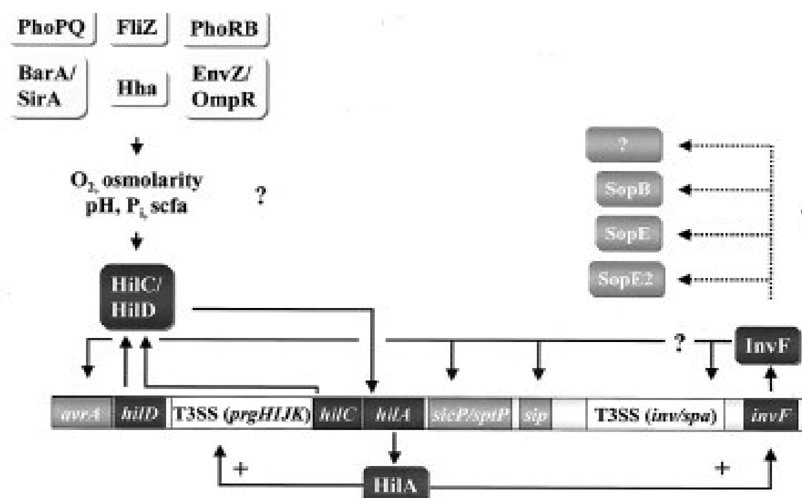

Figure 6: A schematic representation of the regulation of the expression of SPI-1 related genes (Schmidt and Hensel, 2004).

3.2. *Salmonella* Pathogenicity Island 2

Hensel and coworkers (Hensel et al., 1995; Shea et al., 1996) were the first to develop the method of signature tagged mutagenesis (STM), using the murine typhoid fever model of *Salmonella* Typhimurium. This not only resulted in a new revolutionary technique to locate virulence genes that are relevant *in vivo*, it also resulted in the discovery of a second pathogenicity island, called SPI-2. At the same time, Ochman and coworkers (1996) identified SPI-2 as a sequence that was present in *Salmonella enterica*, but absent in *E. coli*. Unlike SPI-1, SPI-2 is absent in *Salmonella bongori*, meaning this PAI was acquired at a later time point than SPI-1. The SPI-2 T3SS did not arise from gene duplication of SPI-1, is phylogenetically only distantly related to the SPI-1 T3SS and both T3SS were acquired in completely independent events (Ochman and Groisman, 1996). SPI-2 has a mosaic structure. The first 25-kb portion of SPI-2 contains at least 32 genes which code for a T3SS, a few effector proteins and their putative chaperones and a 2-component regulatory system. The second portion (15 kb) is not required for virulence and harbors genes for metabolic or unknown functions, such as the tetrathionate reductase, a specific trait that is commonly used for the enrichment and identification of *Salmonella* (Hensel et al., 1999). A common characteristic of SPI-1 and SPI-2 is that only a subset of effector proteins are encoded within the island. The majority of effectors are encoded in distinct loci, scattered over the chromosome (Miao and Miller, 2000). They can be associated with bacteriophages or can even rely in other PAI (ex. SPI-5). In contrast to SPI-1, the exact role and mechanism of action of the effectors of SPI-2 are only vaguely understood. In Table 4, all known SPI-2 related effector genes and their respective functions are summarized.

The function of SPI-2 (reviewed in Waterman and Holden, 2003) is essential for a major hallmark in the pathogenesis of salmonellosis, namely the ability to spread throughout the body and cause a systemic infection. This is especially so in infections caused by host-specific serovars. This feature is linked to the ability of *Salmonella* to survive and replicate inside a variety of host cells, including professional phagocytes like macrophages and dendritic cells. Therefore, it is not surprising to find that environmental stimuli that characterise the SP (a slightly acidic pH or the limitation of inorganic phosphate) are sufficient to induce a rapid up-regulation of SPI-2 genes, through the activation of the SsrA/B regulatory system (Löber et al., 2006). In contrast, *Salmonella* Typhimurium mutant strains that are SPI-2 deficient are attenuated by at least five orders of magnitude compared with the

Table 4: The characteristics of all currently described SPI-2 related effector genes.

Protein	Localization	SsrA/SsrB dependent	Function as effector protein	References
SpiC	SPI-2	Yes	Interferes with vesicular trafficking Possible translocator protein	Uchiya et al., 1999; Freeman et al., 2002
SseF	SPI-2	Yes	Contributes to SIF formation, SCV position and alteration of host endosome aggregation	Guy et al., 2000; Kuhle and Hensel, 2002; Abrahams and Hensel, 2006
SseG	SPI-2	Yes	Contributes to SIF formation, SCV position and alteration of host endosome aggregation	Guy et al., 2000; Kuhle and Hensel, 2002; Abrahams and Hensel, 2006
SifA	Pathogenicity Islet	Yes	Required for SIF formation, SCV integrity and intracellular replication Interferes with MHC-II presentation	Stein et al., 1996 Beuzon et al., 2002 Mitchell et al., 2004
SifB	Pathogenicity Islet	Yes	Unknown	Miao and Miller, 2000
SspH1	Gifsy-3 prophage	No	Inhibits pro-inflammatory pathways	Haraga and Miller, 2003
SspH2	Uncharacterized bacteriophage	Yes	Colocalizes with polymerizing actin	Miao et al., 2003
SlrP	Chromosome	No	Role in mouse virulence	Tsolis et al., 1999
SseI/SrfH	Gifsy-2 prophage	Yes	Colocalizes with polymerizing actin	Miao et al., 2003
SseJ	Uncharacterized bacteriophage	Yes	Acyltransferase Promotes SCV integrity Role in mouse virulence	Ruiz-Albert et al., 2002; Miao et al., 2003; Ohlsen et al., 2005
SopD2	Pathogenicity Islet	Yes	SIF formation; Role in mouse virulence	Brumell et al., 2003; Jiang et al., 2004
PipB	SPI-5	Yes	Localizes to the SCV and SIF's Colonization factor specific for the chick infection model	Morgan et al., 2004; Knodler and Steele-Mortimer, 2005
PipB2	Chromosome	Yes	SIF formation	Knodler and Steele-Mortimer, 2005
SseK1	Pathogenicity Islet	Yes	Unknown	Kujat et al., 2004
SseK2	Pathogenicity Islet	Yes	Unknown	Kujat et al., 2004
SteA	Chromosome	No	Colonization of mouse spleens	Geddes et al., 2005
SteB	Chromosome	No	Putative dipicolinate reductase	Geddes et al., 2005
SteC	Chromosome	Yes	Colonization factor specific for the chick infection model	Morgan et al., 2004; Geddes et al., 2005
GogB	Gifsy-1 prophage	Yes	Unknown	Coombes et al., 2005
SrfA- SrfM	Often phage related	Yes	Unknown; role in apoptosis; manipulating motility of infected cells	Worley et al., 2000; Waterman and Holden, 2003; Worley et al., 2006
SseL	Chromosome	Yes	Delayed cytotoxicity in macrophages	Rytkönen et al., 2007

wild-type strain after oral or intraperitoneal inoculation of BALB/c mice (Shea et al., 1996). Although SPI-2 was formerly believed to play a role only in the systemic phase of infection, it has also been implicated in the development of intestinal pathology (Coombes et al., 2005; Hapfelmeier et al., 2005). Although many cellular phenotypes related to SPI-2 have been described, they are only poorly understood compared to the mechanisms of SPI-1. The SPI-2 related actions can roughly be divided in the escape from intracellular antimicrobial agents, the influence on intracellular growth and the induction of a late type of cell death.
An overview is given below.

SPI-2 mediated intracellular survival
The ability of *Salmonella* to survive inside professional immune cells requires the evasion of several intracellular antimicrobial mechanisms. In contrast to other facultative intracellular pathogens as *Listeria* or *Shigella*, *Salmonella* does not escape from its vacuole to evade the host cell defence mechanisms. Instead, it adapts this potentially dangerous environment to a 'safe niche'. In here, the bacterium is isolated from the hostile extracellular environment (complement, antibodies,…) which allows it to persist intracellularly.
To accomplish this, *Salmonella* has a multitude of SPI-2 dependent ways to create its own secure spot in the SCV. These can be subdivided in 4 major actions: the inhibition of the fusion of lysosomes with the SCV, the inhibition of the NADPH oxidase dependent killing, the inhibition of the localization of inducible nitric oxide synthase to the SCV and the maintenance of the SCV by the assembly of a refined meshwork around it.

The first SPI-2 effector protein to be identified and characterized was SpiC. A lot of the vacuoles containing wild type *Salmonella* Typhimurium do not fuse with endosomes and lysosomes. A mutation in *spiC* resulted in a significantly greater proportion of vacuoles undergoing this fusion, resulting in a survival defect in macrophages and a virulence defect in mice (Uchiya et al., 1999). At a later stage, it was found that a *spiC* mutant strain was deficient in the translocation of other SPI-2 related effectors, so its interference with the vesicular trafficking may also be explained by the lack of translocation of other SPI-2 effector proteins (Freeman et al., 2002). If the latter is the case, the actual effector protein interfering with the lysosome/endosome traffic is not yet identified.

The finding that the virulence of SPI-2 deletion mutants was restored in knockout mice lacking the NADPH oxidase activity, led to the assumption that SPI-2 interfered with the

oxidative burst (Vazquez-Torres et al., 2000). This mechanism normally results in the production of large amounts of ROS, which have a devastating effect on intracellular pathogens. SPI-2 does not inhibit the production of ROS in general, but mediates the exclusion of the NADPH oxidase complex from the phagosomal membrane (Vazquez-Torres et al., 2000; Gallois et al., 2001).

Next to the fast production of ROS, which are bactericidal, macrophages also produce RNI, which have a more sustained bacteriostatic mode of action. The alteration of the inducible nitric oxide reductase (iNOS) localization in macrophages is yet another characteristic of SPI-2. iNOS was shown to be strongly co-localized with SPI-2 mutants, but not with the wild type strain (Chakravorrty et al., 2002).

Garcia-del Portillo and coworkers (1993) first observed that endosomes containing lysosomal membrane glycoproteins (lgp or lamp, lysosome associated membrane proteins) fused with tubules that connected to the SCV. The biogenesis of the SCV depends on the nature of the cell type studied (reviewed by Knodler and Steele-Mortimer, 2003). A univocal theme is that in all cell types the SCV is considered a modified endosomal compartment that diverts from the normal phagocytic pathway. The association with early and late endosomes, but probably not lysosomes, ensures a progressive increase of the SCV membrane surface to accomodate the replicating bacterial cells (Beuzon et al., 2000).
After invasion, the SCV is directed in a SPI-2 independent manner towards a perinuclear localization in close apposition to the Golgi-apparatus (Salcedo and Holden, 2003). The steady-state position of the SCV near the nucleus is maintained through the interference of the SPI-2 effectors SseF and SseG with the host cell microtubule cytoskeleton and the motor-protein recruitment (reviewed by Abrahams and Hensel, 2006).
A few hours after invasion, the intracellular salmonellae induce the formation of stable filamentous structures (*Salmonella* induced filaments; sif’s). The formation of these filaments was found to be dependent on the SPI-2 effector SifA (Stein et al., 1996). Since transport and fusion between late endosomes and lysosomes is controlled by the small GTPase Rab7 and given that SifA has been shown to interact with Rab7, SifA could not only be involved in the formation of sif’s, but also provide a mechanism for influencing SCV fusion with endosomes/lysosomes (Harrison et al., 2004). Recently, it has been shown that SPI-2 effectors also modify the exocytotic transport, which results in the recruitment of secretory vesicles to the vicinity of the SCV (Kuhle et al., 2006).

Complementary to the above described features, F-actin filaments are assembled around the SCV (reviewed by Guiney and Lesnick, 2005). These structures are referred to as VAP (vacuolar-associated actin polymerizations). The formation of these VAP requires a functional SPI-2 T3SS. Two SPI-2 effectors, SspH2 and SseI, have been shown to colocalize with the polymerizing actin cytoskeleton (Miao et al., 2003). However, neither SspH2 nor SseI is essential for the formation of F-actin around the SCV, since single and double *sspH2/sseI* mutants still show normal morphology of the VAP (Miao et al., 2003). Although VAP have been found in different cell types and correlate with the intracellular replication of *Salmonella*, there remains no direct evidence that VAP formation plays an important role during intracellular pathogenesis.

SPI-2 and the intracellular replication paradox

Salmonella is widely known as an intracellular bacterial pathogen that proliferates within vacuoles of mammalian cells. However, recent *in vivo* studies have revealed that the vast majority of infected cells contain very few (three to four) intracellular bacteria in both acute and chronic infections in mice (Sheppard et al., 2003; Monack et al., 2004). In addition, it was found that the intracellular growth is limited in cultured dendritic cells and fibroblasts (Cano et al., 2001; Jantsch et al., 2003). These findings are changing our classical view of *Salmonella* as a fast growing intracellular pathogen and suggest that this pathogen may trigger responses directed to reduce the growth rate within the infected cell.

As SPI-2 is clearly necessary for intracellular survival in macrophages and monocytes (Shea et al., 1996; Cirillo et al., 1998), its function is less clear in other cell types. SPI-2 was shown to be dispensible for bacterial survival or proliferation in dendritic cells (Jantsch et al., 2003), but plays a relevant role in the survival of *Salmonella* in fibroblasts (Cano et al., 2001). The role of SPI-2 in the intracellular colonization of epithelial cells still remains a matter of debate (Brumell, 2002; Paesold et al., 2002; Waterman and Holden, 2003). SPI-2 may help *Salmonella* to finely orchestrate the defences in the infected cell to reach a perdurable and low intracellular growth rate (Tierrez and Garcia-del Portillo, 2005).

The recent findings collected in animal tissues suggest that intracellular *Salmonella* may not experience more than two to three doublings along its lifetime within the infected cell. Cells harbouring more than 10 bacteria represent a minor percentage of the infected cells, even in conditions of an acute lethal infection (Tierrez and Garcia-del Portillo, 2005). The data

collected in the fibroblast infection model also raise the question of whether *Salmonella* persistence is established exclusively in macrophages, especially in animals infected with non host-adapted strains.

Interfering with antigen presentation: key to long term persistence?

As *Salmonella* is able to interfere with both endocytotic and exocytotic transport, it was not surprising to find that *Salmonella* is also able to interfere with the antigen presentation in dendritic cells (Cheminay et al., 2005). Mitchell and coworkers (2004) suggested an additional role for SifA. This SPI-2 effector displayed a down-regulating effect on MHC class II expression. Interference with MHC class I antigen presentation has been described (Qimron et al., 2004), but it was not SPI-2 dependent. Although the exact mechanism of this interference is not understood, it was proposed that a *Salmonella* transporter system might prevent peptide loading of phagosomal MHC class I molecules by flooding the vacuole with competing short peptides. Interestingly, the uptake of *Salmonella* coated with anti-LPS antibodies by dendritic cells, does allow the presentation of *Salmonella*-derived antigens in both MHC-I and MHC-II molecules (Tobar et al., 2004). Even though the exact contribution of these mechanisms to the pathogenesis of salmonellosis is not clear, they might provide an interesting view on persistence.

Delayed type of cell death: SPI-2 and the virulence plasmid acting together?

Salmonella enterica mutant strains that are deficient in the SPI-1 T3SS (see above) can still kill macrophages (Van der Velden et al., 2000; Browne et al., 2002). This type of cell death requires prolonged incubation (up to 24 h) and appears to be dependent on the activity of the SPI-2 T3SS (Van der Velden et al., 2000; Monack et al., 2001). Although the identity of a specific SPI-2 effector is not yet linked to this delayed type of cell death, a candidate protein may be the virulence plasmid encoded protein SpvB (Browne et al., 2002). It is possible that SpvB may be delivered into cells by the SPI-2 T3SS, although evidence to the contrary has been reported (Gotoh et al., 2003). Another study indicated that genes of SPI-2 and the spv locus are crucial for the induction of apoptosis and the prolonged bacterial growth in intestinal epithelial cells (Paesold et al., 2002).

Hsu et al. (2004) suggest that the delayed form of *Salmonella*-induced macrophage cell death may require the stimulation of TLR4. It is becoming increasingly clear that stimulation of the innate immune system by PAMPs such as LPS leads to both anti-apoptotic as well as pro-apoptotic pathways (Hueffer and Galan, 2004; Zeng et al., 2006). It is possible that

Salmonella, through the activity of some SPI-2 T3SS effector proteins in conjunction with SpvB may favour the pro-apoptotic responses that follow TLR activation, thereby triggering programmed cell death (Hueffer and Galan, 2004). Nevertheless, also other virulence genes may play a role in apoptosis induction (Valle and Guiney, 2005; Figure 5). Only very recent, a SPI-2 virulence gene has been described which seems to be directly involved in the delayed cytotoxic effect through its deubiquitinating activity (Rytkönen et al., 2007)

Very little is known about the significance of the different types of macrophage cell death triggered by *Salmonella* as well as their relative contribution to pathogenesis or host defence. It is not known if macrophage cell death is triggered by *Salmonella* to counteract host defence mechanisms or whether it constitutes a host response to halt bacterial replication. It is even less clear whether the different types of macrophage cell death represent evolutionary adaptations of the pathogen or the host. When, where and in what type of cells do the different types of *Salmonella*-induced cell death become predominant? More research will help to understand the biological and immunological significance of these different types of cell death and their relative contribution to the pathogenesis of and defense against *Salmonella* infections (Hueffer and Galan, 2004).

3.3. Other SPI encoded virulence genes

SPI-3

The genetic organization and function of SPI-3 are markedly different from those of the previous SPI's. The locus is less than half the size of SPI-1 or SPI-2, no T3SS is encoded and the overall base composition is similar to that of the core genome (Blanc-Potard et al., 1999). It is a complex structure of different virulence factors with unrelated functions. Furthermore, SPI-3 shows alternate structures in different subspecies and serotypes of *Salmonella* (Amavisit et al., 2003).

The most investigated virulence factors are MgtBC, a high affinity magnesium transport system, which is required for adaptation to the nutritional limitations of the phagosome, the intramacrophage survival and growth in low Mg2+ medium (Blanc-Potard and Groisman, 1997).

Recently, MisL, a fibronectin binding protein of the AIDA-I autotransporter family, was shown to be required for the colonization of the alimentary tracts of chicks (Morgan et al., 2004; Dorsey et al., 2005).

MarT might be a regulating protein, because of the similarities to the ToxR protein of *Vibrio cholerae*, although some important differences between both proteins were noticed (Blanc-Potard, et al., 1999). The first gene of the island, *sugR*, encodes a protein that exhibits closest similarity to a putative ATP binding protein encoded in the genome of a clinical isolate of *E. coli* (Lim, 1992). Finally, the *rhuM*, *rmbA*, *fidL*, *slsA*, and *cigR* gene products do not exhibit sequence similarity to proteins with known functions in the sequence databases (Blanc-Potard, et al., 1999).

SPI-4

SPI-4 is a 25 kb segment located at 92 min on the chromosome of *Salmonella* Typhimurium that might constitute a single operon (Wong et al., 1998). Several putative virulence genes are present, for example a putative type 1 secretion system and open reading frames (ORFs) with weak similarity to RTX toxins. A previously identified locus required for survival in murine macrophages is located in SPI-4 and was the basis for the definition of this segment of DNA as a pathogenicity island (Fields *et al.*, 1986). The publication of the complete genome sequence of *Salmonella* Typhimurium strain LT2 refined the sequence of SPI-4 and reorganized the operon into six ORFs (McClelland *et al.* 2001). The regulation of the expression and the time point of action of SPI-4 during infection is still controversial (Ahmer et al., 1999; Detweiler et al., 2003; Morgan et al., 2004; De Keersmaecker et al., 2005)
Recently, SPI-4 was identified as being necessary for efficient gut colonization in the calf but not in the chick model and the ORFs were renamed as *siiA-F*, for *Salmonella* intestinal infection (Morgan et al., 2004). The role of SPI-4 in the colonization of pigs is not known.

SPI-5

SPI-5 is a small locus of 7.6 kb and may thus rather be called a pathogenicity islet (pathogenicity Island < 10 kb). It was originally identified as a locus important for the enteropathogenicity of *Salmonella* Dublin in intestinal loops in calves (Wood et al., 1998). It encodes effector proteins for both the T3SS of SPI-1 and SPI-2 (Knodler et al., 2002). SopB (*Salmonella* Dublin)/SigD (*Salmonella* Typhimurium) and its putative chaperone PipC/SigE are translocated by the SPI-1 T3SS and are under the control of SPI-1 related regulators (see SPI-1). PipB, however, is translocated by the SPI-2 T3SS and localizes to the SCV and Sifs (See SPI-2; Knodler and Steele-Mortimer, 2005). Except for a role in the enteropathogenicity in calf loops, no concrete role has been established for the remaining genes, PipA, PipD and ORFX.

SPI-6 or SCI

A large locus in the genome of *Salmonella* Typhi has been designated SPI-6 and later the *Salmonella enterica* centisome 7 genomic island (SCI) in *Salmonella* Typhimurium (Parkhill et al., 2001; Folkesson et al., 2002). It contains 37 putative proteins, including the *saf* fimbrial operon and the *sinR* transcriptional regulator. The open reading frames *sciA/Z* encode putative proteins with homologies to virulence-associated proteins in a number of gram-negative bacteria such as *Pseudomonas aeruginosa*, *Yersinia pestis* and enterohemorrhagic *Escherichia coli*. The SCI genomic island is restricted to *Salmonella enterica* subspecies I and deletion of the island results in a reduced invasion of cultured cells (Folkesson et al., 2002). The genes within the 59-kb island could have been acquired at various evolutionary intervals (Anjum et al., 2005).

SPI-7 or MPI

SPI-7 is a PAI specific for *Salmonella* Typhi, *Salmonella* Dublin and *Salmonella* Paratyphi C. It is often referred to as the major pathogenicity island (MPI; Zhang et al., 1997) and is transcribed in macrophages infected with *Salmonella* Typhi (Faucher et al., 2005). One important virulence factor in typhoid pathogenesis is the capsular Vi antigen (Pickard et al., 2003).

The insertion of the SPI-1 translocated SopE protein (see SPI-1) in the SPI-7 of *Salmonella* Typhi, must have occurred rather recently, since it is not present in the SPI-7 of *Salmonella* Dublin and *Salmonella* Paratyphi C. Nevertheless, due to its extreme mobility, the SopE phage (and correlated gene) is also present in *Salmonella* serotypes that lack SPI-7. Another putative virulence factor is the type IVB pilus, encoded by the *pil* genes. The extensive similarities between SPI-7 of *Salmonella* and related loci in plant pathogens and *Pseudomonas aeruginosa* has led to the conclusion that the locus might have been acquired through close contact with environmental bacteria (Pickard et al., 2003).

SPI-8

SPI-8 was identified during whole genome sequencing of *Salmonella* Typhi (Parkhill et al., 2001). Putative virulence factors are genes encoding bacteriocins, but no function has been ascribed yet. The region appears to be specific for *Salmonella* Typhi and it was found to be transcribed in macrophages infected with *Salmonella* Typhi (Faucher et al., 2005).

SPI-9

SPI-9 is another locus that was identified in the genome sequence of *Salmonella* Typhi (Parkhill et al., 2001), but is present in different serotypes, including *Salmonella* Typhimurium (Anjum et al., 2005). Putative virulence factors are a type I secretion system and a large RTX-like protein. The function and contribution of RTX proteins to virulence within *Salmonella* are not yet known. However, RTX proteins located adjacent to type 1 secretory systems are commonly involved in virulence (Anjum et al., 2005).

SPI-10

SPI-10 is a large insertion located at the tRNA *leuX* and contains a cryptic bacteriophage and the *sef* fimbrial genes (Parkhill et al., 2001). Consistent with the fact that *sef* fimbriae are restricted to a limited subset of serotypes (Typhi and Enteritidis), determining host specificity, SPI-10 was transcribed in macrophages infected with *Salmonella* Typhi but was not found in the *Salmonella* Typhimurium genome (Faucher et al., 2005).

SPI-11 and SPI-12

Through whole genome sequencing of *Salmonella* Choleraesuis, Chiu and colleagues (2005) discovered 2 pathogenicity islands containing several important genes. SPI-11 contained, among other genes, *sopB*, *envF*, *msgA*, *pagC* and *pagD*. A similar chromosomal region was found in *Salmonella* Typhimurium already 10 years earlier, but the concept of SPI was not introduced at that time (Gunn et al., 1995). Although there is still some discussion, PagC and MsgA proteins have been assigned a role in intramacrophagal survival (Gunn et al., 1995). PagC may also play a role in the serum resistance phenotype (Nishio et al., 2005). SPI-12 only contains 5 genes, including *msgA* and *narP* and contains only 6.3kb. SPI-12 may thus rather be called a pathogenicity islet (pathogenicity Island < 10 kb).

SPI-13 and SPI-14

The study of Shaha and colleagues (2005) describes the application of a signature-tagged mutagenesis system to identify *in vivo* essential genes of *Salmonella* Gallinarum. Twenty presumptive attenuated mutants were identified, including 2 newly discovered PAI: SPI-13 and SPI-14.

SPI-15, SPI-16 and SP-17

Very recent, Vernikos and Parkhill (2006), were able to identify 3 more SPI's in the genome of *Salmonella* Typhi, using a novel computational method, called Interpolated Variable Order Motifs.

HPI

In addition to the PAI, the High Pathogenicity Island (HPI) of *Yersinia*, encoding an iron uptake system, has been described in *Salmonella*, but not yet in *Salmonella* Typhimurium (Carniel, 2001; Oelschlager et al., 2003).

SGI-1

The characterization of multidrug resistant *Salmonella* Typhimurium DT104 strains has led to the identification of the *Salmonella* Genomic Island 1 (SGI-1; Boyd et al, 2001). Strictly spoken, this region is not a pathogenicity island, but it combines antibiotic resistant factors for ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclins. It has also been identified in other serotypes (Levings et al., 2005) and in porcine *Salmonella* isolates (O'Mahony et al., 2005). In contrast to plasmid-borne antibiotic resistance genes, this chromosomal antibiotic resistance cassette seems to be stable in the absence of selection pressure. Variants have been described (Doublet et al., 2004, Levings et al., 2005).

4. Virulence factors of *Salmonella* playing a role in persistency

Porcine carcasses contaminated with *Salmonella* Typhimurium pose significant public health problems. Prolonged faecal shedding of *Salmonella* in pigs contributes to the contamination level of carcasses. Although the mechanism of prolonged faecal shedding is not yet clarified, a few virulence genes have been identified as playing a role in intestinal colonization and persistence of *Salmonella* Typhimurium in mice.

As SPI-1, SPI-2 and possibly other SPI-related virulence genes, are important factors in the intestinal and/or systemic phase of infection, it can be assumed that these genes may also be important for long term survival in the host, as has been extensively discussed in the chapters above. Here, we intend to review virulence factors important for persistency which are not linked to one of the above mentioned SPI.

Fibronectin binding proteins have been implicated to play a role in the pathogenesis of several bacterial diseases. Although their specific role in the pathogenesis is not always

known, they frequently mediate adherence and entry into mammalian cells (Joh *et al.*, 1999; Schwarz-Linek *et al.*, 2004). A sandwich model has been proposed in which fibronectin acts as a molecular bridge between the fibronectin binding proteins on the pathogen and the integrins on the host cells (Joh *et al.*, 1999; Menzies, 2003). In *Salmonella* Typhimurium, two putative fibronectin binding proteins have been identified: MisL, encoded by SPI-3, and ShdA, encoded in the CS54 Island. Both proteins belong to the autotransporter family. Mutants in both genes are attenuated in a mouse model of *Salmonella* Typhimurium intestinal persistence and are impaired in their ability to colonize the gut (Kingsley *et al.*, 2000; Kingsley *et al.*, 2003; Morgan et al., 2004; Dorsey et al., 2005).

Fimbriae encoded by the *lpf*, *fim*, and *agf* (*csg*) operons mediate attachment of *Salmonella* Typhimurium to epithelial cell lines *in vitro* (Baumler et al., 1996). These *in vitro* data suggest that fimbriae may be involved in intestinal colonization. Whole-genome sequencing has identified 13 operons containing fimbrial gene sequences in the *Salmonella* Typhimurium genome, termed *agf* (*csg*), *fim*, *lpf*, *pef*, *bcf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, *saf*, and *stj* (McClelland et al., 2001). The *Salmonella* Typhimurium *fim* operon directs the assembly of type 1 fimbriae and has been implicated in the colonization of the porcine gut (Althouse et al., 2003). Using genetically susceptible mouse lineages (e.g., BALB/c), the *lpf*, *pef*, and *agf* operons have been implicated in colonization of intestinal tissues (Van der Velden, 1998). In a recent report, *Salmonella* Typhimurium strains carrying deletions in *lpf*, *bcf*, *stb*, *stc*, *std*, or *sth* operons were recovered at significantly reduced numbers from the feces of resistant mouse lineages (Weening et al., 2005). It was concluded that these six fimbrial operons (*lpf*, *bcf*, *stb*, *stc*, *std*, and *sth*) contribute to long-term intestinal carriage of *Salmonella* Typhimurium in genetically resistant mice. Interestingly, type I fimbriae were not found to be important for persistency in mice. In addition, thin aggregative fimbriae and *agfD* regulated O-antigens are believed to play a role in environmental survival (Gibson et al., 2006; White et al., 2006).

Isocitrate lyase, encoded by the *aceA* gene, is required for fatty acid utilization via the glyoxylate shunt. Even though isocitrate lyase is essential for *Salmonella* persistence during chronic infection, it is dispensable for acute lethal infection in mice. This may mean that substrate availability in the phagosome evolves over time, with increasing fatty acid dependence during chronic infection (Fang et al., 2005).

In a study aimed at identifying *Salmonella* genes that demonstrated increased expression within the intracellular environment (Valdivia and Falkow, 1997), a PhoP-regulated gene designated *mig-14*, was identified. Recently, it was shown that *mig-14* contributes to long-term persistence of *Salmonella* in the spleen and mesenteric lymph nodes of chronically

infected mice, even though *mig-14* mutant bacteria can colonize the liver and spleen of mice identically to wild-type bacteria until 5 days after inoculation (Valdivia et al., 2000; Brodsky et al., 2005.).

Numerous other genes have been identified contributing to long term survival in various hosts, but most of these genes are so-called housekeeping genes and are also necessary for *in vitro* survival or for the acute phase of infection (e.g. *aroA*, *purA*, …). Strictly spoken, these genes do not specifically contribute to long term persistency.

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SCIENTIFIC AIMS OF THE STUDY

According to preliminary data, *Salmonella* Typhimurium has become the most important serotype causing salmonellosis in humans in Belgium. Infections with this serotype are mainly associated with the consumption of pork and swine persistently infected with *Salmonella* Typhimurium pose an important public health hazard. When we started our studies, the mechanisms by which *Salmonella* Typhimurium colonizes and persists in pigs were largely unknown. The general aim of this thesis was, therefore, to obtain better insights in these mechanisms. A thorough knowledge of how this pathogen interacts with the porcine host should indeed form the basis for the development and evaluation of efficient monitoring programmes and control measures.

The specific aims were:

1. The selection of a *Salmonella* Typhimurium strain that is able to persistently infect pigs.
2. The development of *in vitro* and *in vivo* models to investigate the pathogenesis of *Salmonella* Typhimurium infections in pigs.
3. To determine the role of genes situated on *Salmonella* Pathogenicity Islands 1 and 2 as well as of the fibronectin binding protein ShdA in the colonization and persistence of *Salmonella* Typhimurium in pigs.

EXPERIMENTAL STUDIES

CHAPTER 1:

Choice of strain used in *Salmonella* Typhimurium pathogenesis research: does it matter?

ABSTRACT

The course of a *Salmonella* infection depends on the relation host species – *Salmonella* strain. Increasing numbers of virulence factors have recently been shown to be host-specific. In addition, numerous genetic transfers between strains of *Salmonella* result in a mosaic of strains harbouring or lacking different virulence factors. Nevertheless, the same standard strains are often used for pathogenesis research in different animal species. In this study we wanted to characterize a standard strain of *Salmonella* Typhimurium often used for research in mice (NCTC 12023) and a porcine field strain (112910a) in a mouse and in a porcine *in vivo* infection model. Seven week old BALB/c mice were intragastrically inoculated and 4-week old piglets were orally inoculated with these *Salmonella* Typhimurium strains. The NCTC 12023 strain was found in significantly higher numbers in the internal organs than the porcine 112910a strain in the mouse at 4 days post inoculation (pi). In contrast, faecal excretion by piglets inoculated with the NCTC 12023 strain was significantly ($p < 0.05$) lower at days 12, 20, 22, 26 and 28 pi compared to that of piglets inoculated with the 112910a strain. At day 28 pi, the pigs inoculated with strain 112910a were infected to a significantly higher extent in the ileum, the contents of the ileum, the caecum and the ileocaecal lymph nodes.

In conclusion, it was shown that different *Salmonella* Typhimurium strains can follow a distinct course of infection in different host species. These findings stress the importance of the appropriate choice of strain in pathogenesis research.

KEYWORDS

Salmonella Typhimurium – pig – mouse – host adaptation

INTRODUCTION

The last decennia, the increase in research on the pathogenesis of salmonellosis resulted in the identification of many virulence genes in *Salmonella* Typhimurium, located mainly in pathogenicity islands (Kingsley and Bäumler, 2002). Research on the pathogenesis of *Salmonella* Typhimurium infections has been conducted mostly in BALB/c mice using a limited number of *Salmonella* Typhimurium standard laboratory strains. A *Salmonella* Typhimurium infection in mice resembles a *Salmonella* Typhi infection in humans, so this typhoid model has been used extensively for pathogenesis studies. In pigs, however, a *Salmonella* Typhimurium infection predominantly is subclinical, but sometimes results in a self-limiting enterocolitis (Fedorka-Cray et al., 2000). Since the course of a *Salmonella* Typhimurium infection in mice (lethal systemic disease) is very different from that in pigs (subclinical local infection), the BALB/c mouse model is not biologically relevant to study the pathogenesis of *Salmonella* Typhimurium infections in pigs or even in man.

The course of a *Salmonella* infection depends on the relation host species – *Salmonella* strain. Increasing numbers of virulence factors have been shown to be host-specific (Tsolis et al., 1999; Pasmans et al., 2003; Morgan et al., 2004). Numerous genetic transfers may take place between different serotypes or strains of *Salmonella* (Zhang et al., 2002; Boyd et al., 2003; Barth et al., 2005), even between different strains of a clonal serotype like *Salmonella* Typhi (Porwollik et al., 2003), resulting in a mosaic of strains harbouring or lacking different virulence factors. This means that the choice of the strain may be of great importance in pathogenesis research.

It was the aim of these studies to characterize the behaviour of a standard laboratory strain of *Salmonella* Typhimurium often used in mice and a porcine field strain of *Salmonella* Typhimurium in both a mouse and a pig *in vivo* model.

MATERIALS AND METHODS

All experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Bacterial strains and growth conditions

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) strain 112910a was isolated from a pig stool sample. A genetically identical strain was also

isolated from pig carcasses originating from the same farm at the slaughterhouse (ILVO, Melle, Belgium). This strain was used as the porcine wild type field strain. The *Salmonella* Typhimurium NCTC 12023 strain was used as a standard laboratory strain (Hensel, 1995; Unsworth et al, 2004).

For the oral inoculation of mice and pigs, the bacteria were grown in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) for 6 h at 37°C. The bacteria were washed three times in phosphate buffered saline (PBS, 2000 x *g* for 10 min at 4°C). The number of viable *Salmonella* bacteria/ml was determined by plating tenfold dilutions on Brilliant Green Agar (BGA; Oxoid, Basingstoke, UK).

Experimental infection of mice

Seven-week-old male BALB/c mice were inoculated using a standard protocol (Cirillo et al., 1998). In short, all feed was withdrawn 12 h before inoculation. Eight mice per group were anaesthetized with isoflurane and subsequently intragastrically inoculated with 5 x 10^8 cfu of one of both *Salmonella* Typhimurium strains in 200 µl of PBS. On day 1 and day 4 post inoculation, 4 animals of each group were killed. For each animal, caecum, spleen and liver were removed for bacteriological analysis. All mice were kept in filter-topped cages with ad libitum food and water.

Experimental infection of piglets

Experimental infections were performed in 4-week-old piglets (commercial closed line based on Landrace), that were negative for *Salmonella* at faecal sampling. They arrived at the facility 14 days before they were inoculated and were divided at random into 3 groups: 2 groups of 10 pigs each (groups 1 and 2) and one negative control group of 3 pigs. All three groups were housed in separate isolation units at 25°C under natural day-night rhythm with ad libitum access to feed and water. Pigs were penned in pairs for the first 5 days and individually for the remainder of the experiment.

The animals of groups 1 and 2 were orally inoculated with 10^7 cfu of one of both *Salmonella* Typhimurium strains in 2 ml HBSS; the negative control group was sham-inoculated with 2 ml PBS.

For the first two days post-inoculation (pi) the rectal temperature was measured twice a day and the clinical condition of the pigs was monitored (anorexia, lethargy, diarrhea). From day 3 till day 7 pi this was performed once a day, and subsequently once every other day until day 28 pi. For the first 5 days, each pig was given a score to describe the consistency of the

faeces. Normal faeces were given score 0, mild diarrhea (loose stools) score 1 and severe diarrhea (watery) score 2.

Fresh faecal samples were taken from each pig on days 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 pi for bacteriological analysis.

On days 5 and 28 pi, 5 pigs of each *Salmonella* inoculated group and 3 control pigs were euthanized. Samples of tonsils, mandibular lymph nodes, lung, heart, liver, spleen, kidney, ileocaecal lymph nodes, jejunum, ileum, caecum and contents of jejunum, ileum and caecum were taken for bacteriological analysis.

Bacteriological analysis

From all samples, 10% (w/v) suspensions were made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was homogenized with a stomacher. The homogenized samples were examined for the presence of the *Salmonella* by plating tenfold dilutions on BGA. If negative at direct plating, the samples were pre-enriched overnight in BPW at 37°C, enriched overnight at 37°C in tetrathionate broth and then plated on BGA. Samples that were negative after direct plating but positive after enrichment, were presumed to contain 83 cfu/g. Samples that remained negative were presumed to have 0 cfu/g.

Statistical analysis

Statistical analysis was performed using a Student's T-test on all results. Differences with a P value ≤ 0.05 were considered significant.

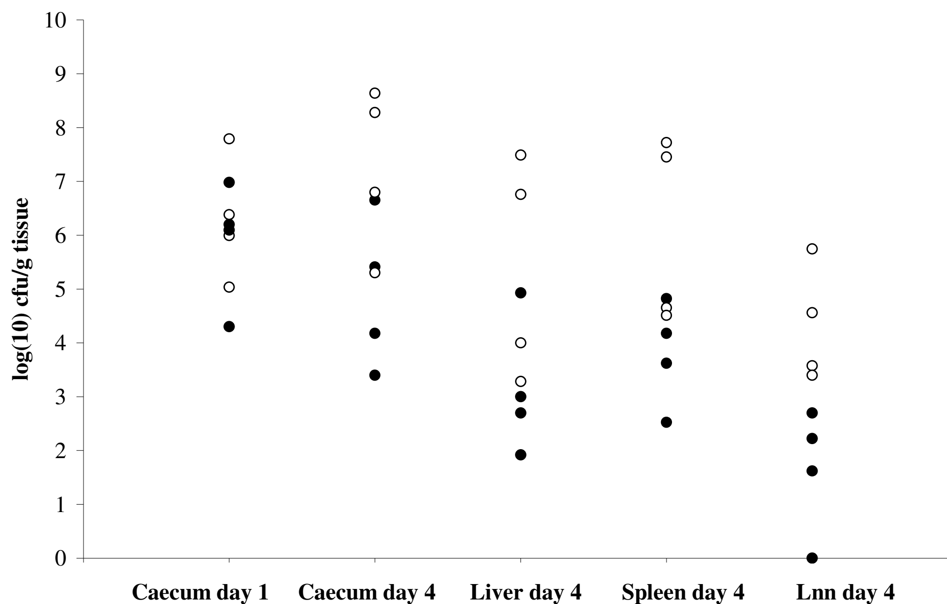
RESULTS

Experimental infection of mice

The results are summarized in Fig. 1. At day 1 pi, the animals of both groups were infected to a similar extent in all sampled organs ($p > 0.05$).

At day 4 pi, 2 mice inoculated with *Salmonella* Typhimurium strain NCTC 12023 succumbed and all remaining mice were euthanized for sampling. At that time, the animals inoculated with strain NCTC 12023 were infected to a significantly higher extent in the spleen, caecum and GALT ($p < 0.05$), and there was a trend towards higher colonization of the liver than in animals inoculated with *Salmonella* Typhimurium strains 112910a ($p = 0.07$).

Figure 1: Dot plot representing the bacterial load in caecum, liver, spleen and mesenterial lymph nodes (Lnn) of BALB/c mice, 1 and 4 days after intragastrical inoculation with +/- 10^8 cfu of either *Salmonella* Typhimurium strain 112910a (●) or strain NCTC 12023 (○).

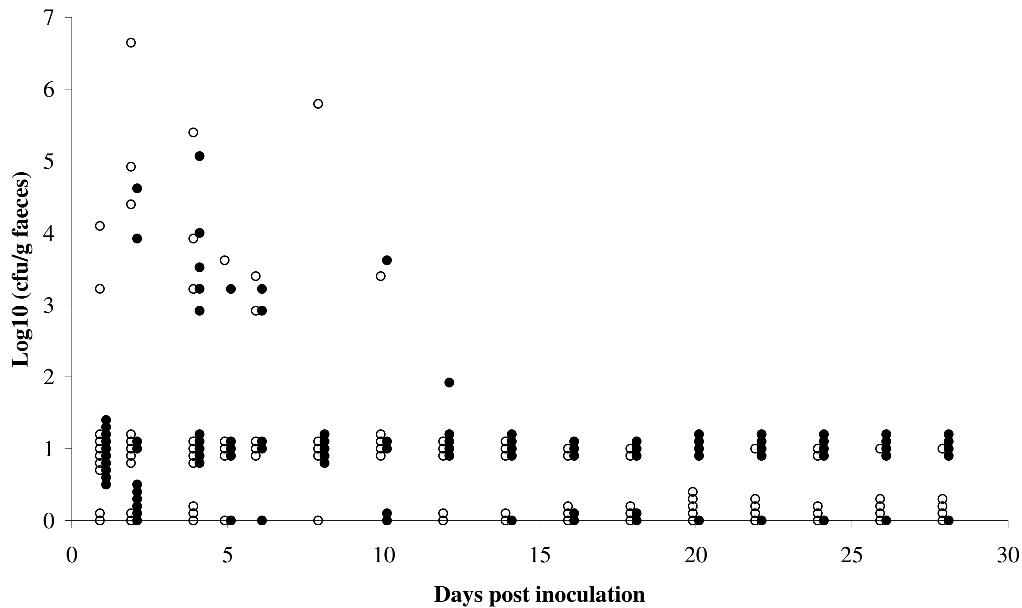


Experimental infection of piglets

Sham inoculated control piglets did not develop disease signs and *Salmonella* was not isolated from any of the samples taken from these animals throughout the experiment.
The course of infection of the piglets inoculated with the porcine field strain was similar to that observed in several preliminary experiments. The animals had a slight increase in body temperature during the first few days after inoculation and some also had mild diarrhoea. At days 3 to 5 pi, a peak in *Salmonella* shedding was noticed in both groups. At day 5 pi, the animals of both groups were infected to the same extent in most of the internal organs (Table 1). The lungs of the animals inoculated with strain NCTC 12023 were significantly more infected, while the ileum, the contents of the ileum and the ileocecal lymph nodes of the animals inoculated with strain 112910a were colonized to a significantly higher extent ($p < 0.05$).

Approximately two weeks after inoculation, the piglets intermittently shed *Salmonella* at enrichment level. Excretion by animals inoculated with the NCTC 12023 strain was significantly ($p < 0.05$) lower from 20 days pi onwards (Fig. 2). At day 28 pi, the animals inoculated with strain 112910a were infected to a significantly higher extent in the ileum, the contents of the ileum, the caecum and the ileocaecal lymph nodes (Table 1).

Figure 2: Dot plot representing the faecal excretion of *Salmonella* Typhimurium after oral inoculation of pigs with +/- 10^7 cfu of either the 112910a strain (●) or the NCTC 12023 strain (○).



DISCUSSION

In these experiments, an often used standard laboratory *Salmonella* Typhimurium strain was found to be markedly more virulent in BALB/c mice, compared to the porcine field strain, while the porcine field strain seemed to be more efficient in inducing a persistent infection in pigs. This finding suggests a degree of adaptation of the *Salmonella* Typhimurium strains to mice and pigs, respectively. The adaptation of the porcine field strain to pigs is not as obvious as the well known host-restriction in other serotypes (eg Typhi in men), but was expressed as a more efficient colonization, predilection for specific organs and the induction of a persistent infection.

Even though *Salmonella* Typhimurium is considered a serotype with a broad host range, several reports have been made of some strains being more adapted to one host species. The most obvious one is the adaptation of phage types 2 and 99 to pigeons. These phage types are highly cytotoxic for pigeon macrophages and induce severe clinical symptoms, while other phage types do not (Pasmans et al., 2003). However, until now, attempts to distinguish the genomes of *Salmonella* Typhimurium pigeon isolates from those of other *Salmonella* Typhimurium strains were not successful (Andrews-Polymenis et al., 2004; Anjum et al., 2005). More subtle variants of host adapted *Salmonella* Typhimurium strains are found in ducks (DT8 and DT46), wild birds (DT40) (Rabsch et al., 2002) and epidemic cattle-

associated strains carrying the *sopE* gene (Mirold et al., 1999). In addition, *in vitro* adaptation of *Salmonella* Typhimurium to mice has also been described (Nilsson, 2004).

	Tissue	*Salmonella* Typhimurium 112910a		*Salmonella* Typhimurium NCTC 12023	
		Frequency	Log_{10} cfu g^{-1} ± stdev	Frequency	Log_{10} cfu g^{-1} ± stdev
Day 5 pi	Mand. lnn.	4/5	1.56 ± 1.11	2/5	0.89 ± 1.50
	Tonsil	3/5	1.33 ± 1.57	4/5	1.51 ± 1.36
	Lung	1/5	0.2 ± 0.44	4/5	0.8 ± 0.45 •
	Heart	2/5	0.4 ± 0.55	2/5	0.4 ± 0.55
	Liver	4/5	0.8 ± 0.45	3/5	0.6 ± 0.55
	Spleen	2/5	0.58 ± 0.86	1/5	0.2 ± 0.45
	Kidney	2/5	0.78 ± 1.27	2/5	0.4 ± 0.55
	Ileocecal lnn.	5/5	3.69 ± 0.62 *	5/5	2.41± 0.85
	Jejunum	5/5	2.97 ± 1.97	4/5	1.64 ± 2.05
	Ileum	5/5	4.92 ± 0.52 *	4/5	2.70 ± 1.67
	Cecum	5/5	3.85 ± 0.68	5/5	3.50 ± 0.99
	Content jejunum	5/5	2.64 ± 1.70	2/5	0.96 ± 1.66
	Content ileum	5/5	4.40 ± 0.73 *	4/5	1.41 ± 1.53
	Content cecum	4/5	2.74 ± 1.56	5/5	1.97 ± 1.37
Day 28 pi	Mand. lnn.	0/5	0 ± 0	0/5	0 ± 0
	Tonsil	2/5	0.76 ± 1.23	2/5	0.98 ± 1.19
	Lung	0/5	0 ± 0	0/5	0 ± 0
	Heart	0/5	0 ± 0	0/5	0 ± 0
	Liver	0/5	0 ± 0	0/5	0 ± 0
	Spleen	0/5	0 ± 0	0/5	0 ± 0
	Kidney	0/5	0 ± 0	0/5	0 ± 0
	Ileocecal lnn.	4/5	1.32 ± 1.34 *	1/5	0.2 ± 0.45
	Jejunum	2/5	0.4 ± 0.55	0/5	0 ± 0
	Ileum	5/5	1.72 ± 1.61 *	2/5	0.4 ± 0.55
	Cecum	5/5	1.45 ± 1.01 *	2/5	0.4 ± 0.55
	Content jejunum	0/5	0 ± 0	0/5	0 ± 0
	Content ileum	5/5	1.58 ± 1.29 *	2/5	0.4 ± 0.55
	Content cecum	5/5	1 ± 0	3/5	0.6 ± 0.55

Table 1: Post mortem bacteriological findings at days 5 and 28 post inoculation of piglets orally inoculated with 10^7 cfu of *Salmonella* Typhimurium strains 112910a or NCTC 12023. The number of positive tissues in relation to the total number of tissues (frequency) and the average number of cfu (log_{10}) ± stdev per gram tissue are shown. Samples only positive after enrichment were rendered a value of 83 cfu g^{-1}. A significantly ($p \leq 0.05$) higher number of bacteria in the organs of piglets inoculated with strain 112910a is indicated with a "*".A significantly ($p \leq 0.05$) higher number of bacteria in the organs of piglets inoculated with strain NCTC 12023 is indicated with a "•".

The increased virulence of the laboratory strain might be explained by the presence or absence of one or more virulence genes (for example mobile genetic elements, including phages, plasmids, and plasmid-like and transposable elements; Kang et al., 2006), or altered expression levels of virulence genes (for example SPI). This was not investigated in the present study, but could be accomplished by using a full genomic indexing of both strains with the micro-array technique on DNA and RNA level.

The two models used in this study represent two distinct strategies of *Salmonella* to colonize a host and to spread to other susceptible hosts. In the BALB/c mouse model, *Salmonella* Typhimurium quickly overgrows the immune system and is spread brusquely by the excretion of high numbers of bacteria, often shortly before death occurs. In swine, *Salmonella* normally "sneaks" in, causing no apparent symptoms and inducing no systemic disease. The bacteria are spread during a longer period, but in smaller numbers. This also means that virulence factors causing systemic disease may differ from those causing a persistent infection and the presence/absence of these genes may differ between different strains of *Salmonella* Typhimurium.

In conclusion, there is no such thing as a single pathogenesis of *Salmonella* infections in general. Both the infected host species and the infecting serotype and even strain influence the course of an infection. This also means that the importance of certain virulence factors of *Salmonella* and the interaction with the immune system of different host species can differ largely. Care should be taken choosing the optimal animal model and relevant strain before pathogenesis studies are conducted.

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CHAPTER 2:

Porcine *in vitro* and *in vivo* models to assess the virulence of *Salmonella enterica* serovar Typhimurium for pigs

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ABSTRACT

Salmonella Typhimurium infections in pigs pose an important human health hazard. One promising control measure is the development of live attenuated vaccine strains using defined knock-out mutants. Preferably, screening of candidate knock-out vaccine strains for attenuation should first be done in models allowing testing of a large number of strains. Thereafter, a limited number of selected strains should be further characterized in an experimental infection model in pigs. It was the aim of the present study to develop such models.

The invasive and proliferative characteristics of *Salmonella* Typhimurium were assessed in both a non-polarized and a polarized porcine intestinal epithelial cell line. Neutrophils obtained from porcine blood were used to study the capacity of *Salmonella* to withstand killing by these phagocytes. The ability to induce an intestinal inflammatory response was investigated in an intestinal loop model. The systemic phase of infection was mimicked by studying the uptake and intracellular survival of *Salmonella* Typhimurium in porcine pulmonary alveolar macrophages and peripheral blood monocytes. These models should allow screening for attenuated strains. For further characterization, an experimental infection model was established, providing extensive data on the course of an oral infection and the optimal time points for evaluation of colonization (day 3-5 pi) and persistency (days 21-28 pi) in pigs.

In conclusion, screening for virulence of *Salmonella* Typhimurium strains with subsequent confirmation for a subset of strains in a well defined experimental infection model would significantly reduce the number of experimental pigs required.

KEY WORDS

Salmonella Typhimurium – pig – screening – models – virulence

INTRODUCTION

Salmonella enterica serovar Typhimurium infections in pigs cause considerable economic losses and public health problems, as pigs are a reservoir of infection for humans (Berends *et al.*, 1996). Until today, measures to control *Salmonella* infections in pigs are often of hygienic origin with no guarantee for a satisfactory result. One promising measure in the combat against *Salmonella* is the development of live attenuated vaccine strains using defined knock-out mutants (Methner et al., 2004; Kirkpatrick et al; 2006; Mohler et al., 2006). Previously, virulence of *Salmonella* strains in pigs was assessed using *in vivo* infection models (Wood *et al.*, 1991; Wood and Rose, 1992; Isaacson and Kinsel, 1992; Fedorka-Cray *et al.*, 1995; Althouse *et al.*, 2003). However, screening candidate knock-out vaccine strains for attenuation, using solely *in vivo* infection models is expensive, time consuming and requires high numbers of experimental animals.

Most of our knowledge concerning interactions of *Salmonella* with the host is derived from experiments in mice or murine cell types. Even though many tools are available for studying *Salmonella* pathogenesis in murine models, these results often cannot be reproduced in other hosts, compromising extrapolation to human and pig disease (Schwartz, 1999).

In vitro studies using primary isolated cells or cell lines of porcine origin are rare (Donné *et al.*, 2005; Boyen *et al.*, 2006a; Schierack *et al.*, 2006). Comparative data from these alternative methods to one another and to those of the *in vivo* infection model are missing. In times of growing animal welfare awareness, there is need for experimental models that replace, reduce or refine the use of experimental animals (Russell and Burch, 1959).

It was the aim of this study to evaluate different *in vitro* and *in vivo* models for the investigation of host-pathogen interactions and virulence of *Salmonella* Typhimurium in pigs. The intestinal phase of infection was reproduced using an invasion and proliferation assay in two porcine intestinal epithelial cell lines, a bacterial killing assay with neutrophils and an intestinal loop model in pigs. For modelling the systemic phase of the infection, a bacterial killing assay with porcine alveolar macrophages and peripheral blood monocytes was used. Finally, the course of a *Salmonella* Typhimurium infection, different clinical score systems and the effect of different infection doses were studied in an experimental infection model in pigs.

MATERIALS AND METHODS

Experimental animals

For the *in vitro* experiments with pulmonary alveolar macrophages (PAM), a total of 3 male crossbred pigs, aged 3-4 weeks, were used. For the *in vitro* experiments with polymorphonuclear cells (PMN) and peripheral blood monocytes (PBM) 1 female hybrid pig, aged 20 weeks, was used. The intestinal loop model was performed on 2 female 6-week-old, farm reared Landrace/Large White cross male piglets. For the *in vivo* experiments, a total of 38 piglets of mixed sexes (commercial closed line based on Belgian landrace), aged 4 weeks were used. All the pigs used in the experiments came from farms with no history of *Salmonella* infections and were negative for *Salmonella* at faecal sampling. Pigs arrived at the facility 7 days before infection as an acclimatization period. They were kept in pairs in isolation units at 25°C under natural day-night rhythm with *ad libitum* access to feed and water in HEPA-filtered stables. Piglets were provided with a rubber ball as environmental enrichment.

The experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Strains and growing conditions

Salmonella serovar Typhimurium strain 112910a, phage type 120/ad, isolated from a pig without clinical signs of salmonellosis, was used in all experiments. The bacteria were stored at –70 °C. The inocula for the intestinal loop model were prepared according to the temperature shift method for *Salmonella.* Cultures in Luria-Bertani broth (LB; Sigma-Aldrich, Steinheim, Germany) were shaken at 130 rpm for 24 h at 25 °C. After diluting twofold and adjusting the OD_{600} nm, the cells were incubated for 2 h at 37 °C, with shaking at 130 rpm. Afterwards, the OD_{600} nm was adjusted with fresh LB once again so that equal densities were obtained and 3 syringes of 5 ml were filled with the strain. The actual number of bacteria/ml was assessed by plating serial dilutions on MacConkey agar (Oxoid, Hampshire, UK) plates.

For all other experiments, the bacteria were grown in brain heart infusion broth (BHI; Oxoid) for 6 h at 37 °C without shaking and then washed twice in phosphate buffered saline (PBS). The number of viable microorganisms was determined by plating tenfold dilutions on Brilliant Green Agar (BGA; Oxoid).

Interactions of *Salmonella* Typhimurium with intestinal epithelial cells

The porcine epitheloid intestinal cell line IPI-2I was derived from the ileum of an adult boar (Kaeffer *et al*., 1993). The polarized porcine epithelial intestinal cell line IPEC-J2 is derived from jejunal epithelia isolated from a neonatal piglet (Rhoads *et al*., 1994; Schierack *et al*., 2006).

The IPI-2I cells were cultured in RPMI (Gibco, Life Technologies, Paisley, Scotland) containing 10 % (v/v) fetal calf serum (FCS; Hyclone, Cramlington, England), 2 mM L-glutamine (Gibco, Life Technologies), 1 mM sodium pyruvate (Gibco, Life Technologies), 100 units nystatin ml^{-1} (Gibco, Life Technologies), 100 units penicillin ml^{-1} and 100 µg streptomycin ml^{-1} (penicillin/streptomycin, Gibco, Life Technologies). The IPEC-J2 cells were maintained on 50% DMEM – 50 % (v/v) F12 medium (Gibco, Life Technologies) with 5 % (v/v) FCS, 1 % (v/v) insulin/transferrin/Na-selenite media supplement (Gibco, Life Technologies) and antibiotics as described above. IPI-2I and IPEC-J2 cells were seeded in 24 well plates at a density of approximately 10^5 cells per well and were allowed to grow to confluency for at least 7 days. These wells were inoculated with *Salmonella* at a multiplicity of infection (MOI) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at 365 x g for 5 min. After 25 min incubation at 37 °C under 5 % CO_2, the wells were washed and fresh medium supplemented with 50 µg ml^{-1} gentamicin (Gibco, Life Technologies, Paisley, Scotland) was added. After an additional 60 min incubation at 37 °C under 5 % CO_2, the wells were washed three times.

To assess invasion, the cells were lysed with 0.25% deoxycholate (Sigma-Aldrich) 90 min after inoculation and 10-fold dilutions were plated on BGA plates.

To assess intracellular growth, the medium containing 50 µg ml^{-1} gentamicin was replaced after the 60 min incubation time with fresh medium supplemented with 15 µg ml^{-1} gentamicin and the number of viable bacteria was assessed 24 hours after inoculation as described above.

All measurements were performed in triplicate and the experiment was carried on at least two occasions. The results were analysed by a paired Student's t-test. A P - value of < 0.05 was considered significant.

Interactions of *Salmonella* Typhimurium with porcine mononuclear cells

The PAM were collected by broncho-alveolar washes and the PBM by ficoll-paque density gradient centrifugation, as described previously (Dom *et al*., 1992a; Donné *et al*., 2005). The cells were maintained in RPMI supplemented with 10 % (v/v) FCS, 0.3 mg ml^{-1}

L-glutamine, 1 mM Na-pyruvate, 1 % (v/v) non-essential amino acids, 10 U ml^{-1} heparin, 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 0.1 mg ml^{-1} kanamycin. After overnight incubation in 96 well plates and washing, the cells were inoculated with *Salmonella* Typhimurium in RPMI at a MOI of 10. The determination of invasion and intracellular replication of *Salmonella* Typhimurium, was carried out using a gentamicin protection assay as described for the intestinal epithelial cell lines. Cells were lysed at 90 min, 3 h or 7 h post inoculation, using 0.5 % (v/v) triton X-100 (Sigma-Aldrich). All measurements were performed in triplicate and the experiment was carried out on 3 occasions. The results were analysed by a paired Student's t-test. A P - value of < 0.05 was considered significant.

Interactions of *Salmonella* Typhimurium with neutrophils

Neutrophils were isolated from heparinised (1500 IU ml^{-1}) blood using discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation according to Dom *et al.* (1992b).

The determination of the percentage of salmonellae killed by PMN was carried out according to Barrio *et al.* (2000) with some modifications. The assay was run in Eppendorf® tubes (Netheler-Hinz GmbH, Hamburg, Germany) in a final volume of 500 µl with the following composition: 400 µl HBSS containing 2.5 x 10^6 ml^{-1} PMN and 100 µl HBSS containing 10^7 cfu ml^{-1} bacteria (mixture sample). Control samples consisted of 400 µl HBSS and 100 µl HBSS with 10^7 cfu ml^{-1} bacteria. The tubes were rotated end-over-end at 37°C for 60 min. A sample (25 µl) was taken at 0 and 60 min pi. The PMN in the mixture samples were lysed with 0.5 % (v/v) triton X-100. The number of bacteria in the mixture and control samples was determined by plating tenfold dilutions on BGA plates. The percentages of killed bacteria were calculated using the formula described by Barrio *et al.* (2000).

Intestinal loop model

Pigs were starved overnight before surgery to empty the intestines. Piglets were sedated for intratracheal intubation using 1 ml per 20 kg azaperone (Stressnil, Janssen Pharmaceutics, Beerse Belgium). Induction of anaesthesia was performed by slow intraveneous injection of 1 ml per 4 kg alphaxolone/alphadolone (Saffan, Schering-Plough Animal Health, Middlesex, UK). Anaesthesia was maintained with 1-3 % isoflurane in conjunction with 1 % pure oxygen, using a closed circuit with CO_2 and isoflurane absorbers. The piglets were kept warm and

heart and respiratory rate were monitored. A continuous ringer-glucose drip was inserted into the dorsal ear vain.

The porcine ligated loop model was carried out as follows. Commencing at the mid ileum, loops of 7-8 cm were ligated using surgical silk, separated by 1 cm spacers. The lumen of the ileum was flushed with 0.9 % (w/v) NaCl before the construction of the loops to remove the intestinal contents. The loops were injected with 1-2 x 10^9 cfu of *Salmonella* Typhimurium. Approximately 10 ml of blood was removed from the piglets to isolate the PMN. The isolated PMN were labelled with ^{111}Indium oxinate and re-injected intravenously. The influx of PMN in the intestinal wall and in the lumen, as assessed by the counts per minute (cpm) emitted from ^{111}Indium-labelled PMN within the loop, was recorded 12 h after injection of the loops using a gamma counter. The results were analysed by a paired Student's t-test. A *P* - value of < 0.05 was considered significant.

Course of a *Salmonella* Typhimurium infection in pigs

Twenty one pigs were randomly divided into 7 groups of 3 piglets, which were orally inoculated with 10^7 cfu *Salmonella* Typhimurium in 2 ml PBS, by syringe. One group of 3 pigs was sham-inoculated orally with 2 ml PBS and served as negative controls. The rectal temperature and clinical condition (anorexia, lethargy, diarrhoea) was monitored and faecal samples were taken directly from the rectum from each pig on several days post inoculation. On day 1, 2, 5, 9, 14, 21 and 28 pi, 3 pigs were euthanized and necropsied. Samples of various organs were taken for bacteriological analysis.

All the samples were stored at -70 °C until further examination. The samples were thawed and weighed, 10 % (w/v) suspensions were prepared in buffered peptone water (BPW; Oxoid, Hampshire, UK) after which the material was homogenized. The homogenized samples were examined for the number of *Salmonella* bacteria per gram by plating tenfold dilutions on BGA. If negative at direct plating, the samples were pre-enriched overnight at 37 °C in BPW, enriched overnight at 37 °C in tetrathionate broth and then plated on BGA. Samples that were negative after direct plating but positive after enrichment were presumed to have 10 cfu g^{-1}. Samples that remained negative were presumed to contain 0 cfu g^{-1}.

Dose-response study

Fourteen pigs were randomly divided into 3 groups of 4 pigs, which were orally inoculated with 10^5 cfu (group 1), 10^7 cfu (group 2) or 10^9 cfu (group3) of *Salmonella*

Typhimurium in 2 ml PBS. The animals were weighed before the inoculation and just before euthanasia. Their rectal temperature and clinical condition (anorexia, lethargy, diarrhoea) was monitored twice daily the first two days pi and daily at day 3 and day 5 pi. On day 5, the pigs were euthanized and necropsied. Samples were taken for bacteriological analysis and bacterial counts in the tissues and faeces were determined as described above.

Results of bacterial analysis and loss of weight for the different groups were statistically analysed using a Kruskal-Wallis test and significant differences ($P < 0.05$), were compared in a Mann-Whitney test. A P - value of < 0.05 was considered significant.

RESULTS

Salmonella Typhimurim invades and replicates in intestinal epithelial cell lines

The results of the invasion and proliferation tests in both epithelial intestinal cell lines are summarized in Figure 1A. Invasion was significantly ($P < 0.05$) higher in the polarized epithelial cell line IPEC-J2 compared to the IPI-2I cell line. Intracellular replication rates were not significantly different.

Salmonella Typhimurium replicates in PAM, but not in PBM

PAM and PBM were invaded to a similar extent. The bacteria replicated quickly in the PAM, but not in the PBM at 7 hours after inoculation. At 24 h pi, the viability of PAM and PBM did not allow accurate titration of intracellular bacteria. The results of the invasion and proliferation tests are summarized in Figure 1B.

Salmonella Typhimurium induces PMN influx and is killed rapidly by PMN

The results obtained in the intestinal loop model are shown in Figure 2. *Salmonella* Typhimurium induced massive attraction of PMN in both the lamina propria and the lumen of the gut. The loops contained a rather small amount of slimy, pus-like substance. The number of PMN was significantly ($P < 0.05$) higher in the loops inoculated with *Salmonella* compared to the control loops. The intra- and interexperimental variation between loops was relatively low.

In the bacterial killing assay with PMN, 60 minutes after addition of *Salmonella* Typhimurium, 45 % (± 11 % stdev) of the bacteria was killed by the PMN.

Figure 1: The invasiveness and intracellular replication of *Salmonella* Typhimurium in intestinal epithelial cells (A) and mononuclear cells (B). In figure A, the results obtained in the polarized cell line IPEC-J2 are shown with the open circles and a full line, the results obtained in the non polarized cell line IPI-2I are shown with the black circles and the dotted line. In figure B, the results obtained in the macrophages are shown with the open circles and a full line, the results obtained in the monocytes are shown with the black circles and the dotted line. The log values of the number of gentamicin-protected bacteria are shown. The results represent the means of at least 2 independent experiments conducted in triplicate ± standard error of the means. An asterisk refers to a significant ($P < 0.05$) difference.

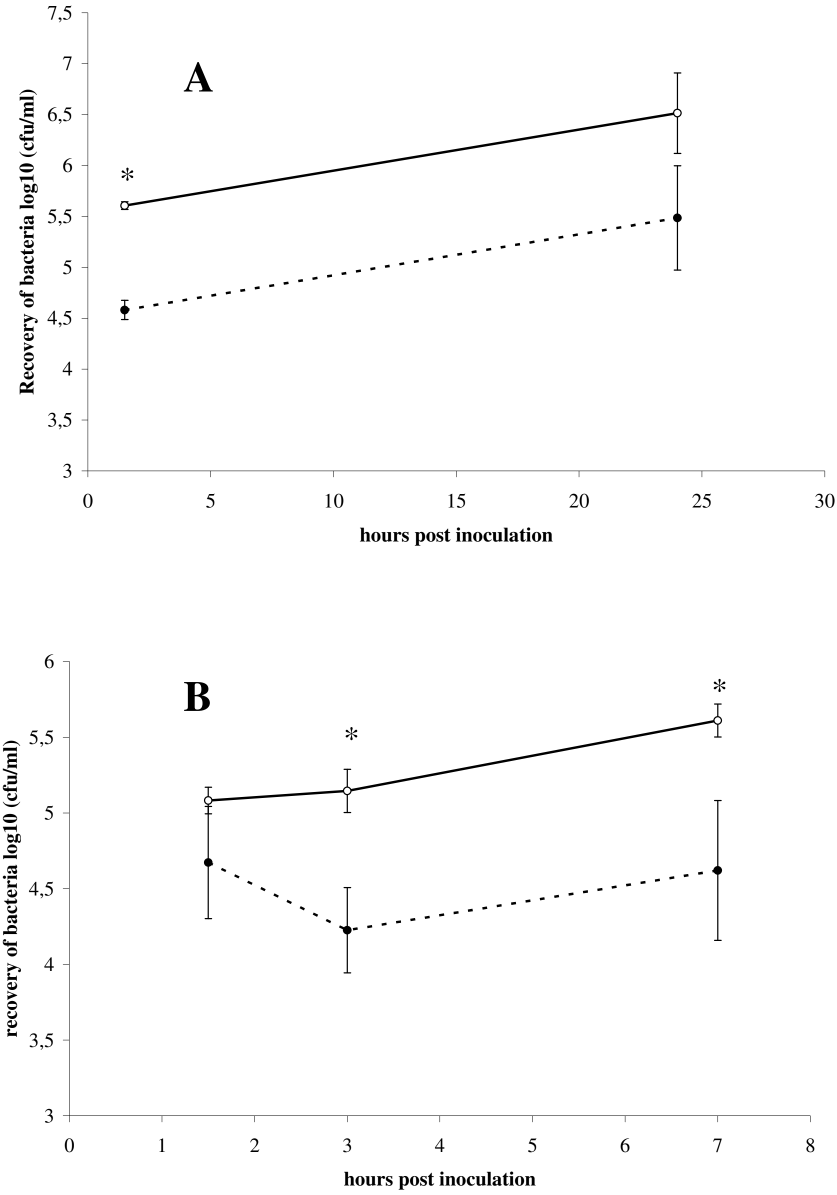
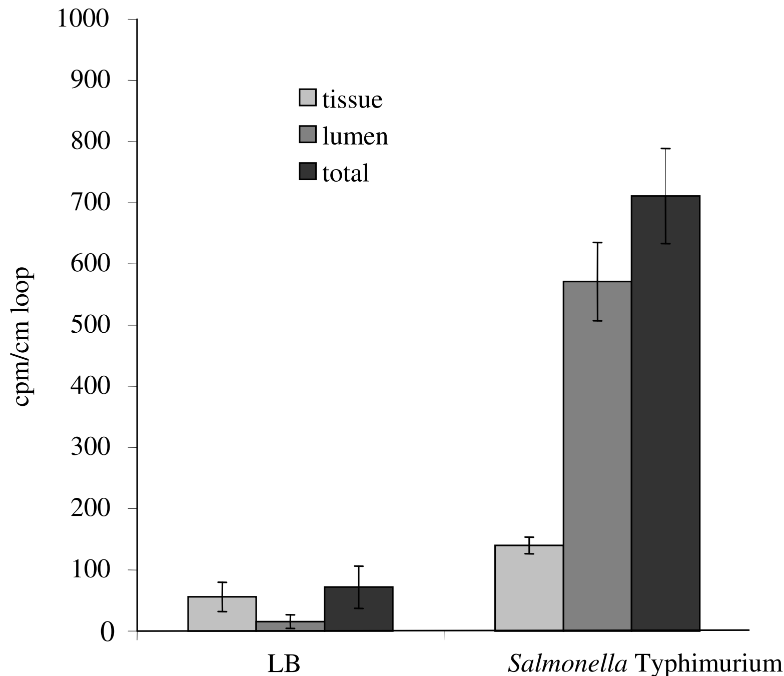


Figure 2: Radio-active γ counts per minute per cm loop as a measure for the early PMN influx elicited by *Salmonella* Typhimurium in porcine intestinal loops. Approximately 1.5 x 10^9 cfu of *Salmonella* Typhimurium were injected into the loops and left for 12 h before analysis. Luria-Bertani broth (LB) served as negative control. Each mean ± SEM is calculated from 3 loops in 2 piglets.

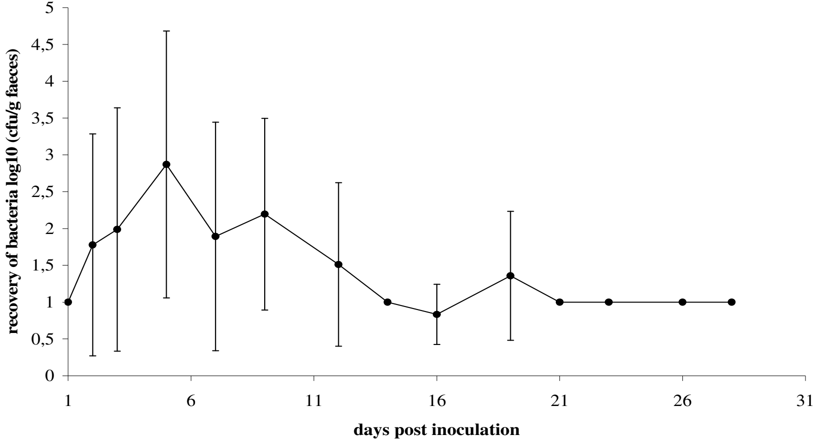


Course of a *Salmonella* Typhimurium infection in pigs

In the *Salmonella* inoculated groups, all pigs were colonized. Mean bacterial counts in faeces were the highest at day 5 pi and pigs were shedding *Salmonella* Typhimurium intermittently until day 28 pi (Fig. 3).The dynamics of the percentage of piglets that shed *Salmonella* Typhimurium at least at enrichment level and of diarrhoea and fever (> 40 °C) are shown in Figure 4. Mean bacterial counts ($\log_{10}$ ± stdev) in the various tissues of the infected pigs are shown in Table 1. Mean bacterial counts in the internal organs were highest at day 5 pi and remained stable at an enrichment level from day 21 pi on. Mean bacterial counts of the intestinal contents were in 16 of the 21 cases lower than bacterial counts of the intestinal wall.

In the negative control group, *Salmonella* Typhimurium was not isolated from faeces or tissues and none of the piglets developed either diarrhoea or fever.

Figure 3: Faecal shedding of pigs inoculated with 10^7 cfu of *Salmonella* Typhimurium, shown as the average number of cfu (log_{10}) ± stdev of *Salmonella* Typhimurium per gram faeces.



Salmonella Typhimurium infection is dose-dependent

In the *Salmonella* inoculated groups all pigs were colonized. Mean bacterial counts (log_{10} ± stdev) in the various tissues of these pigs are shown in Table 2. The results of the clinical scores (diarrhoea, anorexia, fever) and the mean body weight are summarized in Table 3. Significantly ($P < 0.05$) more pigs of group 3 developed diarrhoea than the pigs of group 1 and 2. The loss of weight in group 3 was significantly ($P < 0.05$) higher than in groups 1, 2 and the control group.

The bacterial load in ileum and caecum and in the contents of ileum and caecum of pigs from groups 1 and 2 were significantly ($P < 0.05$) lower than the bacterial load of the pigs from group 3. In the negative control group, *Salmonella* Typhimurium was not isolated from the faeces or tissues at any time point.

Figure 4: Percentages of pigs that excrete *Salmonella* Typhimurium (-■-), showing diarrhoea (-●-) or fever (-Δ-) after inoculation with 10^7 cfu.

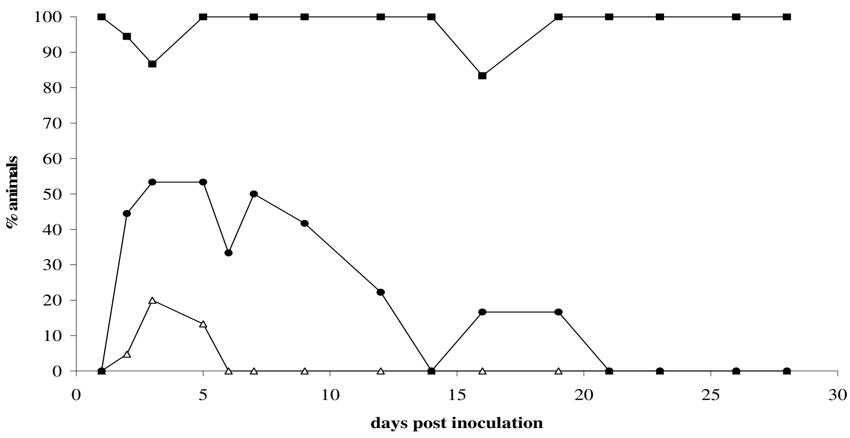


Table 1: Mean log_{10} cfu/g of *Salmonella* Typhimurium in different samples at days 1, 2, 5, 9, 14, 21 and 28 after experimental inoculation with 10^7 cfu of *Salmonella* Typhimurium. The frequency (F) shows the fraction of positive samples in relation to the total number of tissues.

Days post inoculation		1		2		5		9		14		21		28
Samples	F	Log_{10} cfu/g ± stdev	F	Log_{10} cfu/g ± stdev	F	Log_{10} cfu/g ± stdev	F	Log_{10} cfu/g ± stdev	F	Log_{10} cfu/g ± stdev	F	Log_{10} cfu/g ± stdev	F	Log_{10} cfu/g ± stdev
Mand. ln.	2/3	0.67 ± 0.58	2/3	0.67 ± 0.58	3/3	1 ± 0	3/3	1 ± 0	3/3	1 ± 0	3/3	1 ± 0	3/3	1 ± 0
Tonsil	2/3	1.83 ± 1.6	3/3	2 ± 1.73	3/3	2.6 ± 1.38	3/3	2.17 ± 2.02	3/3	2.17 ± 2.03	3/3	1.87 ± 1.51	3/3	1 ± 0
Lung	0/3	0 ± 0	2/3	0.67 ± 0.58	3/3	1 ± 0	2/3	0.67 ± 0.58	2/3	0.67 ± 0.58	2/3	0.67 ± 0.58	0/3	0 ± 0
Heart	0/3	0 ± 0	0/3	0 ± 0	1/3	0.33 ± 0.58	1/3	0.33 ± 0.58	1/3	0.33 ± 0.58	1/3	0.33 ± 0.58	2/3	0.67 ± 0.58
Liver	2/3	0.67 ± 0.58	1/3	0.33 ± 0.58	3/3	1 ± 0	3/3	1 ± 0	2/3	0.67 ± 0.58	1/3	0.33 ± 0.58	0/3	0 ± 0
Spleen	1/3	0.33 ± 0.58	1/3	0.67 ± 0.58	1/3	0.33 ± 0.58	1/3	0.33 ± 0.58	0/3	0 ± 0	0/3	0 ± 0	0/3	0 ± 0
Kidney	0/3	0 ± 0	1/3	0.33 ± 0.58	0/3	0 ± 0	2/3	0.67 ± 0.58	0/3	0 ± 0	0/3	0 ± 0	1/3	0.33 ± 0.58
Mesent. ln.	1/3	0.33 ± 0.58	3/3	1.9 ± 1.55	3/3	1.55 ± 0.94	3/3	1.56 ± 0.96	2/3	0.67 ± 0.58	2/3	0.67 ± 0.58	3/3	1 ± 0
Jejunum	1/3	1.64 ± 2.84	2/3	3.32 ± 2.88	3/3	1.75 ± 1.31	1/3	1.26 ± 2.18	1/3	0.33 ± 0.58	2/3	0.67 ± 0.58	3/3	1 ± 0
Ileum	1/3	0.84 ± 1.46	3/3	1 ± 0	3/3	1.94 ± 1.63	3/3	1.41 ± 0.71	2/3	1.21 ± 1.32	0/3	0 ± 0	3/3	1 ± 0
Caecum	2/3	1.62 ± 1.99	3/3	2.43 ± 1.25	3/3	3.87 ± 1.09	3/3	3.6 ± 0.56	3/3	1 ± 0	2/3	0.67 ± 0.58	3/3	1 ± 0
Contents jejunum	1/3	1.63 ± 2.83	2/3	2.23 ± 2.15	2/3	1.33 ± 1.15	2/3	1.33 ± 1.15	1/3	0.96 ± 1.66	0/3	0 ± 0	2/3	1.33 ± 1.15
Contents ileum	1/3	0.33 ± 0.58	2/3	0.67 ± 0.58	3/3	1 ± 0	2/3	0.67 ± 0.58	1/3	0.33 ± 0.58	3/3	1 ± 0	2/3	0.67 ± 0.58
Contents caecum	1/3	0.8 ± 1.38	2/3	1.5 ± 1.81	3/3	1.95 ± 1.66	3/3	1.59 ± 1.02	2/3	0.67 ± 0.58	3/3	1 ± 0	3/3	1 ± 0

Table 2: Mean $\log_{10}$ cfu/g of *Salmonella* Typhimurium in different tissues at 5 days after experimental inoculation of pigs with either 10^5, 10^7 or 10^9 cfu of *Salmonella* Typhimurium. The frequency (Freq.) shows the fraction of positive samples in relation to the total number of tissues.

Infection dose	10^5 cfu		10^7 cfu		10^9 cfu	
Samples	Freq.	$\log_{10}$ cfu/g ±stdev	Freq.	$\log_{10}$ cfu/g ±stdev	Freq.	$\log_{10}$ cfu/g ±stdev
Mand. ln.	2/3	0.92 ± 0.88	3/4	0.92 ± 0.7	4/4	1.41 ± 0.67
Tonsil	4/4	1.48 ± 0.96	4/4	1.44 ± 0.88	4/4	2.66 ± 1.16
Bronch. ln	1/4	0.25 ± 0.5	0/4	0 ± 0	1/4	0.25 ± 0.5
Lung	1/4	0.25 ± 0.5	3/4	0.75 ± 0.5	4/4	1 ± 0
Heart	0/4	0 ± 0	1/4	0.25 ± 0.5	3/4	0.75 ± 0.5
Liver	3/4	1.06 ± 0.78	4/4	1 ± 0	4/4	1 ± 0
Spleen	4/4	1 ± 0	3/4	0.75 ± 0.5	3/4	0.88 ± 0.64
Kidney	4/4	1 ± 0	3/4	0.75 ± 0.5	2/4	0.71 ± 0.89
Mesent. ln.	1/4	0.48 ± 0.96	3/4	1.21 ± 1.07	4/4	2.4 ± 0.62
Jejunum	2/4	1.06 ± 1.53	4/4	3.26 ± 1.57	3/3	3.51 ± 1.29
Ileum	3/4	1.08 ± 0.94	4/4	1.21 ± 0.29	3/3	3.27 ± 0.4
Caecum	4/4	2.76 ± 1.18	4/4	3.25 ± 0.96	3/3	4.46 ± 0.32
Contents jejunum	2/4	0.73 ± 0.92	3/4	2.35 ± 2.18	3/3	5.12 ± 1.67
Contents ileum	3/4	2.75 ± 0.5	4/4	2.33 ± 1.62	3/3	4.66 ± 0.58
Contents caecum	4/4	1 ± 0	4/4	1.49 ± 0.98	3/3	5.37 ± 0.51

Table 3: Results of the clinical scores (diarrhoea, fever, anorexia) during 5 days post inoculation with either 10^5 (group 1), 10^7 (group 2) or 10^9 (group 3) cfu of *Salmonella* Typhimurium and the mean gain in body weight at 5 days post inoculation. Clinical scores are presented as average fractions of animals showing the respective clinical signs over the 5 day period.

	Average fraction of animals showing			
Group	Diarrhoea	Fever	Anorexia	Mean weight gain
1	0.05	0	0	- 0.14 kg
2	0.1	0.05	0	- 0.13 kg
3	0.45	0.30	0.15	- 0.49 kg
Negative control	0	0	0	+ 0.15 kg

DISCUSSION

In this study, we used several *in vitro* models to assess the virulence of *Salmonella* Typhimurium strains.

A main virulence property of *Salmonella* is the ability to invade non-phagocytic cells. The invasion of intestinal epithelial cells is generally accepted as a major event in the pathogenesis of *Salmonella*-infections (Schlumberger and Hardt, 2005). In this study, invasion was markedly increased in a polarized cell line compared to a non-polarized cell line. Since polarized cell lines more closely approach the *in vivo* situation and since some *Salmonella* virulence features are lost using non-polarized cells (Raffatellu et al., 2005), we suggest using polarized cell lines as much as possible.

One of the most characteristic features of *Salmonella* induced diarrhoea is the massive influx of neutrophils (Tükel et al., 2006). In no longer than 12 hours, the porcine intestinal loop model was able to mimic this trait in a reproducible manner. Regarding the fact that 1 piglet can provide more or less 30 individual loops, one can easily understand that this model has tremendous advantages compared to models using individual pigs. Indeed, in several species including pigs, the intestinal loop model has been used to explore the intestinal inflammation phase during *Salmonella* infections (Wallis et al., 1989; Mehta et al., 1998; Bolton et al., 1999; Boyen et al., 2006). In contrast to the situation in calves (Bolton et al., 1999), we did not find a large volume of watery fluid in the intestines but rather a small amount of slime containing mainly neutrophils, so the measurement of luminal fluids may not be as useful in porcine loops as it is in bovine loops. Some degree of fluid response can be induced, however, using very young piglets (Clarke and Gyles, 1987). Even though a variant form of the intestinal loop model has been used to quantify invasion in intestinal epithelial cells (Uzzau et al., 2001; Meyerholz and Stabel, 2003), we recommend to use a polarized intestinal epithelial cell line, since it requires no animals to be used and it proves to be cheaper and less time consuming. Since Interleukin-8 (IL-8) is a major cytokine in the development of *Salmonella*-induced intestinal inflammation (Tükel et al., 2006), one could try to assess the ability of *Salmonella* strain to induce an intestinal inflammation response by measuring the IL-8 production by either macrophages or epithelial cells (Boyen et al, 2006a; Tükel et al., 2006). However, since also other cytokines play a role in the attraction of neutrophils to the lumen of the gut (Tükel et al., 2006) and since enteritis is the result of an extensive interplay of different receptors on different cell types (Tükel et al., 2006), the intestinal loop model is a much more physiological and reliable model.

Intracellular survival and proliferation inside macrophages has been shown to contribute to persistent infections of *Salmonella* in mice (Buchmeier and Heffron, 1989; Leung and Finlay, 1991). In spite of the fact that the systemic phase of a *Salmonella* Typhimurium infection is hardly as important in pigs as in BALB/c mice, the role of mononuclear cells, for example in lymphoid tissues, in the pathogenesis of *Salmonella* Typhimurium infections in pigs must not be neglected. As in mice (Buchmeier and Heffron, 1989; Hensel et al., 1998), the source of isolation of the mononuclear cells determined the degree of growth or survival of *Salmonella* Typhimurium. In our case, the tissue macrophages allowed intracellular growth, whereas the monocytes were more restrictive. This finding may reflect the loss of important microbicidal functions of resident macrophages compared with blood monocytes (Chitko-McKown et al., 1991). Therefore, it may be useful to include both cell types in *in vitro* assays.

Even though the work with cell lines or single cell suspensions has its advantages, it also holds limitations. One of the most important drawbacks is that these models lack the interplay of different important cell types (Niewold et al., 2006). This may mean that sometimes the use of an *in vivo* infection model is inevitable. The oral infection may be considered the most physiological model. Oral infection of pigs resulted in colonization of and persistence in both the tonsils and the gut, using low, moderate or high inoculation doses. However, it took an inoculation dose of at least 10^7 cfu to render all animals positive for *Salmonella* in all gut samples at day 5 pi. This uniformity may be important for standardization purposes in comparative assays. Inoculation with 10^9 cfu also guarantees uniform colonization, but this extreme high inoculation dose differs greatly from the natural infection, which is often established with low or moderate numbers of *Salmonella* (Loynachan and Harris, 2005). Even though all animals were colonized with 10^7 cfu, only maximum 50 % of the animals displayed signs of diarrhoea and only maximum 20 % had fever, both on days 3 and 5 pi. These results suggest that the oral inoculation model can be used to investigate colonization (at day 5 pi) and even persistence (at day 28 pi), but is not suited as an enteritis model or a systemic disease model, due to low and variable numbers of animals showing clinical signs.

In conclusion, the *in vitro* models and the intestinal loop model evaluated in the present study can be used to screen candidate knock-out vaccine strains for attenuation. A promising selection of strains may then be used for further *in vivo* characterization. The data obtained in the porcine experimental infection model are helpful to determine optimal inoculation dose, time points of sampling and numbers of animals needed in future experimental infection studies.

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CHAPTER 3:

The role of different *Salmonella* virulence genes in the pathogenesis of *Salmonella* Typhimurium infections in pigs

Salmonella Typhimurium SPI-1 genes promote intestinal but not tonsillar colonization in pigs

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ABSTRACT

Salmonella Pathogenicity Island 1 (SPI-1) genes are indispensable for virulence of *Salmonella* Typhimurium in several animal species. The role of SPI-1 in the pathogenesis of *Salmonella* Typhimurium infections of pigs, however, is not well described.

The interactions of a porcine *Salmonella* Typhimurium field strain and its isogenic mutants with disruptions in the SPI-1 genes *hilA*, *sipA* and *sipB* with porcine intestinal epithelial cells was characterized *in vitro* and in a ligated intestinal loop model in pigs. HilA and SipB were essential in the invasion of porcine intestinal epithelial cells *in vitro*. A *sipA* mutant was impaired for invasion using a polarized cell line, but fully invasive in a non-polarized cell line. All SPI-1 mutants induced a significant decrease in influx of neutrophils in the porcine intestinal loop model compared with the wild type strain.

Pigs were orally inoculated with 10^8 colony forming units of both the wild type *Salmonella* Typhimurium strain and its isogenic *sipB*::kan mutant strain. The *sipB* mutant strain was significantly impaired to invade the intestinal, but not the tonsillar tissue, one day after inoculation and was unable to efficiently colonize the intestines and the GALT, but not the tonsils, 3 days after inoculation.

This study shows that SPI-1 plays a crucial role in the invasion and colonization of the porcine gut and in the induction of influx of neutrophils towards the intestinal lumen, but not in the colonization of the tonsils.

Key words

Salmonella Typhimurium – pig – gut - tonsils – SPI-1

INTRODUCTION

Human salmonellosis is frequently associated with *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium; Borch et al., 1996; Berends et al., 1998) and salmonellosis caused by this serovar is mainly associated with the consumption of pork. *Salmonella* Typhimurium is the predominant serovar isolated from pigs in Europe (Anonymous, 2004) and pigs may be subclinically infected. These animals generally carry the bacterium in the tonsils, the intestines and the gut-associated lymphoid tissue (GALT; Fedorka-Cray et al., 2000). Such carriers are a major reservoir of *Salmonella* Typhimurium and pose an important threat to animal and human health Berends et al., 1997; Poppe et al., 1998).

The invasion of intestinal epithelial cells is considered a major step in the pathogenesis of *Salmonella* infections. *Salmonella* Pathogenicity Island 1 (SPI-1) is crucial in the interactions of *Salmonella* with intestinal epithelial cells in several animal species. It encodes a type three secretion system (T3SS-1) which consists of a hollow, needle-like structure and a pore-forming ring (also called translocon) through which effector proteins are transported into the cytoplasm of the infected host cell (Zhou and Galan, 2001). Through direct and indirect modulation of the host cell's cytoskeleton, the bacterium forces the host cell to engulf the attached *Salmonella* bacterium (Zhou and Galan, 2001; Garcia-del Portillo and Finlay, 1994). SPI-1 also plays a central role in the induction of enteritis and diarrhoea. The secretion of pro-inflammatory cytokines, for example IL-8, is a result of the activation of the MAP kinase pathways and the transcription factor NF-κB by T3SS-1 effector proteins (Hobbie et al., 1997; Murli et al., 2001). IL-8, released from the basolateral aspect of infected epithelial cells, plays an important role in the initial movement of neutrophils from the circulation into the subepithelial region (McCormick et al., 1995). The actual transepithelial migration of the polymorphonuclear leucocytes (PMN) into the lumen of the gut is mediated by another cytokine, called the pathogen elicited epithelial chemo-attractant (PEEC), which is secreted on the apical side of the epithelial cells, in response to the SPI-1 effector SipA (Lee et al., 2000). Recently, PEEC has been identified as the key regulator of mucosal inflammation, hepoxilin A_3 (Mrsny et al., 2004).

Most of the findings described above were identified through experiments in mice, cell culture chambers or the calf intestinal loop model. Since the course of a *Salmonella* Typhimurium infection varies greatly between different host species, biological effects of

bacterial virulence factors may differ. Information on the importance of SPI-1 in the pathogenesis of *Salmonella* Typhimurium infections in pigs is scarce. It was the aim of the present study to characterize the role of SPI-1 of a porcine *Salmonella* Typhimurium field strain in the intestinal phase of infection of pigs.

MATERIALS AND METHODS

All animal experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Bacterial strains and growth conditions

Table 1: The strains used in this study.

Strain	Genotype	Source or reference
WT	*Salmonella* Typhimurium 112910a	Donné et al., 2005
WT_{nal}	*Salmonella* Typhimurium 112910a Nal^r	This study
HilA	*Salmonella* Typhimurium 112910a Δ*hilA*	Boyen et al., 2006a
SipA	*Salmonella* Typhimurium 112910a Δ*sipA*	Boyen et al., 2006a
SipB	*Salmonella* Typhimurium 112910a Δ*sipB*	Boyen et al., 2006a
$SipB_{kan}$	*Salmonella* Typhimurium 112910a *sipB*::kan	Boyen et al., 2006a
$SipB_{kan/nal}$	*Salmonella* Typhimurium 112910a Nal^r *sipB*::kan	This study

Salmonella Typhimurium strain 112910a, phage type 120/ad, was isolated from a pig stool sample and has been shown to persist in tonsils, intestines and gut-associated lymph nodes of experimentally infected pigs during at least 28 days (unpublished data). The construction and characterization of mutants with non-polar deletion mutations in the genes encoding the major SPI-1 regulatory protein HilA, the SPI-1 translocator/effector protein SipB and the SPI-1 effector protein SipA have been described before (Boyen et al., 2006a). An invasive, spontaneous nalidixic acid resistant derivative of the wild type strain (WT_{nal}) was used for the mixed infection assay in order to minimise irrelevant bacterial growth when plating out intestinal and faecal samples. The *sipB*::kan mutation of the original strain 112910a (Boyen et al., 2006a) was moved by P22 transduction (Schmieger and Backhaus,

1976) into the WT_{nal} background and enabled us to make a phenotypical distinction (kanamycin resistance) between the wild type strain and the SPI-1 deficient derivative. All mutations were confirmed by PCR and sequencing of the relevant PCR fragment, using the primers as described before (Boyen et al., 2006a). At different stages of the construction, bacteriophage P22 sensitivity was tested to confirm the smooth phenotype.

To obtain highly invasive late logarithmic cultures (Lundberg et al., 1999) for invasion assays, 2 µl of a stationary phase culture was inoculated in 5 ml Luria-Bertani broth (LB; Sigma-Aldrich, Steinheim, Germany) and grown for 5 hours at 37 °C without aeration. The inocula for the intestinal loop model were prepared as described previously (Boyen et al., 2006b). For the oral inoculation of pigs, the bacteria were grown overnight in LB with aeration at 37 °C. The bacteria were washed twice in phosphate-buffered saline (PBS, 2000 x g, 10 min, 4 °C) and diluted in PBS to the appropriate concentrations. The number of viable *Salmonella* bacteria ml^{-1} in the inoculum was determined by plating tenfold dilutions onto Brilliant Green Agar (BGA; Oxoid, Basingstoke, UK).

Cell cultures

The porcine intestinal epitheloid IPI-2I cell line (Kaeffer et al., 1993) and the polarized porcine intestinal epithelial cell line IPEC-J2 (Rhoads et al., 1994) were used for invasion assays. The IPI-2I cells were cultured in RPMI (Gibco, Life Technologies, Paisley, Scotland) containing 10 % (v/v) fetal calf serum (FCS; Hyclone, Cramlington, England), 2 mM L-glutamine (Gibco, Life Technologies, Paisley, Scotland), 1 mM sodium pyruvate (Gibco, Life Technologies, Paisley, Scotland), 100 units nystatin ml^{-1} (Gibco, Life Technologies, Paisley, Scotland), 100 units penicillin ml^{-1} and 100 µg streptomycin ml^{-1} (penicillin/streptomycin, Gibco, Life Technologies, Paisley, Scotland). The IPEC-J2 cells were maintained on 50 % DMEM – 50 % F12 medium with 5 % (v/v) FCS, 1 % (v/v) insulin/transferrin/Na-selenite media supplement (Gibco, Life Technologies, Paisley, Scotland) and antibiotics as described above.

Analysis of secreted proteins

Proteins secreted by *Salmonella* Typhimurium into culture supernatants during growth in LB broth were precipitated using trichloroacetic acid as described previously (Watson et al., 1998). The resuspended proteins (5 µl) were separated on NuPAGE 10 % Bis-Tris gels (Invitrogen) and stained with Coomassie brilliant blue. The presence of SipB in the secreted proteins was probed through transfer of 5 µl of each protein preparation to Hybond ECL

membrane by standard Western blotting techniques and detection of SipB using the anti-SipB monoclonal antibody at 1 µg ml^{-1}. The anti-SipB monoclonal antibody was a kind gift from Ed Galyov, IAH Compton.

Invasion and intracellular survival of *Salmonella* Typhimurium and its SPI-1 mutants in porcine intestinal epithelial cells

IPI-2I and IPEC-J2 cells were seeded in 24 well plates at a density of approximately 10^5 cells per well and were allowed to grow to confluency for at least 7 days. These wells were inoculated with the different *Salmonella* strains at a multiplicity of infection (MOI) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at 365 x g for 5 min. After 25 min incubation at 37 °C under 5% CO_2, the wells were washed and fresh medium supplemented with 50 µg/ml gentamicin (Gibco, Life Technologies, Paisley, Scotland) was added. After an additional 60 min incubation at 37 °C under 5% CO_2, the wells were washed three times.

To assess invasion, the cells were lysed with 0.25% deoxycholate (Sigma-Aldrich, Steinheim, Germany) 90 min after inoculation and 10-fold dilutions were plated on brilliant green agar (BGA) plates.

To assess intracellular growth, the medium containing 50 µg/ml gentamicin was replaced after the 60 min incubation time with fresh medium supplemented with 15 µg/ml gentamicin and the number of viable bacteria was assessed 24 hours after inoculation as described above.

Induction of an inflammatory response by *Salmonella* Typhimurium and its SPI-1 mutants in porcine intestinal loops

Intestinal loops were made in 6-week-old farm-reared Landrace/Large White cross male piglets. This model has been described in detail elsewhere (Wallis et al., 1995; Bolton et al., 1999). In short, the lumen of the ileum was gently flushed with 0.9 % NaCl before the construction of the loops to remove the intestinal contents. Commencing at the distal ileum, a maximum of 30 sequential loops (each 6-7 cm in length and containing both absorptive epithelium and follicle associated epithelium) were ligated using surgical silk, separated by 1 cm spacers. The inoculum injected into each loop, consisting of *Salmonella* Typhimurium 112910a or one of the isogenic SPI-1 mutants, was in the range 1-2 x 10^9 colony forming units (cfu). Approximately 10 ml of blood was taken from the piglets to isolate the PMNs. The isolated PMNs were labelled with ^{111}Indium and reinjected intravenously. The influx of

PMNs in the intestinal wall and in the lumen of the gut, as assessed by the counts per minute (c.p.m.) emitted from ^{111}Indium-labelled PMNs within each loop, was recorded 12 h after injection of the loops using a Wallac 1275 mini gamma counter.

Experimental mixed infection of piglets

Experimental infections were performed in 6-week-old piglets (commercial closed line based on Landrace), from a serologically negative breeding herd and were negative for *Salmonella* at faecal sampling. They arrived at the facility 7 days before they were inoculated and were divided at random into two groups: one group of 10 inoculated pigs and one negative control group of 3 pigs. The piglets were housed in pairs in separate isolation units at 25 °C under natural day-night rhythm with ad libitum access to feed and water.

The 10 experimental animals were orally inoculated with approximately 10^8 cfu of a stationary phase culture of the WT_{nal} and the $SipB_{kan/nal}$ strains in 2 ml PBS in a 1:1 ratio. The negative control group was sham-inoculated with 2 ml PBS.

Fresh faecal samples were taken from each pig on a daily basis for bacteriological analysis. On days 1 and 3 after inoculation, 5 piglets were humanely euthanized. Sham-inoculated piglets were euthanized 3 days after inoculation. Samples of tonsils, liver, spleen, kidney, mesenteric, ileocecal and colonic lymph nodes, jejunum, ileum, cecum and colon were taken for bacteriological analysis. The jejunum, ileum, cecum and colon tissue samples were separated from their contents and were rinsed in PBS. Both the contents and the rinsed tissue samples were bacteriologically examined.

All samples were stored at -70 °C until use. The samples were thawed and weighed, 10% (w/v) suspensions were made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was homogenized with a stomacher. The homogenized samples were examined for the presence of the *Salmonella* strains by plating tenfold dilutions on BGA. All samples were plated in duplicate; once on BGA supplemented with 20 µg/ml nalidixic acid (BGA^{NAL}) and once on BGA supplemented with 20 µg/ml nalidixic acid and 100 µg/ml kanamycin ($BGA^{NAL/KAN}$). If negative at direct plating, the samples were pre-enriched overnight in BPW at 37 °C, enriched overnight at 37 °C in tetrathionate broth and then plated on BGA^{NAL} and $BGA^{NAL/KAN}$.

Salmonella colonies that grew on $BGA^{NAL/KAN}$ were presumed to be the $SipB_{kan/nal}$ strain, colonies that grew on BGA^{NAL} were both the WT_{nal} and the $SipB_{kan/nal}$ strain. The number of colonies on $BGA^{NAL/KAN}$ was subtracted from the number of colonies on BGA^{NAL}, resulting in the number of WT_{nal} colonies. The ratio WT_{nal} / $SipB_{kan/nal}$ was calculated for all

samples derived from each piglet. These data were converted logarithmically prior to the calculation of averages and statistical analysis. The appropriate detection limits were calculated and used to estimate the minimum ratios when samples were not positive after direct plating.

Localization of *Salmonella* Typhimurium in porcine tonsils

Four piglets were orally inoculated with approximately 10^8 cfu of a stationary phase culture of the WT_{nal} as described above. One and 3 days after inoculation, 2 piglets were humanely euthanized. Samples were taken from the tonsils for bacteriological examination and for immunohistochemical analysis. Tonsillar tissue was fixed in 10% formalin, embedded in paraffin and sectioned at 10 µm. The sections were incubated with rabbit polyclonal antiserum to *Salmonella* O_4 somatic antigen (Pro-Lab Diagnostics, Neston, UK) diluted 1:50. Biotinylated goat anti-rabbit antibodies (Zymed Laboratories, San Francisco, USA) diluted 1:500 were added to the tissue sections (45 min, room temperature). Positive cells were stained brown after adding StreptABComplex/HRP (DakoCytomation, Glostrup, Denmark) and diaminobenzidine (Sigma-Aldrich, Steinheim, Germany). Sections were counterstained with haemaluin. As negative controls, sections were stained as described above, using rabbit polyclonal antiserum to *Salmonella* O_9 somatic antigen (Pro-Lab Diagnostics, Neston, UK) as primary antibody. This somatic antigen is not expressed by *Salmonella* Typhimurium. Sections were evaluated using the Leica DM LB2 microscope. Pictures were taken using the Leica DFC 320 camera and the Leica IM50 imaging software.

Statistical analysis

The gentamicin protection assays were carried out in triplicate with three repeats per experiment. The *in vitro* invasion and intestinal loop data were analysed by one-way analysis of variance method, using the SPSS 12.0 software for Windows and Bonferroni corrections were applied. A paired Student's t-test was used to determine whether the log value of the WT_{nal} / $SipB_{kan/nal}$ ratio of the samples was significantly different from the log value of the WT_{nal} / $SipB_{kan/nal}$ ratio of the inoculum.

RESULTS

Secreted protein analysis

Both the wild type strain and its nalidixic resistant derivative express and secrete the full complement of Sip proteins. The *hilA* mutant strain was defective in the secretion of several SPI-1 encoded effector proteins, including SipA, SipB, SipC and SipD. The *sipA* mutant strain was defective in the secretion of the SipA protein exclusively. All *sipB* mutant strains were defective in the secretion of SipB, but showed no altered secretion of other SPI-1 encoded proteins. The results of both the gel electrophoresis and the western blot are shown in Fig 1.

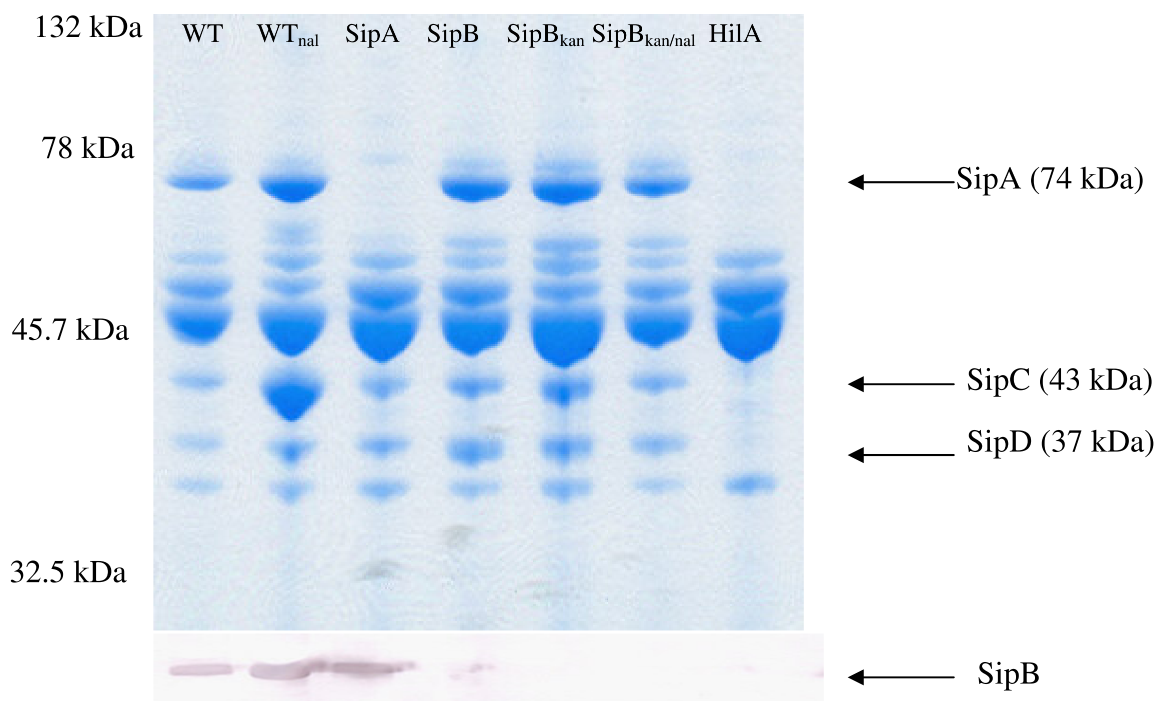


Figure 1: Profiles of proteins secreted by *Salmonella* Typhimurium 112910a and its derivative strains into the culture supernatant. Arrows indicate the molecular weights of SipA, SipC and SipD. The molecular sizes of marker proteins are given on the left. Beneath the respective lanes, the results are shown of the western blots performed on all strains, using an anti-SipB monoclonal antibody.

Invasion in porcine intestinal epithelial cells is SPI-1 dependent

Invasion and intracellular replication of *Salmonella* Typhimurium 112910a and its isogenic mutant strains was compared in porcine IPI-2I and IPEC-J2 cells strains, using a gentamicin protection assay. The results are summarized in Fig. 2. Ninety minutes post inoculation (pi), the wild type strain and its nalidixic resistant derivative showed identical

invasion and survival rates in both cell lines. The HilA, SipB, $SipB_{kan}$ and $SipB_{kan/nal}$ strains invaded the IPI-2I and IPEC-J2 cells respectively 10 times or 1000 times less than the wild type strain (statistically significant, $p < 0.005$). The *sipA* mutant strain did not show any significant differences from the wild type strain ($p > 0.05$) in the IPI-2I cell line, but was significantly ($p < 0.005$) attenuated in invasion using the IPEC-J2 cell line. The SPI-1 mutant strains were not impaired in intracellular replication.

Induction of intestinal inflammation in pigs is SPI-1 dependent

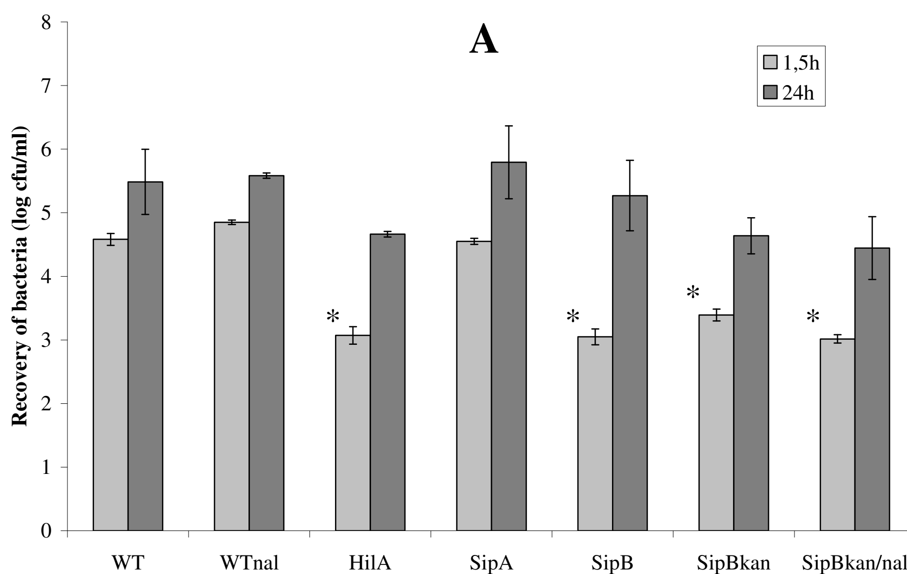
In the porcine intestinal loop assays, inoculation with the wild type strain resulted in intestinal inflammation 12 hours after inoculation as quantified by influx of ^{111}In-labelled neutrophils. Although the number of neutrophils in the tissue was only modestly increased over control loops, the number of neutrophils in the lumen was increased by more than 100-fold compared to the sham-inoculated loops. The inflammatory response in the intestinal loops inoculated with the SPI-1 mutant strains was abolished. Although there was a slight increase in the induction of a neutrophil influx, especially in the lumen, compared to the control loops, this was significantly lower ($p < 0.005$) than in the loops inoculated with the wild type strain. These results are shown in Fig. 3.

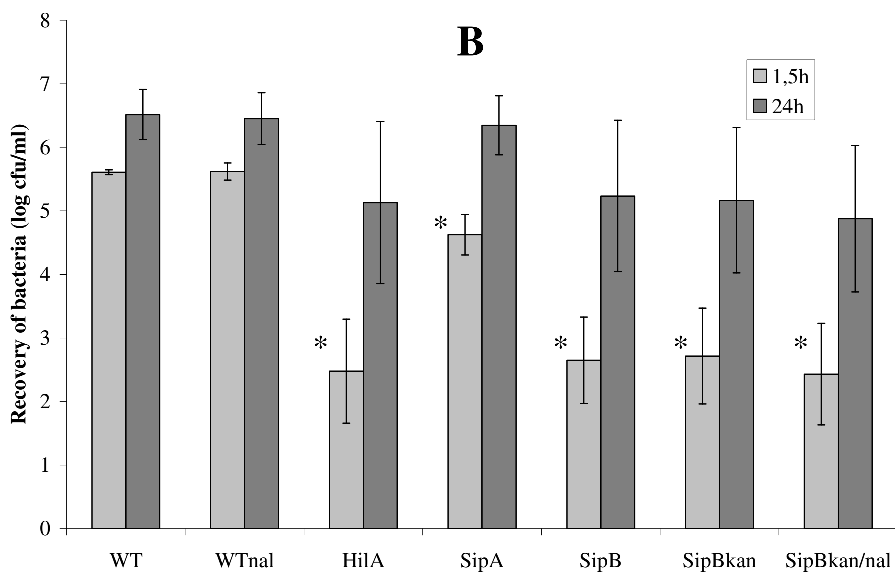
SPI-1 is necessary for efficient invasion of the porcine gut

The ability of the WT_{nal} and $SipB_{kan/nal}$ strains to colonize porcine tissues were quantified following oral inoculation of 6 week old piglets

One day after inoculation, the WT_{nal} was recovered in numbers at least two orders of magnitude higher than the $SipB_{kan/nal}$ strain in the jejunal, ileal and cecal wall, the jejunal contents and the ileocecal lymph nodes. In these organs, the output ratio WT_{nal} / $SipB_{kan/nal}$ was significantly higher ($p < 0.05$) than in the inoculum. In contrast, the ileal and cecal contents and the tonsils were colonized to a similar extent by both strains. The numbers of bacteria in the liver, spleen, kidney, mesenteric lymph nodes and colon were very low, impairing a meaningful quantitative comparison between both strains. The average log values of the ratio $WT_{nal}/SipB_{kan/nal}$ of 5 piglets are summarized for all samples in Table 2 and Fig. 4.

Figure 2: The invasiveness and intracellular replication of all *Salmonella* Typhimurium strains described in this paper in IPI-2I (A) and IPEC-J2 (B) cells. The log values of the number of gentamicin protected bacteria are shown. The results represent the means of three independent experiments conducted in triplicate ± standard error of the means for the IPEC-J2 cell line and a representative of three independent experiments conducted in triplicate for the IPI-2I cell line. An asterisk refers to a significantly lower invasion relative to the wild type strain ($p < 0.01$).





SPI-1 is necessary for efficient colonization of the porcine gut, but not the tonsils

Three days pi, the intestines and the GALT of all piglets were colonized by the WT_{nal} strain, with very high numbers of bacteria in the ileum and cecum. The $SipB_{kan/nal}$ strain was found in markedly lower numbers in the intestinal wall, in the intestinal contents and in the GALT than the WT_{nal} strain. In these samples, the output ratio WT_{nal} / $SipB_{kan/nal}$ was significantly higher ($p < 0.05$) than in the inoculum. In contrast, the tonsils were colonized to a similar extent by both strains. The numbers of bacteria in the liver, spleen, kidney and colonic lymph nodes were very low, impairing a meaningful quantitative comparison between both strains. The average log values of the ratio WT_{nal} / $SipB_{kan/nal}$ for all samples of 5 piglets are summarized in Table 2 and Fig. 4.

Salmonella was not isolated from any of the samples taken from the control animals throughout the experiment.

In stained sections of the tonsils, immunopositive bacilli, but also immunonegative cocci, were frequently seen attached to the surface of the epithelium. No immunopositive cells were seen intracellularly in the basal layers of the epithelium or in the lymphoid tissue. Representative pictures are shown in Fig. 5.

Figure 3: Influx of PMN in the porcine intestinal loop model. The results represent the means of 2 independent experiments conducted in triplicate ± standard error of the means. The loops inoculated with the SPI-1 mutant strains all induced significantly lower numbers of cpm/cm loop compared to the loops inoculated with the wild type strain ($p < 0.005$).

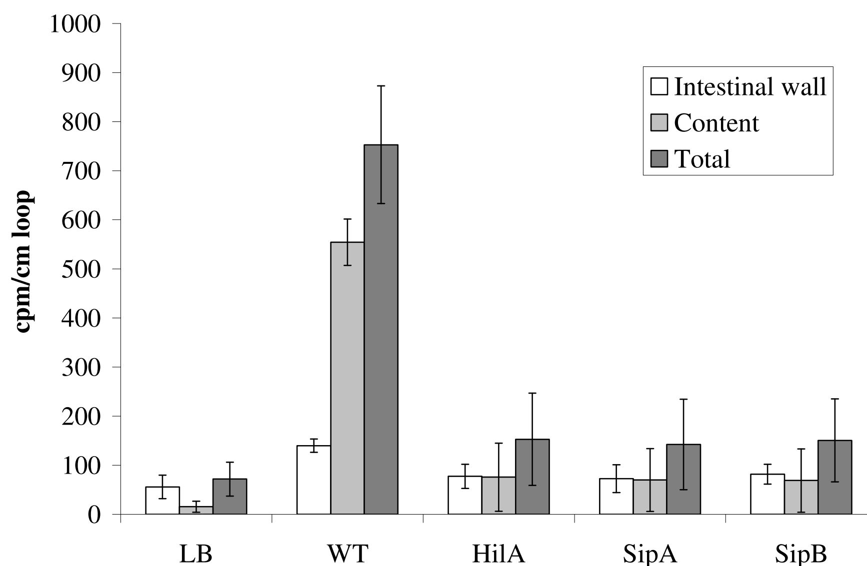


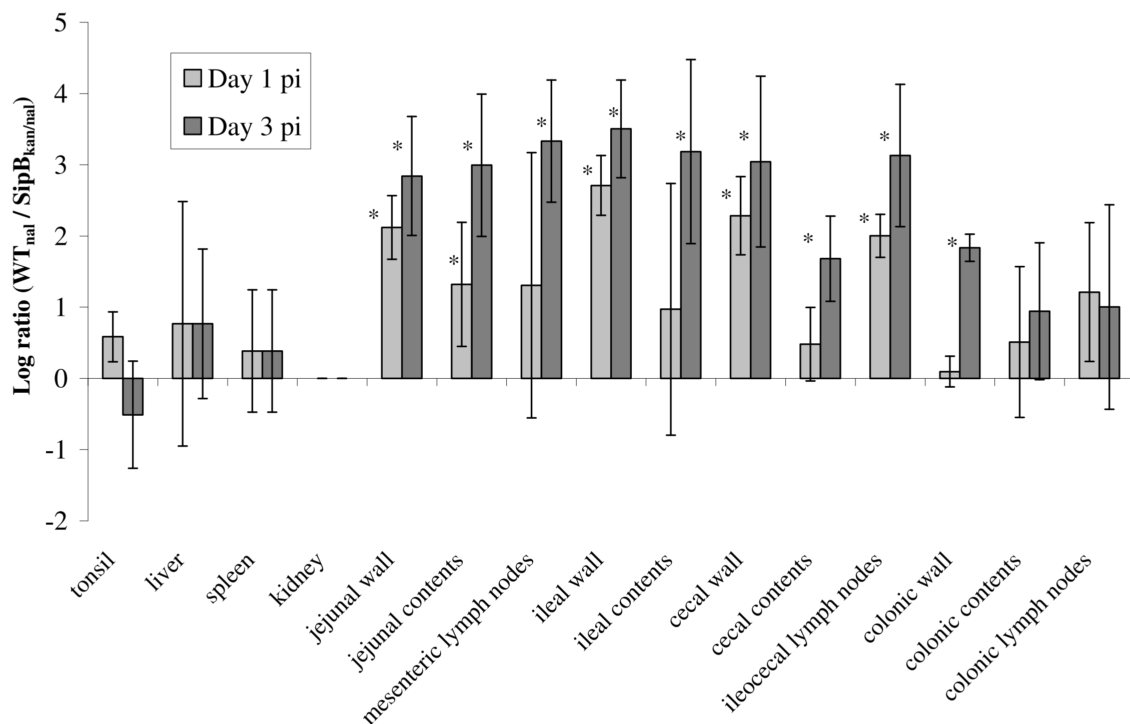
Table 2: Post mortem bacteriological findings at day 1 and day 3 post inoculation of piglets inoculated with an equal mixture of WT_{nal} and $SipB_{kan/nal}$. Number of positive tissues in relation to the total number of tissues (frequency) and the average number of cfu (log_{10}) ± stdev per gram tissue are shown. Samples only positive after enrichment were rendered a value of log 83 cfu g^{-1} and negative samples of log 1 cfu g^{-1}.

		WT_{nal}		$SipB_{kan/nal}$	
	Tissue	Frequency	Log_{10} cfu/g ± stdev	Frequency	Log_{10} cfu/g ± stdev
Day 1 pi	Tonsil	5/5	4.94 ± 0.70	5/5	4.35 ±0.57
	Liver	3/5	1.02 ± 0.83	1/5	0.34 ± 0.68
	Spleen	1/5	0.34 ± 0.68	0/5	0 ± 0
	Kidney	1/5	0.34 ± 0.68	1/5	0.34 ± 0.68
	Mesenterial lnn.	3/5	1.65 ± 1.61	1/5	0.38 ± 0.77
	Ileocecal lnn.	5/5	2.30 ± 0.74	1/5	0.34 ± 0.68
	Colonic lnn.	4/5	1.46 ± 0.76	1/5	0.34 ± 0.68
	Jejunal wall	5/5	1.99 ± 0.47	0/5	0 ± 0
	Ileal wall	5/5	5.21 ± 0.45	5/5	2.46 ± 0.50
	Cecal wall	5/5	4.34 ± 0.52	5/5	1.93 ± 0.36
	Colonic wall	5/5	1.88 ± 0.27	5/5	1.74 ± 0.09
	Contents jejunum	4/5	1.57 ± 0.89	1/5	0.34 ± 0.68
	Contents ileum	5/5	5.32 ± 0.29	5/5	4.35 ± 1.37
	Contents cecum	5/5	3.03 ± 0.78	5/5	2.51 ± 0.75
	Contents colon	5/5	2.65 ± 0.87	5/5	2.05 ± 0.50
Day 3 pi	Tonsil	5/5	3.68 ± 0.94	5/5	4.19 ± 0.54
	Liver	2/5	0.77 ± 0.94	0/5	0 ± 0
	Spleen	1/5	0.38 ± 0.77	0/5	0 ± 0
	Kidney	0/5	0 ± 0	0/5	0 ± 0
	Mesenterial lnn.	5/5	3.72 ± 0.79	1/5	0.34 ± 0.68
	Ileocecal lnn.	5/5	3.90 ± 0.24	2/5	0.72 ± 0.89
	Colonic lnn.	4/5	1.77 ± 1.00	2/5	0.68 ± 0.83
	Jejunal wall	5/5	3.77 ± 1.31	2/5	0.88 ± 1.12
	Ileal wall	5/5	5.39 ± 0.82	4/5	1.80 ± 1.06
	Cecal wall	5/5	4.90 ± 0.85	3/5	1.80 ± 1.64
	Colonic wall	5/5	2.22 ± 0.60	1/5	0.34 ± 0.68
	Contents jejunum	5/5	3.38 ± 1.35	1/5	0.34 ± 0.68
	Contents ileum	5/5	5.52 ± 0.86	4/5	2.34 ± 1.31
	Contents cecum	5/5	3.17 ± 1.13	3/5	1.45 ± 1.25
	Contents colon	5/5	2.42 ± 1.00	3/5	1.39 ± 1.32

DISCUSSION

Deletion of the gene encoding the major SPI-1 regulator HilA causes a serious decrease in expression of the SPI-1 encoded genes (Altier, 2005). Alternatively, a *Salmonella* Typhimurium strain lacking the translocator SipB does not influence SPI-1 expression levels, but renders the effector translocation inefficient, because of a deficient SPI-1 type three secretion system (T3SS; Zhou et al., 2001). Both mutations constructed independently in this study resulted in a significant decrease of invasion of porcine enterocytes and in the abolishing of the influx of PMN in a porcine intestinal enteropathogenesis assay. This confirms the role of SPI-1 in the intestinal phase of *Salmonella* Typhimurium infections of pigs. The role of SPI-1 in the intestinal phase of infection has been shown earlier for *Salmonella* Choleraesuis in pigs (Lichtensteiger and Vimr, 2003) and for *Salmonella* Typhimurium and *Salmonella* Dublin in calves (Zhang et al., 2002; Galyov et al., 1997). Recently, Grondahl and coworkers (2005) found that an *invH* mutant strain, although not impaired for invasion, was attenuated for the induction of an inflammatory response in pigs.

Figure 4: Recovery of bacteria from various organs of 5 piglets at days 1 and 3 after inoculation with an equal mixture of WT_{nal} and $SipB_{kan/nal}$. The log value of the ratio of the number of cfu/g sample of WT_{nal} and $SipB_{kan/nal}$ is given as the mean ± standard deviation. An asterisk indicates that the output ratio was significantly different ($p < 0.05$) from that present in the inoculum.



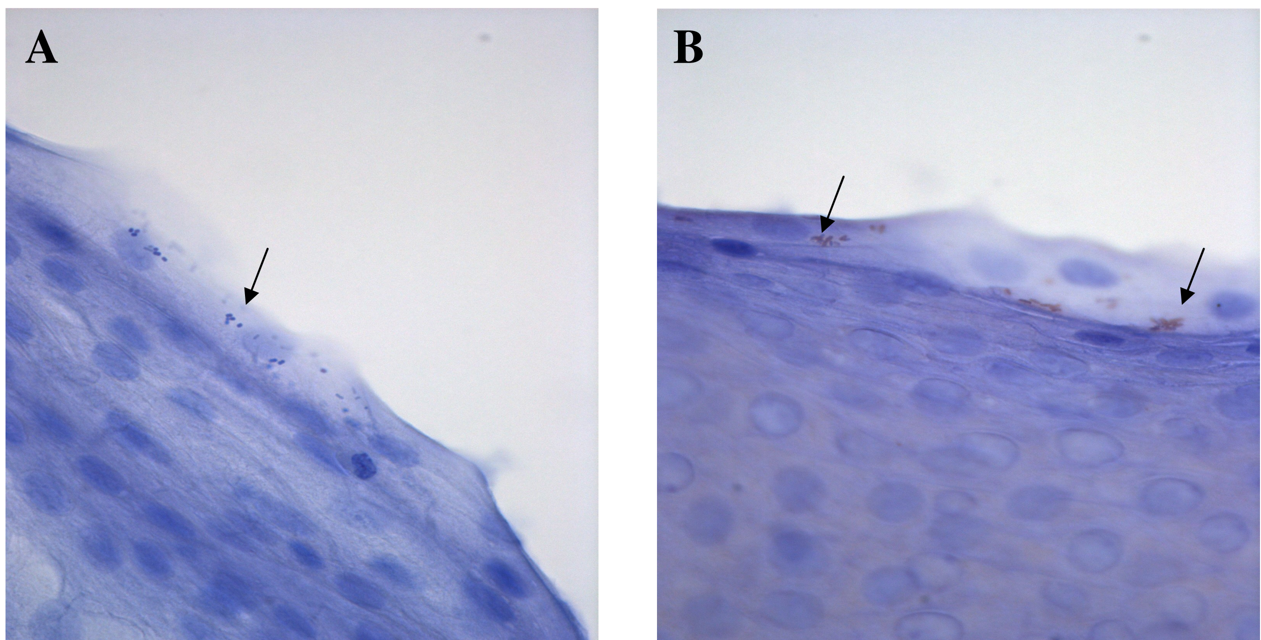
Although the *sipA* mutant strain was attenuated for invasion in the polarized epithelial cell line, it was fully invasive in the non-polarized epitheloid cell line. This remarkable result reflects the recent findings in human cell lines (Raffatellu et al., 2005). The *sipA* mutant strain elicited an inflammatory response similar to that of the non-invasive *hilA* and *sipB* mutant strains in the porcine intestinal loop model. Although the *sipA* mutant strain is not fully invasive, it is still significantly more invasive than the SPI-1 deficient strains. The *sipA* mutant strain induced neutrophil influx similar to that induced by *hilA* and *sipB* mutant strains, might be explained by its role as indirect chemo-attractant, since it induces the excretion of hepoxilin A_3 (Lee et al., 2000; Mrsny et al., 2004). This finding is in agreement with the inflammatory scores in bovine intestinal loops as assessed by microscopic examination of thin sections (Zhang et al., 2002). No difference was seen between the intestinal changes induced by the *sipA* and *sipB* mutant strains based on the infiltration of PMN in the intestinal tissue. Nevertheless, a *sipA* mutant strain induced more fluid accumulation than the *sipB* mutant strain in bovine intestinal loops (Zhang et al., 2002). This could not be confirmed in our experiments, as the degree of fluid accumulation in porcine loops is much less than in bovine loops and impairs an accurate estimate of the fluid accumulation.

To our knowledge, this is the first published report describing the use of a mixed infection model to assess the quantitative importance of a virulence factor of *Salmonella* Typhimurium in the pig. Mixed inoculum assays are more capable of discriminating differences in the ability of strains to colonize the host because the substantial variation in colonization observed between piglets is overcome by considering the ratio of the two strains in a single animal. In this way, it allowed us to reduce the number of animals needed to detect significant differences between two *Salmonella* strains. This model may be applicable to study other virulence genes or even serotypes in the future.

To evaluate the *in vivo* importance of SPI-1 in the short-term colonization of *Salmonella* Typhimurium in pigs, we chose to work with a mutant with a Kan insertion in *sipB* as the importance of HilA as a regulator of the expression of genes in the *Salmonella* pathogenicity island SPI-4 is still controversial (Ahmer et al. 1999; Detweiler et al., 2003; Morgan et al., 2004). Although SPI-4 has a major role in influencing intestinal colonization of cattle, it does not in the colonization of chickens (Morgan et al., 2004). It is not yet known if SPI-4 may affect porcine colonization. To our knowledge, a mutation in *sipB* has not been linked to other than SPI-1 related effects. Hence, the observed *in vivo* effects can be attributed entirely to the absence of a functional T3SS-1.

The *in vivo* attenuation of the SPI-1 deficient mutant strain at 24 hours after inoculation may be ascribed to its decreased capacity to invade the epithelial cells. This is supported by the fact that the wild type strain / SPI-1 mutant strain ratio was more than 100 in the gut tissue, while no significant changes in this ratio were noticed in the contents of the same gut samples compared to the ratio in the inoculum. It may be concluded that a functional SPI-1 secretion system is necessary for the efficient invasion of the porcine intestinal wall.

Figure 5: Light microscopical images from immunohistochemically stained sections of the tonsils obtained from inoculated piglets. In Figure 4A, Immunonegative cocci and in Figure 4B, immunopositive bacilli are shown attached on the surface of the superficial epithelium from a section of tonsil taken at 3 days p.i. Approximate magnification is 1000x.



The SPI-1 deficient strain was unable to persistently colonize the intestines and the associated lymphoid tissue, as shown three days after inoculation. At this stage of infection, it is less straightforward to ascribe this attenuation completely to the defect in invasion. Although the lack of efficient intestinal invasion will probably account for the largest part of the colonization defect observed only a few days after inoculation, other SPI-1 related effects may also play a role. Indeed, SPI-1 has been assigned functions that are important after the phase of invasion in epithelial cells (Steele-Mortimer et al., 2002), although this was not seen in porcine intestinal epithelial cells in our experiments, in macrophages (Hersch et al., 1999; Mukherjee et al., 2001; Boyen et al., 2006a; Drektrah et al., 2006) and in dendritic cells (Van der Velden et al., 2003). It can be concluded that intestinal invasion probably plays an important role in the colonization of the porcine gut.

Both the wild type strain and the SPI-1 deficient strain were present in high and comparable numbers in the porcine tonsils at days 1 and 3 pi. This is in sharp contrast with the intestinal findings. The lack of an invasive defect at day 1 pi may be partially explained by our experimental design, in which the bacteria of the inoculum were grown under conditions that do not allow high SPI-1 expression levels. While SPI-1 expression is gradually induced in the lumen of the gut, this may not be the case in the oral cavity. The fact that the colonization defect was still not detectable 3 days pi may point out that SPI-1 mediated invasion is not a major issue in the colonization of tonsils. The crypts of the porcine tonsils are delineated with lympho-epithelium containing various professional phagocytes (Horter et al., 2003), so SPI-1 mediated invasion may not be a prerequisite to massively enter the tonsillar tissue. Besides, salmonellae may colonize the tonsils by adhering to the epithelium, remaining extracellularly (Horter et al., 2003). Indeed, stained sections of inoculated tonsils showed various immunopositive bacteria attached to the surface of the epithelium, but no immunopositive cells were seen intracellularly in the basal layers of the epithelium or in the lymphoid tissue. The observation that a SPI-1 deficient strain does not show a colonization defect in the tonsils may have important implications and deserves further research.

In conclusion, we have shown that SPI-1 plays a crucial role in the invasion of porcine intestinal epithelial cells by *Salmonella* Typhimurium, thereby contributing to the efficient short term colonization of the porcine gut, and to the induction of influx of neutrophils into the intestinal lumen. However, the invasive and colonizing defect of a SPI-1 mutant strain was absent in the tonsils.

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Role of SPI-1 in the interactions of *Salmonella* Typhimurium with porcine macrophages

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ABSTRACT

Salmonella Pathogenicity Island 1 (SPI-1) genes are indispensable for virulence of *Salmonella* Typhimurium in mice after oral challenge. These genes mediate invasion in intestinal epithelial cells and induce cell death in murine macrophages. The role of SPI-1 in the pathogenesis of *Salmonella* Typhimurium infections in food producing animals is not known. It was the aim of the present study to characterize the interactions of a porcine *Salmonella* Typhimurium field strain and its isogenic mutants in the SPI-1 genes *hilA*, *sipA* and *sipB* with porcine macrophages. SPI-1 was found to be important in the invasion of porcine pulmonary alveolar macrophages (PAM) and the induction of the formation of spacious phagosomes. Both early and delayed cytotoxicity were seen in PAM, but only the early cytotoxicity was SPI-1 dependent. Exposure of PAM to *Salmonella* Typhimurium induced the production of reactive oxygen species (ROS) and interleukin-8, but no differences were noticed between the induction mediated by the wild type strain and its SPI-1 mutant strains. In conclusion, invasion of porcine macrophages and the induction of early, but not delayed, cytotoxicity by *Salmonella* Typhimurium is SPI-1 dependent. SPI-1 dependent invasion, however, is not a prerequisite to induce a pro-inflammatory response.

KEY WORDS

Salmonella Typhimurium – pig – macrophage – SPI-1

INTRODUCTION

Salmonellosis in humans is one of the most important bacterial zoonotic diseases in developing as well as developed countries (Graham et al., 2000; Dalton et al., 2004). In Europe, human salmonellosis is most frequently associated with *Salmonella enterica* subspecies *enterica* serovars Enteritidis and Typhimurium (Anonymous, 2004). These serovars are mainly associated with the consumption of eggs, chicken meat and pork. In the European countries, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is by far the dominant serovar isolated from pigs (Anonymous, 2004). In most cases, *Salmonella* Typhimurium will subclinically colonize the pigs, without causing obvious symptoms (Fedorka-Cray et al., 2000). *Salmonella* Typhimurium is rarely responsible for the septicaemic form of salmonellosis in pigs (Fedorka-Cray et al., 2000). This is in contrast with *Salmonella* Choleraesuis. The latter however is very infrequently isolated in Europe (Fedorka-Cray et al., 2000; Anonymous, 2004). Nevertheless, *Salmonella* Typhimurium is capable of causing severe enterocolitis in pigs. This enterocolitis is characterized by a high morbidity rate, but a low mortality rate (Wilcock and Schwartz, 1992). Infected pigs can shed *Salmonella* for at least 28 weeks (Wood et al., 1989). These carrier pigs are a vast reservoir of *Salmonella* Typhimurium and pose an important threat to animal and human health (Berends et al., 1997; Poppe et al., 1998).

Virulence factors situated on *Salmonella* Pathogenicity Island 1 (SPI-1) are important for the interactions of standard *Salmonella* Typhimurium laboratory strains with intestinal epithelial cells (reviewed by Zhou and Galan, 2001) and macrophages (Monack et al., 2001; Takaya et al., 2005). Most studies on the interaction of *Salmonella* with these cells were carried out with murine cells. Since the course of a *Salmonella* Typhimurium infection in mice is markedly different from that in pigs, biological effects induced by bacterial virulence factors such as SPI-1, may differ. However, data relating to the role of SPI-1 in the interactions of *Salmonella* Typhimurium with macrophages from food producing animals are scarce. It has been suggested that macrophages may play an important role in the generation of the long term carrier state in pigs (Santos and Bäumler, 2004), but a thorough investigation of the interaction between *Salmonella* Typhimurium and pig macrophages is not yet described. It was therefore the aim of the present study to characterize the interactions of a porcine *Salmonella* Typhimurium field strain with porcine macrophages, with special emphasis on the role of SPI-1.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Salmonella Typhimurium strain 112910a was isolated from a pig stool sample and has been shown to persist in tonsils, intestines and gut-associated lymphoid tissue (GALT) of experimentally infected pigs during at least 28 days (unpublished data). This strain was used for the construction of deletion mutants in the genes encoding the major SPI-1 regulatory protein HilA, the SPI-1 translocator/effector protein SipB and the SPI-1 effector protein SipA.

To obtain highly invasive late logarithmic cultures (Lundberg et al., 1999) for invasion assays, spacious phagosome formation, early cytotoxicity assays, Il-8 production and ROS production, 2 µl of a stationary phase culture of the *Salmonella* Typhimurium strain 112910a and its isogenic deletion mutants was inoculated in 5 ml Luria-Bertani broth (LB; Sigma-Aldrich, Steinheim, Germany) and inoculated for 5 hours at 37°C without aeration.

To suppress the SPI-1 mediated early cytotoxic effects in the intracellular survival and late cytotoxicity assays (Lundberg et al., 1999), stationary phase cultures were used. They were obtained by aerated, overnight culture in LB.

In order to obtain high and comparable numbers of intracellular bacteria of both the wild type strain and its isogenic mutant strains, the bacteria used in the intracellular survival and delayed cytotoxicity assays, were opsonized unless otherwise stated. For opsonization, approximately 5 x 10^7 CFU of the *Salmonella* strains were suspended in 1 ml RPMI (Gibco, Life Technologies, Paisley, Scotland) with 2mM L-glutamine (Gibco, Life Technologies, Paisley, Scotland), 1mM sodium pyruvate (Gibco, Life Technologies, Paisley, Scotland) and 10% serum from *Salmonella* free pigs and incubated at 37°C for 20 minutes.

Construction of the deletion mutants

Deletion mutants in virulence genes situated on SPI-1 were constructed according to the one-step inactivation method, using a linear PCR product, first described by Datsenko and Wanner (2000), with some modifications (Donné et al., 2005). Primers used to create the gene-specific linear PCR fragments are summarized in Table 1. In short, these primers and the template plasmid pKD4 DNA were utilized to amplify the linear fragment that was used for the substitution of the gene of interest. The helper plasmid pKD46, encoding the λ Red recombinase was introduced into *Salmonella* Typhimurium strain 112910a by electroporation. Subsequently, the gene-specific linear PCR fragments were introduced into the resulting strain *Salmonella* Typhimurium 112910a (pKD46), again by electroporation. Substitution of

the target genes by a kanamycin resistance cassette was selected for on LB medium containing kanamycin. The substituted loci were transduced into a fresh wild type background using bacteriophage P22HT*int* (Schmieger and Backhaus, 1976). After the elimination of the antibiotic resistance cassette, using the helper plasmid pCP20 encoding the FLP recombinase, the gene was deleted from the start codon till the stop codon. A scar of 83 bp was left in the resulting strains. This was confirmed by sequencing the relevant PCR fragment. At different stages of the construction, bacteriophage P22 sensitivity was tested to confirm the smooth phenotype.

Table 1: Primers used in this study to create the deletion mutants.

Primers	Sequences
hilA forward	5’-TTGTATTAGTTATTATAACTTTTCACCCTGTAAGAGAATACACTATTATCTGTGTAGGCTGGAGCTGCTTC-3’
hilA reverse	5’-CAACCAGATTACGATGATAAAAAAATAATGCATATCTCCTCTCTCAGATTCATATGAATATCCTCCTTAG-3’
sipA forward	5’-TGGAAACCGCCAAAAGCTTCCTGCAAGGATAACAGAAGAGGATATTAATATGTGTAGGCTGGAGCTGCTTC-3’
sipA reverse	5’-TTTTTGACTCTTGCTTCAATATCCATATTCATCGCATCTTTCCCGGTTAACATATGAATATCCTCCTTAG-3’
sipB forward	5’-CTAAAAACGGCGGAGACAGAGCAGCACAGTGAACAAGAAAAGGAATAATTTGTGTAGGCTGGAGCTGCTTC-3’
sipB reverse	5’-ATTCCCACATTACTAATTAACATATTTTTCTCCCTTTATTTTGGCAGTTTCATATGAATATCCTCCTTAG-3’

Isolation of porcine pulmonary alveolar macrophages

Porcine pulmonary alveolar macrophages (PAM) were isolated by broncho-alveolar washes from lungs of euthanized 3-4 week old piglets, obtained from a *Salmonella*-negative farm, as previously described (Dom et al., 1994). Cells derived from three piglets were pooled and frozen in liquid nitrogen in 1 ml aliquots at approximately 10^7cells/ml. These experiments were performed according to animal welfare guidelines and were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Purity of the PAM was checked by flow cytometry, using 74.22.15 anti-SWC3 mouse monoclonal antibody. SWC3 is defined as a specific myelomonocytic antigen of 230 kDa. Forward scatter and side scatter of macrophages were analysed in a FACScalibur flow cytometer (Becton Dickinson, San Jose, California) using CELLQuest software. Viability of the cells was estimated using a trypan blue exclusion test.

Prior to seeding the PAM, frozen aliquots were thawed in phosphate-buffered saline (PBS) with 10% foetal calf serum (Hyclone, Cramlington, England) at 4°C. Cells were washed 3 times in PBS and cultured in RPMI (Gibco, Life Technologies, Paisley, Scotland)

containing 2 mM L-glutamine (Gibco, Life Technologies, Paisley, Scotland) and 1 mM sodium pyruvate (Gibco, Life Technologies, Paisley, Scotland).

Invasion and intracellular survival assays

Macrophages were seeded in 24 well plates at a density of approximately 5 x 10^5 cells per well and were allowed to attach for at least 2 hours. These wells were inoculated with the different *Salmonella* strains at a multiplicity of infection (moi) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at 365 x g for 5 min. After 25 min incubation at 37°C under 5% CO_2, the wells were rinsed and fresh medium supplemented with 100 µg/ml gentamicin (Gibco, Life Technologies, Paisley, Scotland) was added. After an additional 30 min incubation at 37°C under 5% CO_2, the wells were rinsed again three times.

For the invasion assays, the macrophages were lysed with 1% Triton-X100 (Sigma-Aldrich, Steinheim, Germany) for 10 min and 10-fold dilutions were plated on brilliant green agar (BGA) plates. To assess intracellular growth, the medium containing 100 µg/ml gentamicin was replaced after the 30 min incubation time with fresh medium supplemented with 15 µg/ml gentamicin and the number of viable bacteria was assessed 6 hours after infection as described above.

Spacious phagosome formation

In order to follow up the formation of spacious phagosomes in PAM, the cells were seeded and infected as described above with the wild type strain and its isogenic SPI-1 mutant strains. Non-infected cells were used as controls. After the indicated time points, the cells were rinsed carefully with PBS and fixed with a solution of 10% formaldehyde in PBS. Phase contrast micrographs were taken at a magnification of 400 using a Nikon Eclipse TS100 light microscope and a Minolta Dimage 7 Hi digital camera.

Cytotoxicity assays

Macrophages were seeded in 96 well plates at a density of approximately 10^5 cells per well and were allowed to attach for at least 4 hours. The wells were inoculated with the different strains at a moi of 10:1 after opsonization as described above. Six and 20 hours after inoculation, the wells were carefully rinsed. The surviving adherent cells were fixed with 10% formaldehyde in PBS and stained with 1% crystal violet in PBS. The absorption at wavelength 650 nm was read on a microplate reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland) as a measure for cell detachment. The percentages of *Salmonella* induced

cytotoxicity for the wild type strain and its isogenic mutant strains were calculated using the following formula:

$$\% \text{ cytoxicity} = 100 \times \frac{(c - b) - (a - b)}{(c - b)}$$

In this formula:

a = OD_{650} derived from the wells inoculated with *Salmonella* Typhimurium

b = OD_{650} derived from blank wells

c = OD_{650} derived from uninfected control wells

For comparison, the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes Europe, Leiden, The Netherlands), using calcein AM as fluorescent marker for viable cells, was used according to the manufacturers guidelines. In short, PAM were seeded and inoculated as described for the crystal violet cytotoxicity assay. After the appropriate time points, the cells were washed with PBS to remove interfering extracellular esterases. Viable cells were dyed with 200 µl of a 1 µM calcein solution in PBS during 45 minutes. Fluorescence was measured using a Fluoroscan Ascent Fl (Thermo Labsystems, Helsinki, Finland) and the excitation/emission filters of 485/527 nm respectively. As a positive control PAM maintained in culture medium were used and as a negative control, PAM incubated for 30 minutes with 70% methanol in PBS were used.

Macrophage chemiluminescence

Reactive oxygen species (ROS) production was measured using a chemiluminescence technique with Lucigenin (Sigma Biosciences, St. Louis, USA) diluted in HBSS to final assay concentrations of 400 µM as chemiluminogenic probe (Donné et al., 2005).

In short, PAM were seeded in 96-well plates at 10^6 cells per well in 100 µl RPMI. Spontaneous CL (PAM without triggering agents) was recorded for 10 minutes. The CL reaction was started by adding 50 µl/well of the bacterial suspensions at 10 bacteria per macrophage and the production of ROS was measured during 1 h. The CL response is expressed as Relative Light Units per minute (RLU). As a positive control, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma Biosciences, St. Louis, USA). PAM seeded in RPMI were used as negative controls.

Interleukin-8 production

Porcine macrophages were seeded and inoculated as described above. Four hours after infection, supernatant was collected, filtered and stored at -80°C. A commercial ELISA kit (cytoscreen swine Il-8, Biosource International, Camarillo, USA) was used to quantify the Il-8 production. The absorption at wavelength 450 nm was read on a microplate reader (Multiscan MS, Labsystems) as a measure for Il-8 production. A standard curve was prepared using dilution series of porcine Il-8 provided in the ELISA kit. *Salmonella* Typhimurium lipopolysaccharide (LPS; Sigma-Aldrich, Steinheim, Germany) was added as a positive control at 10 µg/ml, negative control wells were incubated with culture medium only.

Statistical analysis

All experiments were carried out in triplicate with three repeats per experiment, unless otherwise stated. In assays with high inter-experimental variabilities, the results were divided by the mean of the respective experiment to reduce inter-experimental variations. The data were analysed by one-way analysis of variance methods, using the SPSS 12.0 software for Windows. Bonferroni corrections were applied for all results.

RESULTS

Macrophage yield

The average yield of pooled PAM isolated from 3 piglets was 4 x 10^8 cells. The purity varied between 90% and 97% and the viability was more than 95%.

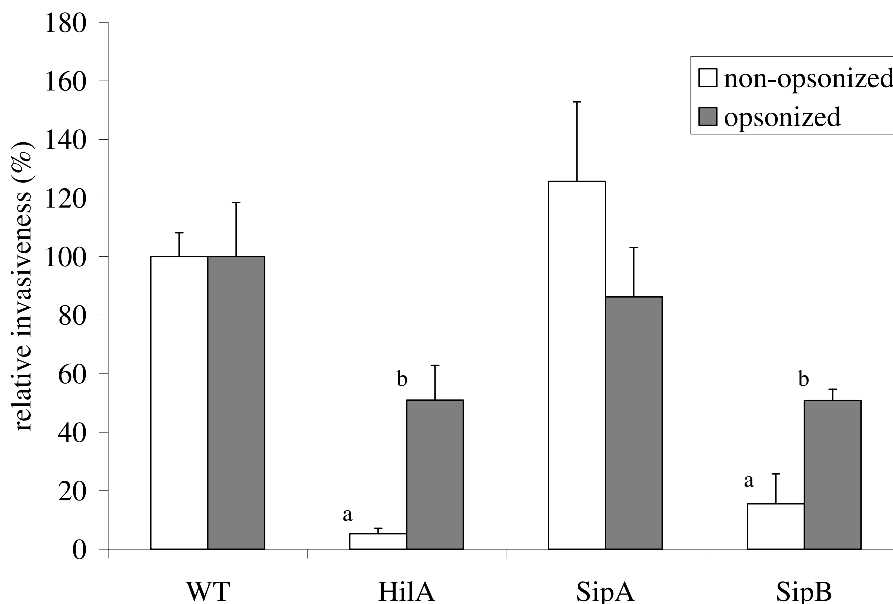
Invasion, but not replication, of *Salmonella* Typhimurium in PAM is hilA and sipB, but not sipA dependent

Invasion of the *Salmonella* Typhimurium 112910a wild type strain in porcine PAM was compared with the invasion of its isogenic deletion mutant strains in a gentamicin protection assay. The results are summarized in Figure 1. One hour post inoculation, the *hilA* and *sipB* mutant strains invaded the macrophages approximately 10 times less than the wild type strain (statistically significant, $p < 0.005$). The *sipA* mutant strain did not show any significant differences with the wild type strain ($p > 0.05$). Opsonization with serum derived from *Salmonella*-free pigs resulted in a partial loss of the differences between the invasion rates of the wild type strain and its isogenic SPI-1 mutants, as visualized in Figure 1.

The invasion rates of the *hilA* and *sipB* mutant strains were nevertheless still significantly reduced compared to the opsonized wild type strain ($p < 0.05$).

The SPI-1 mutant strains did not show significant differences in intracellular replication compared to the wild type strain (data not shown).

Figure 1. The invasiveness of the *Salmonella* Typhimurium 112910a wild type strain and its isogenic SPI-1 mutant strains in PAM with (grey bars) and without (white bars) opsonization with pig serum is shown. The invasiveness of the opsonized and non-opsonized strains are expressed as percentages relative to the invasiveness of the opsonized and non-opsonized wild type strain respectively and no conclusions can be drawn concerning absolute numbers of intracellular bacteria between both groups. The results represent the means of at least 3 independent experiments conducted in triplicate and their standard error of the means. Superscript (a) refers to significantly lower invasion relative to the unopsonized wild type strain ($p < 0.005$); superscript (b) refers to significantly lower invasion relative to the opsonized wild type strain ($p < 0.05$).

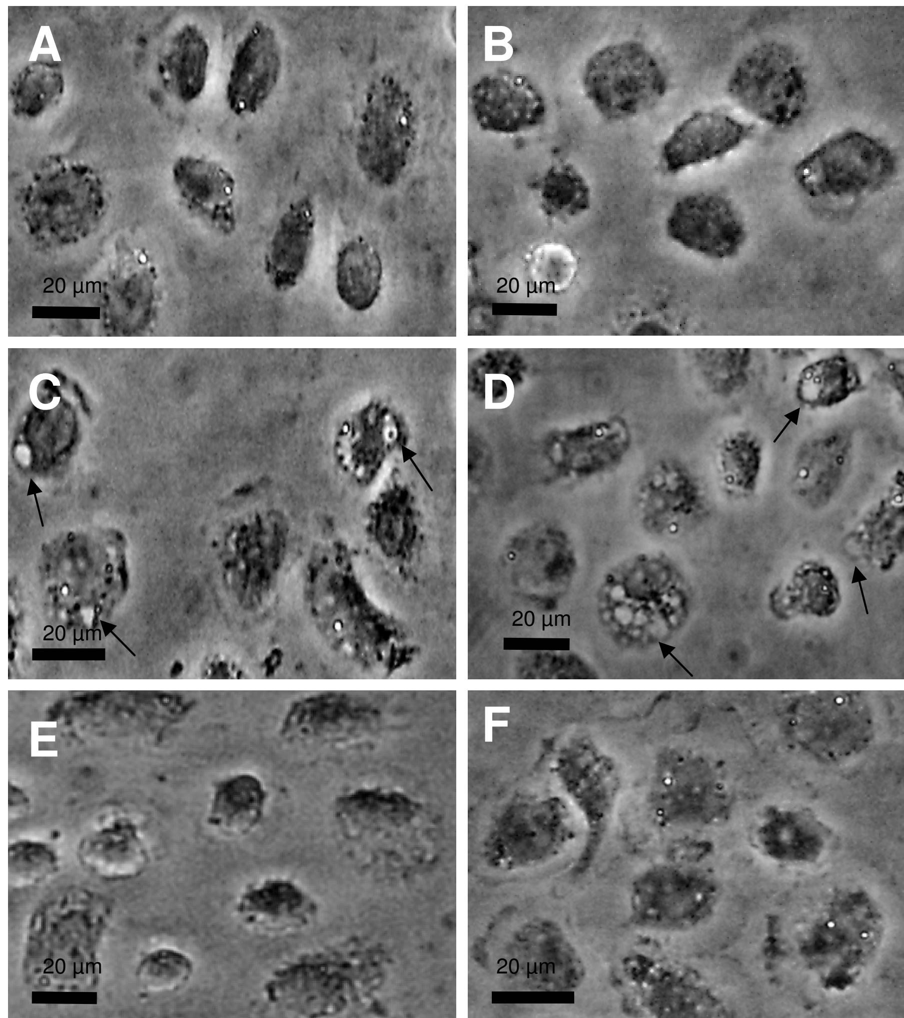


The formation of spacious phagosomes in PAM is hilA and sipB, but not sipA dependent

From five minutes after inoculation, the salmonellae were associated with the macrophages and some early spacious phagosomes were apparent. No spacious phagosomes were seen in the negative control cells. The number of spacious phagosomes was greatly reduced in macrophages infected with the *hilA* mutant or the *sipB* mutant strain. No differences were noticed between the wild type and the *sipA* mutant. The spacious phagosomes remained visible throughout the experiment. Porcine macrophages can be heavily infected, showing numerous phagosomes or very large vacuoles containing multiple

salmonellae. In Figure 2, pictures are shown of PAM inoculated with the wild type strain and the *hilA* mutant strain, compared with sham-inoculated PAM, 5 minutes and 2 hours post inoculation. Pictures of PAM inoculated with the *sipA* or *sipB* mutant strains are not shown.

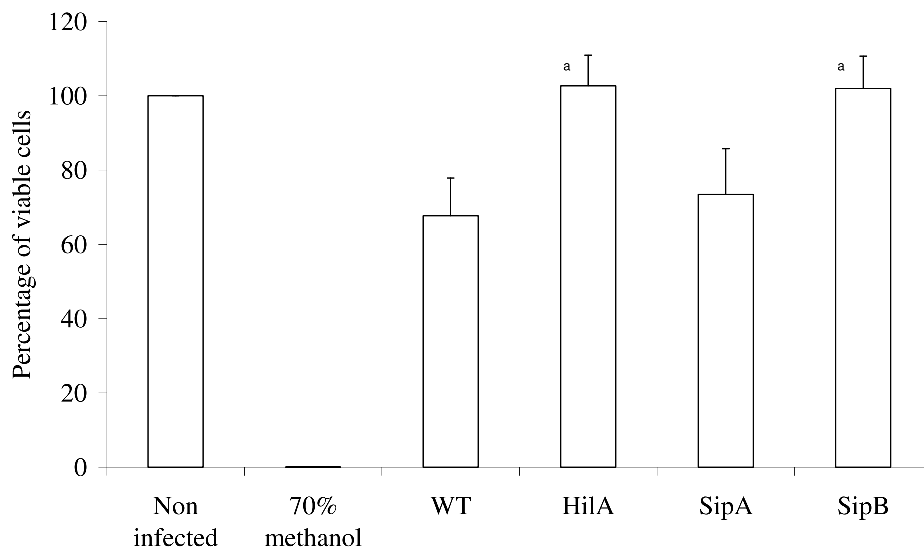
Figure 2. Pictures of PAM cells sham inoculated (A, B), inoculated with *Salmonella* Typhimurium 112910a (C, D) and inoculated with 112910a*ΔhilA* (E, F). Pictures were taken after an incubation time of 5 min (A, C, E) and 2h (B, D, F) respectively. The spacious phagosomes are indicated by an arrow.



Early *Salmonella*-induced cytotoxicity of PAM is hilA and sipB, but not sipA dependent

Six hours after inoculation, no significant cytotoxicity was noticed in the crystal violet cytotoxicity assay compared to non-inoculated wells (data not shown). In the fluorescent calcein viability assay, however, approximately 40% less viable PAM were found in the wells inoculated with the wild type strain and the *sipA* mutant strain. The wells inoculated with the *hilA* and *sipB* mutant strains showed significant higher numbers of viable cells ($p < 0.005$) compared to the PAM inoculated with the wild type strain. The results are shown in Figure 3.

Figure 3. The Percentage of viable PAM 6h after the inoculation with *Salmonella* Typhimurium 112910a wild type strain and its isogenic SPI-1 mutant strains, as measured by the fluorescent calcein viability assay.. The results are expressed relative to the amount of viable cells after 6 h incubation in PAM culture medium. As a control for 100% viable cells, non inoculated PAM were maintained in culture medium. PAM incubated for 30 minutes with 70% methanol in PBS were used as a control for dead cells. The results represent the means of 2 independent experiments conducted in 4 fold and their standard error of the means. Superscript (a) refers to significantly higher numbers of viable cells compared to the wells inoculated with the wild type strain ($p < 0.005$).



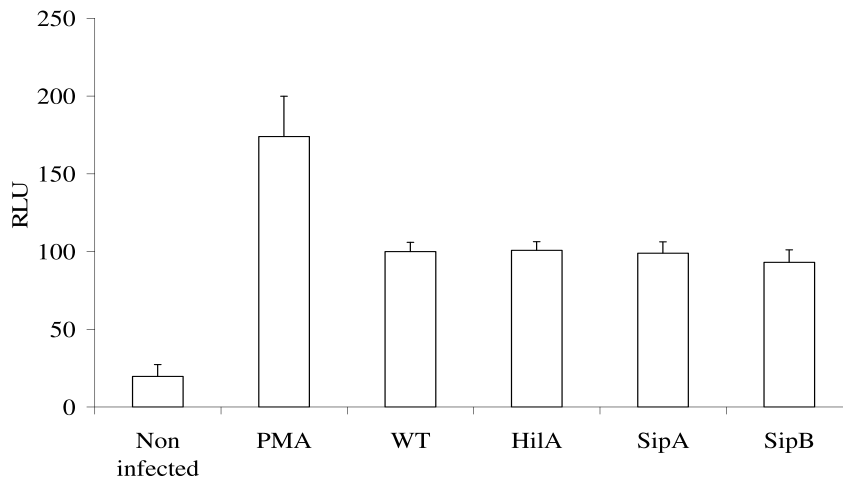
Delayed *Salmonella*-induced cytotoxicity of PAM is SPI-1 independent

In contrast to the observations 6 hours after incubation, approximately 50% of the macrophages were detached from the wells in the crystal violet cytotoxicity assay when they were inoculated with the *Salmonella* Typhimurium wild type strain for 20 hours. No significant differences ($p > 0.05$) were seen between the wild type strain and any of the SPI-1 deletion mutant strains (data not shown). Comparable results were obtained with the Calcein AM viability assay (data not shown).

ROS production by PAM is SPI-1, and thus invasion independent

The porcine macrophages were able to produce ROS after stimulation with phorbol 12-myristate 13-acetate (PMA) and after inoculation with *Salmonella* Typhimurium 112910a. The results are summarized in Figure 4. No significant differences ($p > 0.05$) were noticed between the wild type strain and any of the SPI-1 mutant strains.

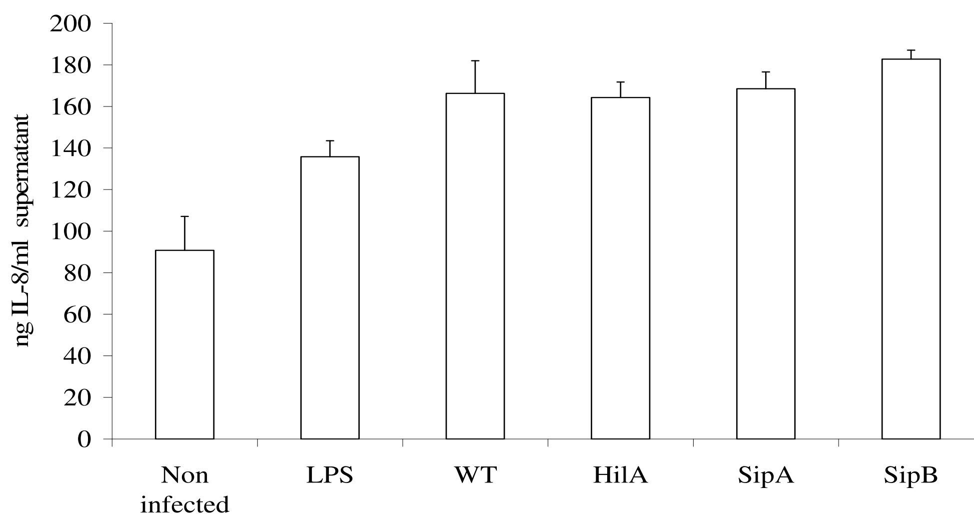
Figure 4. The ROS production of PAM after stimulation with the *Salmonella* Typhimurium 112910a wild type strain and its isogenic SPI-1 mutant strains. As a standard stimulus phorbol 12-myristate 13-acetate (PMA) was used and non inoculated PAM in RPMI were used as a negative control. The results represent the means of at least 3 independent experiments conducted in triplicate and their standard error of the means.



Interleukin-8 production is SPI-1, and thus invasion independent

The PAMs produced increased amounts of IL-8 after stimulation with both LPS and viable *Salmonella* Typhimurium. There were no significant differences ($p > 0.05$) between the wild type strain and its isogenic SPI-1 mutant strains. These results are illustrated in Figure 5.

Figure 5. The Il-8 production of PAM after stimulation with the *Salmonella* Typhimurium 112910a wild type strain and its isogenic SPI-1 mutant strains. As a standard stimulus *Salmonella* LPS was used and non inoculated PAM in culture medium were used as a negative control. The figure shows the results of a representative of 3 experiments conducted in triplicate.



DISCUSSION

The virulence genes of *Salmonella* Typhimurium situated on SPI-1 are necessary for invasion of non-phagocytic (reviewed in Zhou and Galan, 2001; Altier, 2005) and phagocytic cells (Monack et al., 1996; Forsberg et al., 2003). The importance of SPI-1 in the invasion of porcine macrophages *in vitro* was documented in our study, since both a mutation in a major regulator (*hilA*) and in a translocator (*sipB*) of SPI-1, leading to a deficient SPI-1 type three secretion system (TTSS), resulted in a 10-fold decrease of invasion and associated formation of spacious phagosomes. Jepson et al. (2001) found that a mutant in *sipA* is less invasive in Madin-Darby canine kidney (MDCK) cells than its parental strain at the earliest stages of infection. But this effect was no longer apparent 15 min after inoculation. This is in agreement with the obtained results, since we did not find any significant differences in invasion with the wild type strain 1 hour after inoculation. As in other host species, opsonization with negative serum reduces the differences in invasion (Buchmeier and Heffron, 1991; Watson et al., 2000). Although the invasion of *Salmonella* in macrophages and phagocytosis of *Salmonella* by macrophages has been studied *in vitro* (Forsberg et al., 2003; Valle and Guiney, 2005), the relative importance of both phenomena *in vivo* is not clear. Also, the contribution of complement mediated and antibody mediated opsonization *in vivo* is not known and needs further study.

Salmonella is capable of inducing cell death in macrophages in 2 stages (reviewed by Hueffer and Galan, 2004). An early SipB dependent form of cell death occurs within a few minutes to hours after infection (Monack et al., 1996; Schwan et al., 2000; Valle and Guiney, 2005). Salmonellae that do not cause this rapid cell death and reside in the phagocytic vacuole can trigger a SPI-2 or *spv* dependent cell death 12 to 24 hours after infection (Libby et al., 2000; Van der Velden et al., 2000; Monack et al., 2001). With the use of calcein AM as a fluorescent marker for viable cells, a marked difference in early cytotoxicity was found between the wild type strain and SPI-1 TTSS deficient mutant strains. These differences were less obvious when the crystal violet cytotoxicity test was used. When PAM were given more time to die and detach (as in the delayed cytotoxicity test), both assays led to comparable results. The crystal violet cytotoxicity test has been used with success to detect the cytotoxic effect of *Salmonella* Typhimurium on murine macrophages (Monack et al., 1996). However, while early macrophage cytotoxicity is very fast and explicit in murine macrophages, the cell death induction in porcine PAM was less pronounced and dying PAM did not seem to detach

from the wells as easily. This may explain why the crystal violet cytotoxicity test failed to measure the early cytotoxicity in PAM.

Murine macrophages respond to *Salmonella* infection by the production of reactive oxygen species (ROS) during the respiratory burst, potentially resulting in the destruction of the invading bacteria (Tomita et al., 1981; Vazquez-Torres et al., 2000). Exposure of porcine PAM to viable salmonellae also resulted in the induction of ROS production. The SPI-1 mutants induced ROS production similar to the wild type. Induction of ROS may thus be invasion independent. Since no inducible reactive nitrogen intermediates (RNI) production can be detected in porcine PBM (Donné et al., 2005), the effect of SPI-1 on the RNI production in PAM was not investigated.

Interleukin-8, secreted from the basolateral side of infected epithelial cells, is an important chemokine in the pathogenesis of salmonellosis. It plays a key role in the initial movement of neutrophils from the circulation into the subepithelial region of the gut in several animal species, including swine (McCormick et al., 1995; Cho and Chae, 2003; Trebichavsky et al., 2003; Zeng et al., 2003). Macrophages are able to produce IL-8 as well, in response to stimulation of toll-like receptors (Zeng et al., 2003; Zughaier et al., 2005). In our model, invasion was not a prerequisite to induce the secretion of Il-8 by macrophages. Since the toll-like receptors are situated on the cell membrane of the macrophage, this is not surprising.

In summary, invasion of porcine macrophages and the induction of early, but not delayed, cytotoxicity by a *Salmonella* Typhimurium field strain is SPI-1 dependent. SPI-1 mediated invasion, however, is not a prerequisite for the production of ROS or Interleukin-8 by porcine macrophages upon contact with *Salmonella* Typhimurium.

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A limited role for Ssra in persistent *Salmonella* Typhimurium infections in pigs

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ABSTRACT

Virulence genes regulated by the SsrA/B system are indispensable for systemic disease in BALB/c mice. The role of this regulating system in the pathogenesis of *Salmonella* Typhimurium infections in pigs is not documented. In the present study, the interactions of *Salmonella* Typhimurium and an *ssrA* deletion mutant were compared *in vitro* and *in vivo*. The *ssrA* mutant strain displayed decreased *Salmonella* Pathogenicity Island 2 (SPI-2) expression levels, showed a replication defect in mouse macrophages and was attenuated in a mouse model after oral inoculation. Using real time RT-PCR and a porcine intestinal loop model, it was shown that the *ssrA* mutant strain was not significantly attenuated in overall virulence and SPI-1 expression in specific. Flowcytometric analysis demonstrated that the *ssrA* mutant strain was defective in intracellular replication in porcine macrophages. After oral inoculation of piglets with the wild type strain or the *ssrA* mutant strain, the animals of both groups excreted *Salmonella* and were colonized by *Salmonella* to the same extent. In an intravenous mixed infection model, the *ssrA* mutant strain was defective in the colonization of several organs. These results suggest that the *ssrA* gene of *Salmonella* Typhimurium plays a limited role in the persistent colonization of pigs.

KEY WORDS

Salmonella Typhimurium – pig – macrophage – ssrA

INTRODUCTION

Salmonellosis is one of the leading zoonoses in the world. Human infections with non-typhoidal *Salmonella* are generally foodborne and are a major public health concern. Over the last two decades, the emergence of *Salmonella* strains carrying multiple antibiotic resistance genes has led to an increased risk for hospitalization, invasive illness, and death. This is particularly the case for *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium; Velge et al., 2005).

Second to *Salmonella* Enteritidis, *Salmonella* Typhimurium is the most common serotype associated with human salmonellosis in Europe (Fisher et al., 2004). Due to a drop in *Salmonella* Enteritidis infections in poultry in 2005 and 2006, the relative importance of *Salmonella* Typhimurium infections in pigs will increase the next few years. *Salmonella* Typhimurium is the most frequently isolated serotype from pigs and pork (Anonymous, 2004). Infected pigs can shed *Salmonella* for at least 28 weeks (Wood et al., 1989). These carrier pigs are a vast reservoir of *Salmonella* Typhimurium and pose an important threat to animal and human health (Berends et al., 1997). The mechanism underlying this carrier state of infection is unknown.

The virulence genes located on *Salmonella* Pathogenicity Island 2 (SPI-2), which encodes a type III secretion system (T3SS), may play a role in the persistence of *Salmonella* in food-producing animals since these genes are indispensable for the induction of systemic disease and persistence in mice (Cirillo et al., 1998; Hensel et al., 1998). The two-component regulatory system, *ssrAB*, responds to environmental signals (Löber et al., 2006) and controls the expression of the type III secretion system and the secreted effector proteins. Through interaction with the intracellular traffic of macrophages and dendritic cells, the injected SPI-2 effector proteins manage to create a safe niche for the salmonellae inside the phagocyte.

It becomes increasingly clear that the pathogenesis of *Salmonella* infections varies depending on the host - strain combination (Pasmans et al., 2003, Morgan et al., 2004). Therefore, pathogenesis studies that use both the host species of interest and relevant *Salmonella* strains are of crucial importance.

The aim of the present study was to determine the contribution of SsrA to the colonization and persistence of pigs by a *Salmonella* Typhimurium field strain.

MATERIALS AND METHODS

Bacterial strains and plasmids

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) strain 112910a was isolated from a pig stool sample on a pig farm with a persistent *Salmonella* problem and was used as the wild type strain (WT) in which all mutant strains were constructed. The construction and characterization of the non-polar deletion mutant in the major SPI-1 regulatory protein HilA, has been described before (Boyen et al., 2006a,b). A mutant strain in which *ssrA* was deleted (designated SsrA) was constructed accordingly. Briefly, *ssrA* was first substituted by a PCR-modified kanamycin resistance cassette with the aid of a helper plasmid encoding the λ Red system. Primers used to create the PCR- modified resistance cassettes for the *ssrA* gene were 5’-TAGTGATCAAGTGCCAAAGATTTTGCAACAGGCAACTGGAGGGAAGCATTTGTGTAGGCTGGAGCTGCTTC-3’ and 5’-AAGATCTTATATTCTTTCATTTTGCTGCCCTCGCGAAAATTAAGATAATACATATGAATATCCTCCTTAG-3’. The mutant allele was subsequently transduced, using bacteriophage P22HT*int*, in a fresh wild type *Salmonella* background to discount effects on virulence due to unlinked mutations. In the last step, the antibiotic resistance cassette was eliminated using the helperplasmid pCP20. The targeted gene was completely deleted from the start codon till the stop codon. This was confirmed by sequencing.

For all *in vitro* experiments and the loop assays, the WT and SsrA strains were used. For the *in vivo* assays, an invasive, spontaneous nalidixic acid resistant derivative (WT_{nal}) was used. The *ssrA*::kan mutation was moved by P22 transduction into the WT_{nal} strain, resulting in the $SsrA_{kan/nal}$ strain. This strain was used in the mixed *in vivo* assay. For the oral infection model, the kanamycin resistance cassette was eliminated as described above, resulting in the $SsrA_{nal}$ strain. At different stages of the construction, bacteriophage P22 sensitivity was tested to confirm the smooth phenotype.

For flowcytometric analysis, fluorescence microscopy and confocal imaging, the pFPV25.1 plasmid expressing green fluorescent protein under the constitutive promoter of *rpsM* was used (Valdivia and Falkow, 1996).

Cell cultures and growing conditions

The mouse macrophage cell line RAW 264.7 or porcine pulmonary alveolar macrophages (PAM) were used for all experiments. PAM were isolated and kept in culture as previously described [Dom et al., 1994; Boyen et al., 2006a).

For intracellular survival assays, stationary phase cultures were obtained following growth overnight with aeration in Luria-Bertani broth and opsonized with serum obtained from *Salmonella* negative pigs. For the mouse infection model, bacterial strains were grown to stationary phase, washed in phosphate buffered saline (PBS) and resuspended at a concentration of ~ 2.5 x 10^9 CFU/ml in PBS. The inocula for the oral infection models and the intestinal loop model were prepared as described previously (Boyen et al., 2006c). For the intravenous infection assay, overnight cultures of both strains were assembled, washed three times in PBS and resuspended to obtain ~ 3 x 10^8 CFU/ml in PBS.

SPI-1 and SPI-2 expression studies

The relative expression levels of the *hilA* (encoding a major SPI-1 regulator), *sipA* (encoding a SPI-1 effector), *ssrA* (encoding a major SPI-2 regulator) and *sifB* (encoding a T3SS-2 secreted effector) genes of the *Salmonella* Typhimurium wild type strain and the *ssrA* mutant strain were compared in LB broth. A *hilA* mutant strain was used as an internal control.

Total RNA was extracted from 1 ml of the bacterial culture using the RNeasy Mini Kit and the RNAprotect Bacteria reagent (Qiagen, Valencia, USA) followed by a DNase treatment. To quantify gene expression, the quantitative real time reverse transriptase PCR method (qRT-PCR) according to Botteldoorn et al. (2006) was used. Primers used are shown in Table 1. As control house keeping genes 16S rRNA, *gmk* and *rpoD* were used. The normalisation factor (NF) was calculated using the GeNorm software (Vandesompele, 2002).

Table 1: Primers used for quantitative real time PCR (5'- 3' directed)

gene	Nucleotide sequence forward primer	Nucleotide sequence reverse primer
hilA	GGTTCAATCCGAGAGTCTGCAT	AGGCCAAAGGGCGCATA
sipA	GGCTTGCGTGCGGAAATA	ATCGCTACATTGCGCTTTCA
ssrA	GGCCAGTGAGCGATGTAGTA	AAATCCCCTTTACATTAACAGCATTG
sifB	GAGGCAACATCACATAATTCCTTTATATAT	GCTCITTCTTTACGTTACTATGGGAAA

Intestinal loop model

To check the behaviour of the *ssrA* mutant strain during the intestinal phase of infection, an intestinal loop model was performed on 6-week-old piglets. This model has been described in detail elsewhere (Watson et al. 1995; Boyen et al., 2006b). In short, intestinal loops of 6-7 cm in length were ligated and inoculated with 1-2 x 10^9 CFU *Salmonella* Typhimurium 112910a or its isogenic deletion mutants in *hilA* and *ssrA*. Approximately 10 ml of blood was removed from the piglets to isolate the polymorphonuclear leucocytes (PMNs). The isolated PMNs were labeled with ^{111}Indium and reinjected intravenously. The influx of PMNs in the intestinal wall and in the lumen of the gut, as assessed by the counts per minute (cpm) emitted from ^{111}Indium-labelled PMNs within each loop, was recorded 12 h after injection of the loops. A *hilA* mutant strain was used as an internal control.

Flow cytometric analysis of intracellular replication

To assess the intramacrophagal replication deficit of the *ssrA* mutant strain, RAW 264.7 cells or PAM were seeded in 25 cm^2 culture flasks at a density of approximately 5 x 10^6 cells per flask and were allowed to attach overnight. The cells were inoculated with the wild type strain or the *ssrA* mutant strain carrying the pFPV25.1 plasmid, at a multiplicity of infection (moi) of 1:1. To synchronize the infection, the flasks were centrifuged at 365 x g for 5 min. After 25 min incubation at 37°C under 5% CO_2, the cells were washed and fresh medium supplemented with 100 µg/ml gentamicin was added. After additional 60 min incubation at 37°C under 5% CO_2, the cells were washed. To assess the initial bacterial load, cells were released using trypsin and maintained on ice, protected from light until use. To assess intracellular growth, fresh medium supplemented with 15 µg/ml gentamicin was added and cells were released and handled as described 6 hours after inoculation.

Flow cytometric measurements were made using a FACScanto™ cytometer (Becton-Dickinson, Erembodegem, Belgium). Macrophages were discriminated from bacteria and debris based on forward (FSC) and side (SSC) light scatter. GFP fluorescence was recorded using the FL1 channel (emission wavelength: 515-545 nm). Data were expressed in arbitrary units and both the average fluorescence and the median fluorescent value of infected macrophages were calculated from the fluorescence histograms using the FACSDiva software (Becton-Dickinson, Erembodegem, Belgium).

Experimental infection of BALB/c mice

Seven-week-old female BALB/c mice were randomly divided in 2 groups, anesthetized with isoflurane and inoculated orally with ca. 1 x 10^8 CFU of *Salmonella* Typhimurium 112910a or *Salmonella* Typhimurium 112910aΔ*ssrA*. On day 1 and day 4 after inoculation, a subset of animals of both groups were humanely killed. For each animal, cecum, spleen and liver were removed, homogenized and the number of CFU/g tissue was determined on brilliant green agar (BGA) plates.

Experimental infections of piglets

In the experimental infection studies, 5-week-old piglets, obtained from a serologically negative breeding herd, that were negative for *Salmonella* at fecal sampling were used. Oral experimental infections were performed in piglets as described before (Boyen et al., 2006c). In short, animals of groups 1 and 2 were orally inoculated with ca. 1 x 10^7 CFU of *Salmonella* Typhimurium 112910a and *Salmonella* Typhimurium 112910aΔ*ssrA* respectively in 2 ml PBS. Group 3, the negative control group, was sham-inoculated with 2 ml PBS. The rectal temperature and the clinical condition were monitored and fresh fecal samples were collected on several days for bacteriological analysis. On days 5 and 28 pi, 5 piglets of each group were euthanized. Samples of tonsils, mandibular lymph nodes, lung, heart, liver, spleen, kidney, ileocecal lymph nodes, jejunum, ileum, cecum and contents of jejunum, ileum and cecum were taken for bacteriological analysis. All samples were examined for the presence of the *Salmonella* strains by plating tenfold dilutions on BGA supplemented with 20 µg/ml nalidixic acid. If negative at direct plating, the samples were pre-enriched in Buffered Peptone Water (BPW), enriched in tetrathionate broth and plated on BGA supplemented with 20 µg/ml nalidixic acid. Samples that were negative after direct plating but positive after enrichment were presumed to contain 83 CFU/g. Samples that remained negative were presumed to contain 0 CFU/g.

For the mixed infection assay, 11 experimental animals were intravenously inoculated with approximately 1.5 x 10^8 CFU of each strain of a 1:1 mixture of the WT_{nal} and the $SsrA_{kan/nal}$ strains in 0.5 ml PBS. The negative control group consisted of 3 piglets which were sham-inoculated with PBS. The *Salmonella*-inoculated piglets were euthanized on days 1 (5 piglets) and 3 (6 piglets) after inoculation. Sham-inoculated piglets were euthanized 3 days after inoculation. Samples of tonsils, lung, liver, spleen, kidney, bronchial, mesenterial and ileocecal lymph nodes, ileum and cecum were taken for bacteriological analysis. The ileum and cecum tissue samples were separated from their contents and were rinsed in PBS. Both

the contents and the rinsed intestinal samples were bacteriologically examined. All samples were processed as described before (Boyen et al., 2006b). The ratio WT_{nal} / $SsrA_{kan/nal}$ was calculated for all samples derived from each piglet. These data were converted logarithmically prior to statistical analysis. The appropriate detection limits were used to estimate the minimum ratios when samples were not positive after direct plating.

Ethical considerations

The animal work presented here was approved by the Ethical committee of the Faculty of Veterinary Medicine of the Ghent University (EC 2004/103 and EC 2006/104) and in the UK were conducted according to the requirements of the Animal (Scientific Procedures) Act 1986.

Statistical analysis

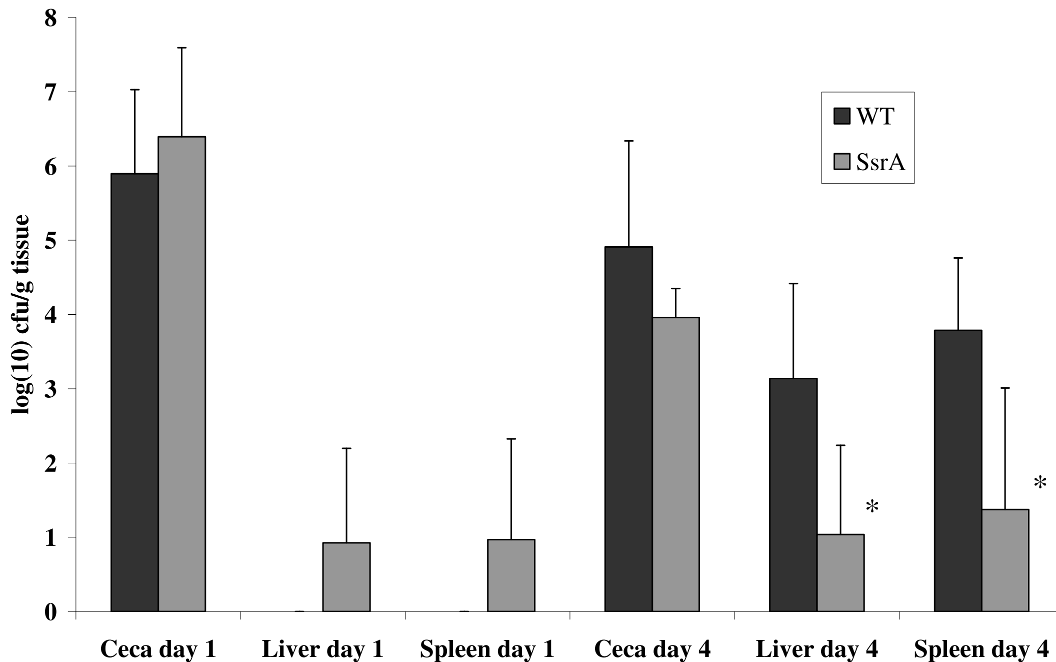
All *in vitro* experiments were carried out in triplicate with three repeats per experiment, unless otherwise stated. The data from the intestinal loops were analyzed by one-way analysis of variance methods, using the SPSS 12.0 software for windows. A Student's t-test was used to determine whether the flow cytometric fluorescence values of both strains differed and to determine whether the log value of the WT_{nal} / $SipB_{kan/nal}$ ratio of the samples was significantly different from the log value of the WT_{nal} / $SipB_{kan/nal}$ ratio of the inoculum. In the oral infection assay, statistical analysis was performed using a non-parametric Kruskal-Wallis test. Differences with a P value ≤ 0.05 were considered significant.

RESULTS

The *ssrA* mutant strain is attenuated in a BALB/c infection model

SPI-2 deficient *Salmonella* Typhimurium strains are severely attenuated in virulence in a BALB/c infection model. Deletion of the *ssrA* gene resulted in a colonization defect in this model, as shown in Figure 1. Although the *ssrA* mutant strain and the wild type strain were found in similar numbers in the murine ceca 1 day after inoculation, the *ssrA* mutant strain was found in lower numbers in the ceca 4 days after inoculation, though not statistically significant ($P > 0.05$). The *ssrA* mutant strain was found in significantly ($P < 0.05$) lower numbers in liver and spleen 4 days after inoculation.

Figure 1: Recovery of bacteria from the ceca, liver and spleen from 4 BALB/c mice at days 1 and 4 after oral inoculation with either WT_{nal} or $SsrA_{nal}$. The results represent the mean log values of the number of cfu per gram tissue and their standard deviation. An asterisk refers to a significantly lower number of $SsrA_{nal}$ cfu compared to the wild type strain ($p < 0.05$).



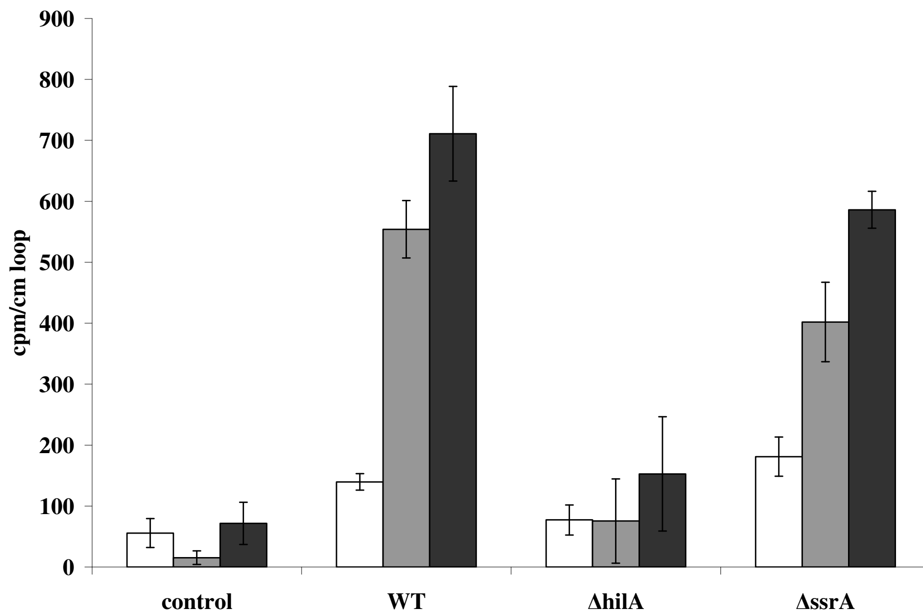
The *ssrA* mutant strain is defective in SPI-2 expresssion

To check the deletion of *ssrA* and the expression of downstream T3SS-2 -secreted effector genes, the expression levels of *ssrA* and *sifB* (Miao and Miller, 2006) were measured using qRT-PCR. The *ssrA* gene was expressed in the wild type strain and the *hilA* mutant strain, but not in the *ssrA* mutant strain. The expression of *sifB* was considerably decreased in the *ssrA* mutant strain. Both the expression of *hilA* (a major SPI-1 regulator) and *sipA* (coding for a SPI-1 effector protein) were dramatically diminished in the *hilA* mutant strain, but not in the *ssrA* mutant strain (results not shown).

The *ssrA* mutant strain elicits an intestinal inflammatory response

In the porcine intestinal loop assays, the virulence of the wild type strain and the *ssrA* mutant strain in the intestinal phase of the infection were compared. Twelve hours post inoculation, both the wild type strain and the *ssrA* mutant strain, but not the *hilA* mutant strain, induced intestinal inflammation. There was no statistically significant difference in neutrophil influx between the wild type strain and the *ssrA* mutant strain ($P > 0.05$). These results are shown in Figure 2.

Figure 2: Radio-active γ counts per minute per cm loop as a measure for the early PMN influx elicited by *Salmonella* Typhimurium strains in porcine intestinal loops. White bars represent the influx of neutrophils into the gut tissue, light grey bars the influx into the lumen of the gut and dark grey bars the total neutrophil influx into the gut. Loops inoculated with the wild type strain and the *ssrA* mutant strain contained significantly ($P < 0.05$) more neutrophils than the control loops and the loops inoculated with the *hilA* mutant strain. The wild type strain did not induce significantly more neutrophil influx than the *ssrA* mutant strain ($P > 0.05$).



The *ssrA* deletion mutant shows impaired intracellular replication in murine and porcine macrophages

At time point 0 h, both the mean fluorescence and the median fluorescent value of the infected RAW 264.7 cells and PAM were similar for the wild type strain and the *ssrA* mutant strain. At 6 h after inoculation, however, both the mean fluorescence and the median fluorescent value of the infected macrophages was significantly higher in the cells infected with the wild type strain, compared to the cells infected with the *ssrA* mutant strain. These results are shown in Table 2.

The *ssrA* deletion mutant is not impaired in porcine colonization after oral inoculation

Sham-inoculated control piglets did not develop disease signs and *Salmonella* was not isolated from any of the samples taken from these animals throughout the experiment.
The animals from both *Salmonella*-inoculated groups had a slight increase in temperature during the first few days after inoculation and some presented with mild diarrhea.

Table 2: Mean and median fluorescent values of infected RAW 264.7 cells and porcine macrophages (PAM) at 0 h and 6 h after inoculation. The average values of 3 independent experiments ± sd are shown. Both the mean and median fluorescent values of the cells infected with the *ssrA* mutant strain at 6 h pi were statistically significant lower ($P < 0.05$) than the values of the cells infected with the wild type strain and are indicated with a "*".

	Mean fluorescence ± sd		Median fluorescent value ± sd	
RAW 264.7	0 h	6 h	0 h	6 h
WT	1165 ± 110	3236 ± 488	831 ± 26	1862 ± 189
ΔssrA	1073 ± 124	1594 ± 298*	801 ± 57	1042 ± 166*
PAM	0 h	6 h	0 h	6 h
WT	744 ± 84	1028 ± 202	594 ± 42	710 ± 126
ΔssrA	749 ± 59	674 ± 122*	578 ± 38	531 ± 74*

The animals of both groups showed a similar fecal excretion pattern (data not shown).

Four days pi, a peak in shedding was noticed in both groups. Two weeks after inoculation, the piglets intermittently shed *Salmonella* at enrichment level. Although during the last few days of the experiment, the *ssrA* mutant strain was shed by fewer animals, this difference was not significant due to the low level of shedding reached at this stage of the infection.

At days 5 and 28 pi, the animals of both groups were infected to the same extent in the gut and gut-associated lymphoid tissue, as well as in the internal organs (Table 3). No significant differences ($P > 0.05$) were seen.

The *ssrA* deletion mutant is attenuated after intravenous inoculation of piglets

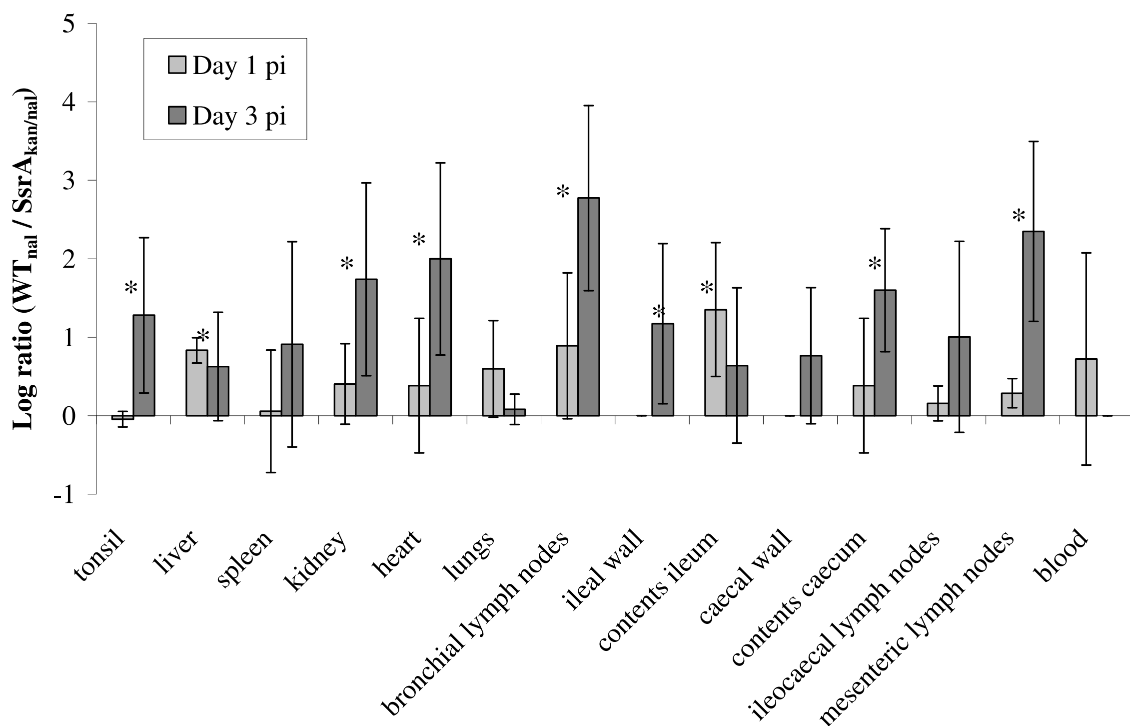
One day after inoculation, both strains were recovered from all pigs inoculated intravenously with a 1:1 mixture of the WT_{nal} and $SsrA_{kan/nal}$ strains. The numbers of bacteria in the blood, the heart, the tonsils and the gut samples were very low, impairing a meaningful quantitative comparison between both strains in these organs. Bacteria were found in relatively higher numbers, although still low, in the liver, spleen, kidney, lungs and bronchial lymph nodes. In these organs, except for the liver, the output ratio WT_{nal} / $SsrA_{kan/nal}$ was not significantly different ($P > 0.05$) from the ratio in the inoculum.

Table 3: Post mortem bacteriological findings at days 5 and 28 after oral inoculation of piglets with 1 x 10^7 CFU of the wild type *Salmonella* Typhimurium strain or the *ssrA* mutant strain. The number of positive tissues in relation to the total number of tissues (frequency) and the average number of CFU ($\log_{10}$) ± sd per gram tissue are shown. Samples only positive after enrichment were given a value of 83 CFU/g.

		Wild type strain		*ssrA* mutant strain	
	Tissue	Frequency	Log_{10} CFU/g ± sd	Frequency	Log_{10} CFU/g ± sd
Day 5 pi	Mand. ln.	4/5	1.56 ± 1.11	3/5	0.98 ± 1.17
	Tonsil	3/5	1.33 ± 1.57	4/5	3.14 ± 1.77
	Lung	1/5	0.2 ± 0.44	2/5	0.4 ± 0.55
	Heart	2/5	0.4 ± 0.55	1/5	0.2 ± 0.45
	Liver	4/5	0.8 ± 0.45	2/5	0.4 ± 0.55
	Spleen	2/5	0.58 ± 0.86	1/5	0.2 ± 0.45
	Kidney	2/5	0.78 ± 1.27	2/5	0.6 ± 0.55
	Ileocecal ln.	5/5	3.69 ± 0.62	5/5	4.04 ± 0.79
	Jejunum	5/5	2.97 ± 1.97	5/5	3.30 ± 1.48
	Ileum	5/5	4.92 ± 0.52	5/5	5.17 ± 1.39
	Cecum	5/5	3.85 ± 0.68	5/5	3.40 ± 2.29
	Content jejunum	5/5	2.64 ± 1.70	4/5	1.18 ± 1.04
	Content ileum	5/5	4.40 ± 0.73	5/5	3.92 ± 2.74
	Content cecum	4/5	2.74 ± 1.56	4/5	2.53 ± 2.70
Day 28 pi	Mand. ln.	0/5	0 ± 0	2/5	0.74 ± 1.18
	Tonsil	2/5	0.76 ± 1.23	4/5	1.88 ± 1.74
	Lung	0/5	0 ± 0	1/5	0.2 ± 0.45
	Heart	0/5	0 ± 0	1/5	0.2 ± 0.45
	Liver	0/5	0 ± 0	1/5	0.2 ± 0.45
	Spleen	0/5	0 ± 0	2/5	0.4 ± 0.55
	Kidney	0/5	0 ± 0	0/5	0 ± 0
	Ileocecal ln.	4/5	1.32 ± 1.34	4/5	0.98 ± 0.68
	Jejunum	2/5	0.4 ± 0.55	2/5	0.4 ± 0.55
	Ileum	5/5	1.72 ± 1.61	5/5	1.18 ± 0.41
	Cecum	5/5	1.45 ± 1.01	2/5	1.04 ± 0.79
	Content jejunum	0/5	0 ± 0	1/5	0.2 ± 0.45
	Content ileum	5/5	1.58 ± 1.29	4/5	0.8 ± 0.45
	Content cecum	5/5	1 ± 0	2/5	0.4 ± 0.55

Three days pi, neither strain could be recovered from the blood samples. The number of WT_{nal} bacteria found in most of the organs was comparable with the number of WT_{nal} bacteria found 1 day after inoculation. The number of $SsrA_{kan/nal}$ bacteria, however, showed an overall decrease. The number of positive lymphoid tissues (tonsils, gut associated lymphoid tissue) was markedly lower for the *ssrA* mutant strain. For the kidney, heart, ileal wall and the bronchial and mesenteric lymph nodes, this resulted in an output ratio WT_{nal} / $SsrA_{kan/nal}$ that was significantly higher ($P < 0.05$) than in the inoculum. The numbers of bacteria in the tonsils, lungs and contents of ileum and cecum were very low, impairing a meaningful quantitative comparison between both strains. The average log values of the ratio WT_{nal} / $SsrA_{kan/nal}$ for all samples are summarized in Figure 3.

Figure 3: Recovery of bacteria from various organs of piglets at days 1 and 3 after intravenous inoculation with an equal mixture of WT_{nal} and $SsrA_{kan/nal}$. The log value of the ratio of the number of CFU/g sample of WT_{nal} and $SsrA_{kan/nal}$ is given as the mean ± standard deviation. An asterisk indicates that the output ratio was significantly different ($P < 0.05$) from that present in the inoculum.



DISCUSSION

In this report we characterized an *ssrA* deletion mutant in a porcine field strain of *Salmonella* Typhimurium. The deletion was confirmed, both at the transcriptional level and the phenotypic level, using qRT-PCR, a RAW replication model and a BALB/c mouse model. Recently it has been found that the expression of SPI-2 encoded genes is regulated exclusively through the induction of the SsrA/B regulatory system (Löber et al., 2006). This means that disabling the SsrA/B system undeniably results in the loss of the SPI-2 T3SS. Nevertheless, it can not be ruled out that the SsrA/B system also regulates genes which are not associated to SPI-2. Using qRT-PCR, it was confirmed that the expression of the SPI-2 secreted effector gene *sifB* was abolished in the *ssrA* mutant strain. The overall virulence and, specifically, the expression of SPI-1 were checked in different assays, confirming that the attenuation in mice is linked to the deletion of *ssrA*.

Using GFP-expressing bacteria, it was shown that the *ssrA* mutant strain was slightly attenuated in intracellular replication in PAM. This defect was more subtle compared to the defect that was observed in mouse macrophages. However, it can not be ruled out that the difference observed in this assay, may also be attributed to the nature of the cells used (primary cells vs. cell line).

In the first part of this pathogenesis study, we chose to perform a single *in vivo* infection assay that resembled the natural infection, which is often established with low or moderate numbers of *Salmonella* (Loynachan and Harris, 2005), to be able to investigate the biological relevance of SsrA in the course of infection in the field. The fact that very few differences were seen in the long-term colonization of pigs orally inoculated with an *ssrA* mutant strain and its isogenic parental wild type strain may come as a surprise. However, a closer look at pathogenesis studies performed in various host species may shed a clearer light on this matter. In laboratory mice, SsrA-regulated genes have an important impact on the pathogenesis of *Salmonella* Typhimurium infections, particularly on the systemic phase of the infection (Cirillo et al., 1998; Hensel et al., 1998), but also on the enteric phase (Hapfelmeier et al., 2005). Data obtained in food-producing animals, however, are scarce. The role of SsrA-regulated genes in host-restricted/adapted serotypes seems consistent in the literature: they are important virulence factors for systemic disease and as a consequence for colonization of the host. For example, for *Salmonella* Gallinarum and *Salmonella* Pullorum, which cause severe systemic disease in fowl, SPI-2 is a prerequisite for virulence and colonization in chickens

(Jones et al., 2001; Wigley et al., 2002). In mice and calves, SPI-2 genes are required for virulence in *Salmonella* Choleraesuis (Dunyak et al., 1997) and *Salmonella* Dublin (Bispham et al., 2001) infections respectively. In broad host range serotypes, however, like *Salmonella* Enteritidis and *Salmonella* Typhimurium (except in NRAMP$^{-/-}$ mice), the importance of SPI-2 is less described. In calves, a *Salmonella* Typhimurium SPI-2 mutant strain was attenuated in colonization after oral inoculation in a signature-tagged mutagenesis assay (Coombes et al., 2005), but was still able to cause lethal infections (Tsolis et al. 1999). In a recent report, SPI-2 was found to play a role in the colonization of *Salmonella* Typhimurium in calves, but not in chickens (Morgan et al., 2004). In addition, a screening of 7,680 *Salmonella* Enteritidis mutants for attenuation in a chicken macrophage infection model resulted in the detection of mutations in several flagellar, LPS and SPI-1 associated genes, but not in SPI-2 genes (Zhao et al., 2002). The role of SPI-2 in the enteric phase of a *Salmonella* Typhimurium infection in food-producing animals also seems negligible. In a rabbit intestinal loop model and in calves, *Salmonella* Typhimurium SPI-2 mutant strains induced inflammation and fluid accumulation to the same extent as the wild type strain (Tsolis et al., 1999; Everest et al., 1999). Considering these reports, the results shown here are not as surprising as expected at first sight.

It is generally accepted that mixed inoculum assays are more capable of discriminating differences in the ability of strains to colonize the host. Using a mixed inoculum assay and a high inoculation dose, we could indeed show that an *ssrA* mutant strain is attenuated for the colonization of internal organs of pigs after intravenous injection. These findings are consistent with the intramacrophagal replication defect of the *ssrA* mutant strain we observed *in vitro*. The mixed intravenous infection protocol, however, does not fit closely to the natural route of infection and bacteria may reach their host cells in a more artificial manner. Recently, it has been shown that the magnitude of the intracellular SPI-2 gene expression is dependent on the mechanism of internalization by macrophages (Drektrah et al., 2007). This phenomenon may additionally explain the differences that were seen in both *in vivo* experiments.

In conclusion, we have shown that an *ssrA* mutant of a porcine field strain of *Salmonella* Typhimurium is fully capable of colonizing pigs and to establish a long term persistent infection after oral inoculation. Using an intravenous mixed infection model, however, the *ssrA* mutant strain was defective in the colonization of several internal organs. This work contributes to the recent insights in the serotype- and host-dependent pathogenesis of salmonellosis in food producing animals.

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The fibronectin binding protein ShdA is not a prerequisite for long term faecal shedding of *Salmonella* Typhimurium in pigs

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ABSTRACT

The aim of this study was to assess the contribution of ShdA in faecal shedding of *Salmonella* Typhimurium in pigs. Pigs were orally inoculated with a *Salmonella* Typhimurium wild type field strain or its isogenic *shdA* mutant strain. For the first few days after inoculation, the s*hdA* mutant strain was excreted more, induced more pronounced diarrhoea and was found in higher numbers of infected internal organs. No effect on long-term shedding was found. In a porcine intestinal loop model, the wild type strain and *shdA* mutant strain did not show any differences in the induction of neutrophil influx into the intestinal wall and lumen. In conclusion, we have shown that a *Salmonella* Typhimurium deletion mutant in s*hdA* is more virulent during the first days after inoculation and is not significantly impaired in persistence or prolonged shedding in pigs.

KEY WORDS

Salmonella Typhimurium – pig – shdA – fibronectin binding protein

INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is an important zoonotic agent. The prolonged excretion of *Salmonella* Typhimurium in pig faeces is a major risk factor both for human and animal health (Berends *et al.*, 1997; Beloeil *et al.*, 2004). It has been estimated that 5-30% of finisher pigs originally infected may still excrete *Salmonella* at the end of the finishing period, and this percentage can double in periods of stress, for example during transport and lairage (Berends *et al.*, 1996). The mechanisms leading to the carrier state or to prolonged faecal shedding in pigs are unknown.

The CS54 Island has been characterized as an important locus for intestinal colonization and prolonged shedding in mice (Kingsley *et al.*, 2000; Kingsley *et al.*, 2003). The most important component of this island is ShdA, an outer membrane protein of the autotransporter family, which is expressed solely in the intestine. Its passenger domain mediates adhesion to fibronectin possibly through a heparin-mimicking binding (Kingsley *et al.*, 2002; Kingsley *et al.*, 2004). Fibronectin binding proteins are common in bacterial pathogens and mainly in Gram positive bacteria. Although their specific role in pathogenesis is not always known, they frequently mediate adherence and entry into mammalian cells (Joh *et al.*, 1999; Schwarz-Linek *et al.*, 2004). A sandwich model has been proposed in which fibronectin acts as a molecular bridge between the fibronectin binding proteins on the pathogen and the integrins on the host cells (Joh *et al.*, 1999; Menzies, 2003).

Kingsley *et al.* (2002) demonstrated that a *Salmonella* Typhimurium strain harbouring a mutation in s*hdA* was shed in reduced numbers and for a shorter period of time in the faeces of mice compared to its isogenic parent strain. Although these studies are of great value, they were exclusively conducted in mice and the role of ShdA in the pathogenesis of salmonellosis in other animal species was not investigated. It was, therefore, the purpose of the present studies to determine the role of this fibronectin binding protein in the persistence and shedding of *Salmonella* Typhimurium in the pig.

MATERIALS AND METHODS

All experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Bacterial strains and growth conditions

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) strain 112910a phage type 120/ad, was isolated from a pig stool sample and was used as the wild type strain. The s*hdA* deletion mutant was constructed in this strain.

The inocula for the intestinal loop model were prepared as follows. Cultures in LB medium were shaken at 130 rpm for 18 hours at 25 °C. After diluting twofold with fresh LB, the cells were incubated for 2 hours at 37 °C, while shaking at 130 rpm. Afterwards, 3 syringes of 5 ml were filled with cultures of each strain. The actual number of bacteria ml^{-1} was assessed by plating serial dilutions on McConkey agar (Oxoid, Basingstoke, UK) plates.

For the oral inoculation of pigs, the bacteria were grown in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) for 6 h at 37 °C. The bacteria were washed twice in phosphate buffered saline (PBS, 2000 x *g* for 10 min at 4 °C). After a third wash with sterile Hanks' balanced salt solution (HBSS) with Ca^{2+}/Mg^{2+} (Gibco, Life Technologies, Paisley, Scotland), the bacteria were resuspended in 10 ml HBSS with Ca^{2+}/Mg^{2+}. The number of viable *Salmonella* bacteria ml^{-1} was determined by plating tenfold dilutions on Brilliant Green Agar (BGA; Oxoid, Basingstoke, UK).

Construction of a non polar shdA deletion mutant

The deletion mutant in *shdA* was constructed according to the one-step inactivation method first described by Datsenko and Wanner (2000) and slightly modified for use in *Salmonella* as described before (Boyen et al., 2005). Primers were designed using the nucleotide sequence with accession number AE008813. The primers *shdA*-P1 *5'aaagggaaatttaaaaattgaacaggacttacagtattgtctggagcgcctgtgtaggctggagctgcttc3'* (primer homologous with sequence upstream of ATG startcodon) and *shdA*-P2 *5'tgtcattcgcctcaaaacgggcagggaacacccgcccggttttgtctaaccatatgaatatcctccttag3'* (primer homologous with sequence downstream of stopcodon) were constructed. These primers and the plasmid pKD4 DNA were utilized to amplify the linear fragment, containing the antibiotic resistance gene *kan*, that was used for the substitution of the gene. The helper plasmid pKD46, encoding the λ Red recombinase, was introduced into *Salmonella* by electroporation, followed by selection on LB agar supplemented with 100 mg carbenicillin l^{-1} (Duchefa Biochemie, Haarlem, The Netherlands). Substitutions of the genes by a kanamycin resistance gene were obtained by electroporation of competent *Salmonella* (pKD46) cells, cultured in presence of 0.2 % (w/v) arabinose, with the linear PCR fragment and selection on LB medium containing 100 mg kanamycin l^{-1} (Duchefa Biochemie, Haarlem, The Netherlands). This was

transduced in a fresh wild type background using bacteriophage P22HT*int* (Schmieger, Backhaus, 1976). Finally, using the helper plasmid pCP20, *shdA* was deleted from the start codon till the stop codon. A scar of 83 bp was left in the resulting strain 112910aΔ*shdA*. This was confirmed by sequencing the relevant PCR fragment. At different stages of the construction, bacteriophage P22 sensitivity was tested to confirm the smooth phenotype.
Two primer pairs were designed: one internal primer pair to confirm the presence of the *shdA* gene, as used in a previous study (Pasmans *et al.*, 2003), and a second primer pair located outside the *shdA* gene, to confirm the substitution and the deletion.

Experimental infection of pigs

Experimental infections were performed in 4-week-old piglets (commercial closed line based on Landrace), that were originating from a serologically negative breeding herd and were negative for *Salmonella* at faecal sampling. They were divided at random into 3 groups: 2 groups of 10 pigs and one negative control group of 6 pigs. All three groups were housed in separate isolation units at 25 °C under natural day-night rhythm with ad libitum access to feed and water. Pigs were penned in pairs for the first 5 days and individually for the remainder of the experiment.

The animals were orally inoculated with 10^7 cfu of *Salmonella* Typhimurium in 2 ml HBSS. Group 1 was inoculated with *Salmonella* Typhimurium 112910a, group 2 was inoculated with *Salmonella* Typhimurium 112910aΔ*shdA* and group 3, the negative control group, was sham-inoculated with 2 ml PBS.

For the first two days post-inoculation (pi) the rectal temperature was measured twice a day and the clinical condition of the pigs was monitored (anorexia, lethargy, diarrhoea). From day 3 till day 7 pi this was performed once a day, and subsequently once every other day until day 28 pi.
Fresh faecal samples were taken from each pig on days 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 pi for bacteriological analysis.

On day 5 and day 28 pi 5 pigs of each *Salmonella* inoculated group and 3 control pigs were euthanized. Samples of tonsils, mandibular lymph nodes, bronchial lymph nodes, lung, heart, liver, spleen, kidney, ileocaecal lymph nodes, jejunum, ileum, caecum and contents of jejunum, ileum and caecum were taken for bacteriological analysis.

All samples were stored at -70 °C until use. The samples were thawed and weighed, 10% (w/v) suspensions were made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was homogenized with a stomacher. The homogenized samples

were examined for the presence of the *Salmonella* strains by plating tenfold dilutions on BGA. If negative at direct plating, the samples were pre-enriched overnight in BPW at 37 °C, enriched overnight at 37 °C in tetrathionate broth and then plated on BGA. Samples that were negative after direct plating but positive after enrichment, were presumed to contain 10 cfu g^{-1}. Samples that remained negative were presumed to have 0 cfu g^{-1}. *Salmonella* colonies isolated from each group were analyzed for the presence or absence of the *shdA* gene by means of PCR to exclude cross contamination between groups. Statistical analysis was performed using a non-parametric Kruskal-Wallis test. Differences with a P value ≤ 0.05 were considered as significant.

Intestinal loop model

The intestinal loop model was performed on 6-week-old farm-reared Landrace/Large White cross male piglets. This model has been described in detail elsewhere (Wallis *et al.*, 1995). In short, commencing at the distal ileum, a maximum of 30 sequential loops (each 6-7 cm in length and containing both absorptive epithelium and follicle associated epithelium) were ligated, separated by 1 cm spacers. The lumen of the ileum was gently flushed with 0.9% NaCl before the construction of the loops to remove the intestinal contents. The inoculum injected into each loop, consisting of *Salmonella* Typhimurium 112910a and *Salmonella* Typhimurium 112910aΔ*shdA* respectively, was in the range 1-2 x 10^9 c.f.u. Approximately 10 ml of blood was removed from the piglets to isolate the polymorphonuclear leucocytes (PMNs). The isolated PMNs were labelled with ^{111}Indium and reinjected intravenously. The influx of PMNs in the intestinal wall and in the lumen of the gut, as assessed by the counts per minute (c.p.m.) emitted from ^{111}Indium-labelled PMNs within each loop, was recorded 12 h after injection of the loops using a Wallac 1275 mini gamma counter. The data were analyzed using a paired t-test, with the SPSS 12.0 software for Windows. A P-value≤ 0.05 was considered significant.

RESULTS

The course of infection of the piglets inoculated with the wild type strain was similar to several preliminary experiments. Sham inoculated control piglets did not develop disease signs and *Salmonella* was not isolated from any of the samples taken from these animals throughout the experiment.

shdA mutant strain is more virulent early after inoculation

During the first days after oral inoculation of the piglets, the *shdA* mutant strain was shed in higher numbers than the wild type strain (Fig. 1). On days 1 and 2 pi, the difference between the wild type strain and the *shdA* mutant strain was significant ($p \leq 0.05$). In accordance with the excretion data, diarrhoea in the group of piglets inoculated with the *shdA* mutant strain was more pronounced (Table 1). At day 5, the number of infected internal organs (bronchial lymph nodes, lung, heart, liver, spleen and kidney) of the piglets inoculated with the *shdA* mutant strain (21/30) was higher than the number of infected internal organs of the piglets inoculated with the wild type strain (10/30), even though the average number of cfu per gram tissue was not significantly different (Table 2). The post mortem bacteriological results are summarized in Table 2. One piglet inoculated with the *shdA* mutant strain died suddenly at day 4 pi. The internal organs were massively infected (>10^6 cfu gram^{-1} tissue) with *Salmonella*.

Days p.i.	wild type strain	*shdA* mutant strain
1	0/10	5/10
2	0/10	3/10
3	1/10	4/10
4	2/10	4/10
5	0/10	3/9

Table 1: The number of clinically affected piglets in relation to the total number of piglets during the first 5 days after inoculation with either the wild type strain or the *shdA* mutant strain are shown.

shdA mutant strain is not impaired in long-term colonization

From day 8 until day 28, no significant differences in mean faecal shedding were noticed between piglets inoculated with the mutant and wild type strain (Fig. 1). From day 18 on, all animals of both groups shed *Salmonella* intermittently at enrichment levels. At day 28, all animals of both groups were positive for *Salmonella* in the ileum and caecum and only the tonsils were colonized in significantly higher numbers in the piglets inoculated with the *shdA* mutant strain. The post mortem bacteriological results are summarized in Table 2.

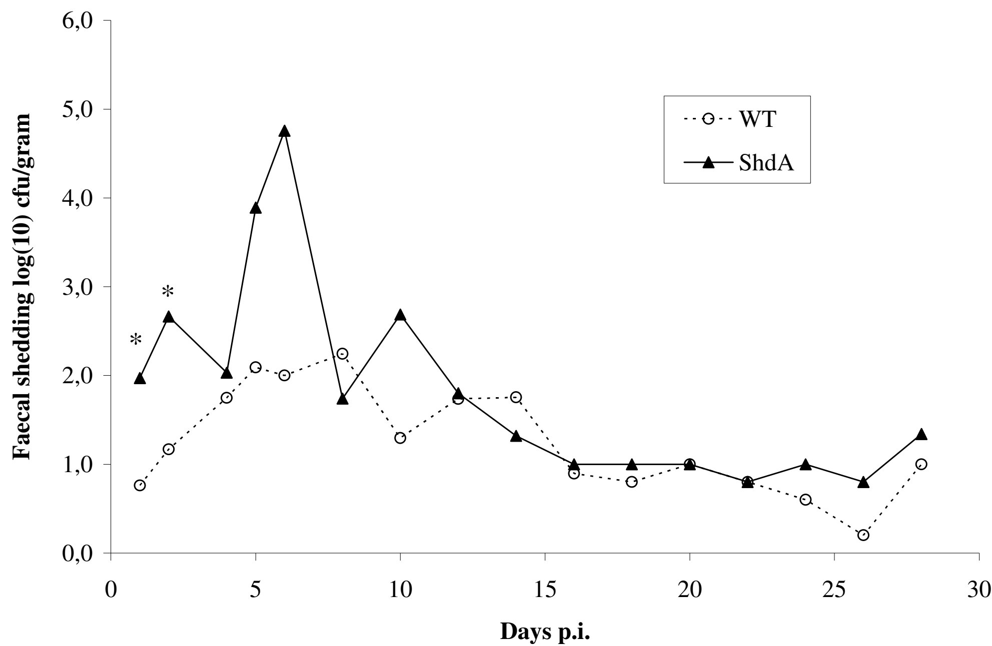
Table 2: Post mortem bacteriological findings at day 5 and day 28 post inoculation of piglets inoculated with 10^7 cfu of the wild type strain or the *shdA* mutant strain. Number of positive tissues in relation to the total number of tissues (frequency) and the average number of cfu (log_{10}) ± stdev per gram tissue are shown. Samples only positive after enrichment were rendered a value of log 10 cfu g^{-1}. A significantly ($p \leq 0.05$) higher mean score of the 112910aΔ*shdA* strain compared to the wild type strain is indicated with a "*".

		Wild type strain		*shdA* mutant strain	
	Tissue	Frequency	Log_{10} cfu/g ± stdev	Frequency	Log_{10} cfu/g ± stdev
Day 5 pi	Mand. ln.	4/5	1.16 ± 0.72	5/5	2.35 ± 1.69
	Tonsil	4/5	1.75 ± 1.22	5/5	2.81 ± 1.96
	Bronch. ln.	0/5	0 ± 0	2/5	0.25 ± 0.5
	Lung	3/5	0.6 ± 0.55	4/5	0.75 ± 0.5
	Heart	2/5	0.4 ± 0.55	3/5	0.50 ± 0.58
	Liver	4/5	0.8 ± 0.45	5/5	1.00 ± 0
	Spleen	1/5	0.2 ± 0.45	4/5	0.75 ± 0.5
	Kidney	0/5	0 ± 0	3/5	1.21 ± 1.81
	Ileocecal ln.	5/5	2.59 ± 1.06	5/5	3.07 ± 0.29
	Jejunum	5/5	2.77 ± 1.92	5/5	2.60 ± 1.22
	Ileum	5/5	4.64 ± 1.09	5/5	4.52 ± 0.94
	Cecum	5/5	4.49 ± 0.52	5/5	4.12 ± 0.61
	Content jejunum	5/5	2.5 ± 2.17	5/5	2.08 ± 1.46
	Content ileum	5/5	3.75 ± 2.28	5/5	4.88 ± 1.68
	Content cecum	4/5	3.14 ± 1.87	5/5	3.50 ± 1.43
Day 28 pi	Mand. ln.	4/5	0.8 ± 0.45	5/5	1 ± 0
	Tonsil	5/5	1 ± 0	5/5	2,79 ± 1.64 *
	Bronch. ln.	0/5	0 ± 0	1/5	0,2 ± 0.45
	Lung	0/5	0 ± 0	2/5	0,4 ± 0.55
	Heart	0/5	0 ± 0	0/5	0 ± 0
	Liver	0/5	0 ± 0	1/5	0,2 ± 0.45
	Spleen	0/5	0 ± 0	2/5	0,4 ± 0.55
	Kidney	2/5	0.4 ± 0.55	1/5	0,2 ± 0.45
	Ileocecal ln.	5/5	1 ± 0	4/5	0,8 ± 0.45
	Jejunum	1/5	0.2 ± 0.45	3/5	0,6 ± 0.55
	Ileum	5/5	1 ± 0	5/5	1 ± 0
	Cecum	5/5	1 ± 0	5/5	1 ± 0
	Content jejunum	2/5	0.4 ± 0.55	0/5	0 ± 0
	Content ileum	4/5	0.8 ± 0.45	4/5	0,8 ± 0.45
	Content cecum	5/5	1 ± 0	5/5	1 ± 0

shdA mutant does not induce an increased neutrophil influx in the gut

In the loop assays, infections with the wild type strain and the *shdA* mutant strain both resulted in enteritis 12 hours after inoculation. The loops contained a rather small amount of slimy, pus-like substance, which made it impossible to quantify the fluid accumulation in the lumen. The *shdA* mutant strain did not show any significant differences in the induction of neutrophil influx in the intestinal wall or in the lumen of the gut compared to its isogenic wild type strain, 12 hours after inoculation (Fig. 2).

Figure 1. The mean log(10) cfu gram^{-1} faeces per piglet (n = 10 at days 1,2 and 4; n = 5 at the remaining days) after oral inoculation of piglets with 10^7 cfu of the wild-type strain 112910a (○) and 112910aΔ*shdA* (▲) respectively. A significant ($p \leq 0.05$) higher mean faecal score of the 112910aΔ*shdA* strain is indicated with a "*".



DISCUSSION

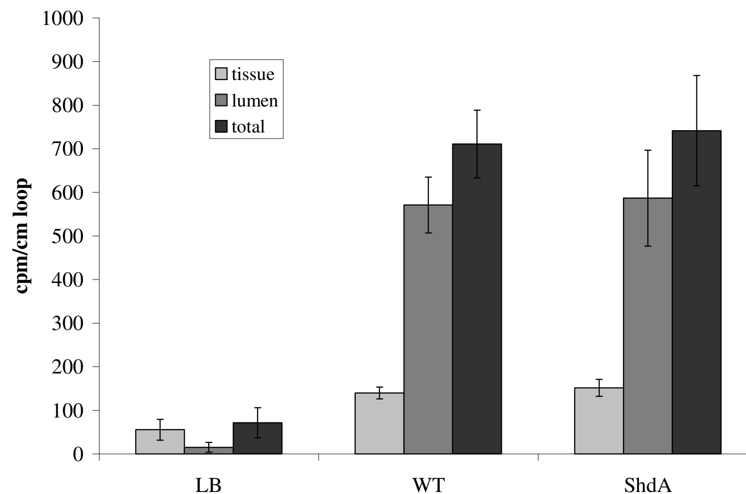
The increased faecal excretion levels during the first few days after inoculation of the piglets inoculated with the *shdA* mutant strain are consistent with the results of Kingsley *et al.* (2003), who reported a threefold increase in excretion of the *shdA* mutant strain at day 5 after inoculation of CBA/J mice with an equal mixture of the wild type strain and the *shdA* mutant strain.

Although Kingsley *et al.* (2000, 2003) found a reduction in excretion of the *shdA* mutant strain in comparison with its isogenic wild type strain by 15 days after inoculation of CBA/J mice, we did not observe any signs of decreased shedding of the *shdA* mutant strain until the end of the experiment (28 days pi). It is becoming more and more obvious that the pathogenesis of *Salmonella*-infections and the importance of some virulence factors are strongly host-related (Pasmans *et al.*, 2003; Morgan *et al.*, 2004). As a consequence, the extrapolation of results from pathogenesis studies in one host species to another host is not always possible. In this respect, it might be possible that ShdA has a different role in the pathogenesis of *Salmonella* Typhimurium infections in porcine and murine hosts. It must be stated, however, that the experiments of Kingsley and coworkers were conducted in a competitive infection model. This type of infection model is capable of detecting small differences in colonization capacities between strains because the rather large variation between animals is overcome by considering the ratio of the 2 strains in 1 animal. The disadvantage of this model is, however, that no conclusions can be drawn concerning the development of disease or diarrhoea. Since mice do not develop an acute intestinal phase of infection, this was not a determining factor for choosing the single infection model for the work in mice. In pigs, the intestinal phase of *Salmonella* infections is definitely important.

Although it was found that fibronectin could play an important role in the invasion of *Salmonella* in epithelial cells (Walia *et al.*, 2004), the *shdA* gene is expressed solely in the gut and it has not been proven to be induced by any *in vitro* condition (Kingsley *et al.*, 2002). Therefore, it was impossible to use a relevant *in vitro* model to investigate the role of ShdA in the invasion of porcine epithelial intestinal cells.

Even though still speculative, altered interactions with the epithelium of the gut could explain the increased colonization capacity of the *shdA* mutant strain in the early phase of infection. Moreover, loss of fibronectin binding proteins has led to an increased virulence in various other bacteria (McElroy *et al.*, 2002; Nyberg *et al.* 2004).

Figure 2. Radio-active γ counts per minute per cm loop as a measure for the early PMN influx elicited by *S*. Typhimurium strains in porcine intestinal loops. Approximately 1.5×10^9 CFU of the wild-type strain 112910a (WT) and 112910a∆*shdA* (ShdA) was injected into each loop and left for 12 h before analysis. Luria-Bertani broth (LB) was used as a negative control. Each mean is calculated from 3 loops in 2 piglets and is presented with the SEM.



In summary, we have shown that a *Salmonella* Typhimurium *shdA* deletion mutant is not significantly impaired in persistence in pigs in a single infection model. It is excreted in higher numbers during the first days after inoculation, possibly because of altered interactions with the intestinal epithelial cells. The role of fibronectin as adherence factor, as non-specific host defense factor or as important modulating factor in inflammatory processes remains an intriguing field of research in host-pathogen interactions.

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GENERAL DISCUSSION

Porcine carcasses are an important source for *Salmonella* infections in humans. Due to an apparent and spectacular drop in *Salmonella* Enteritidis infections in poultry in 2005 and 2006 (Davies et al., 2004; Collard et al., 2007), the relative importance of *Salmonella* Typhimurium infections in pigs will probably increase the next few years. In addition, the increasing multiple antimicrobial resistance associated with strains belonging to this serotype can pose an important human health hazard in the future (Butaye et al., 2006). Both European and national governments try to anticipate this menace by starting monitoring programmes and coordinating control measures on farm and in the slaughterhouse.

The implementation of efficient control measures is impaired by the lack of knowledge on host-pathogen interactions in pigs. This knowledge may not only provide insights in the efficacy of certain measures or products, it can also provide fundamental keys to improve these control measures. In addition, ongoing research may help in fine-tuning the present monitoring programmes.

Salmonella pathogenesis research

Research on the pathogenesis of *Salmonella* Typhimurium infections has been conducted mostly in BALB/c mice (NRAMP1$^{-/-}$) using *Salmonella* Typhimurium laboratory strains. Since a *Salmonella* Typhimurium infection in these mice resembles a *Salmonella* Typhi infection in humans, this typhoid model has been used extensively to study the pathogenesis of *Salmonella* infections. However, since the course of a *Salmonella* Typhimurium infection in BALB/c mice (lethal systemic disease) is very different from that in pigs (subclinical local infection), the mouse model is not biologically relevant to study the pathogenesis of *Salmonella* Typhimurium infections in pigs. Recently, a mouse model has been created for intestinal pathogenesis research, using germ free mice or NRAMP1$^{+/+}$ or NRAMP1$^{-/-}$ mice pretreated with streptomycin (Barthel et al., 2003; Stecher et al., 2005; Stecher et al., 2006). Even though these models offer several important advantages for pathogenesis researchers in terms of tools, the biological relevance of these “artificial models” for food producing animals and even humans may be questionable.

In order to investigate the pathogenesis of persistent infections, a strain that is able to induce a persistent infection in pigs after experimental inoculation is needed. Therefore, the

characteristics of a field strain, isolated from a pig that was persistently infected, were compared with the characteristics of a standard laboratory strain often used in mice. The standard laboratory strain was markedly more virulent in a BALB/c mouse model, compared to the porcine field strain, while the porcine field strain seemed to be more efficient in inducing a persistent infection in pigs. This difference may point to the phenomenon of host-specificity. Some serotypes are very host specific, causing disease in only one or a limited number of animals, whereas others can infect a broad range of host species (Uzzau et al., 2000). However, differences in host-specificity may be present also within a given serotype (Nilsson et al., 2004). This host-specificity may be expressed as a more efficient colonization, predilection for specific organs (for example *Salmonella* Enteritidis and the chicken reproductive tract) and the induction of persistent infections. This means, however, that there is no such thing as a pathogenesis of *Salmonella* infections in general. Both the infected host and the infecting serotype or even strain influence the course of an infection. This also means that the importance of certain virulence factors of *Salmonella* and the interaction with the immune system of different host species can differ greatly (Morgan et al., 2004; McMeechan et al., 2006). Several virulence genes of *Salmonella* Typhimurium have been assigned to play an important role in the pathogenesis of salmonellosis in laboratory mice. In our studies, we wanted to determine the role of these virulence factors in the pathogenesis of *Salmonella* Typhimurium infections in pigs.

It was found that only some, but not all of the investigated virulence factors, important for inducing disease in BALB/c mice, were also important in the pathogenesis of *Salmonella* Typhimurium infections of pigs. The *Salmonella* pathogenicity islands, and more specifically SPI-1 and SPI-2, are generally accepted as being crucial virulence factors for *Salmonella* pathogenesis in mammals, including man. However, until now, 17 pathogenicity islands have been described, and this number will probably keep rising the next few years. It is getting more and more obvious that the distribution and biological significance of these islands may vary greatly between different serotypes or even strains. The presence/absence or regulation of expression of these virulence factors may contribute to a host specific pathogenesis.

***Salmonella* Typhimurium in pigs: to invade or not to invade?**

The present results suggest that *Salmonella* Typhimurium uses two distinct sites for colonization of pigs: the tonsils on the one hand and the intestine and associated lymph nodes

on the other hand. Since these colonization sites have anatomically and physiologically diverse structures and functions, it may not come as a surprise that *Salmonella* shows distinct interactions with both organs.

Salmonella Typhimurium is using at present unidentified virulence factors to colonize the tonsillar epithelium, most probably without actively invading the cells. Indeed, a non-invasive strain (Δ*sipB*; a SPI-1 deficient mutant strain) was perfectly capable of colonizing the tonsils. The tonsils are often heavily infected in pigs and should, therefore, not be underestimated as a source of increased *Salmonella* load during slaughter (Kühnel and Blaha, 2004). The mode of colonization of the tonsils is in sharp contrast with the colonization of the intestine. Active invasion, using the SPI-1 T3SS, was shown to be of critical importance for efficient colonization of the porcine gut. A non-invasive strain (Δ*sipB*) was found to be substantially attenuated in colonization of the gut and the associated lymphoid tissue.

In order to combat *Salmonella* infections in pigs, measures that interfere with both tonsillar and intestinal colonization will probably be needed. Since the mechanisms of colonization of these important sites seem to be very different, the control measures should be designed accordingly.

Even though no thorough research has been done concerning the control of a *Salmonella* infection at the tonsillar level, some control measures used nowadays may show to be effective. The administration of acidified drinking water in pig farms has been shown to lower the prevalence of serologically positive pigs (Van der Wolf et al., 2001). However, no experimental assays have been performed using acidified drinking water, nor has the effect on the colonization of the different organs been investigated. One might expect these products to exert an immediate effect in the oral cavity and on the colonization of the tonsils. This has to be further explored in the future.

Considering the importance of invasion in the colonization of the gut, one could expect that any measure that interferes with this invasion step will decrease the bacterial load in the gut. Indeed, using coated butyric acid we were able to lower intestinal colonization and bacterial shedding in pigs (unpublished results) as has also been described in poultry (reviewed by Van Immerseel et al., 2006).

Do macrophages matter?

Macrophages are the cells of interest for *Salmonella* Typhimurium to disseminate into the internal organs of BALB/c mice. The bacteria replicate rapidly intracellularly and cause the systemic phase of the infection, while interfering with the antibacterial mechanisms of the macrophages and inducing cell death. Liver, spleen and lymphoid tissue are the target organs and act as a continuous source of bacteria, which spread all over the body, including the gut. The long term presence of the bacteria in BALB/c mice, therefore, is a result of systemic dissimination and replication in the internal organs and ultimately often results in death of the animal. In pigs, however, sporadic bacteria present in liver and spleen shortly after experimental inoculation, do not seem to replicate fast and are even cleared from these organs a few weeks after inoculation. Nevertheless, the bacteria are still found in the gut and gut-associated lymph nodes. At these sites, macrophages can be important players when it comes to long term persistency. Keeping these differences in mind, the pathogenesis of persistency in pigs and in mice, will probably be very different. Also the virulence genes used by *Salmonella* may obviously differ.

In order to evade the antibacterial mechanisms of murine macrophages, two important pathogenicity islands of *Salmonella* Typhimurium have been described: SPI-1 and SPI-2. SPI-1 is not only crucial for intestinal invasion, but also plays a role in the interactions of *Salmonella* Typhimurium with murine macrophages as inducer of apoptosis. Therefore, we evaluated the role of SPI-1 in the interactions of *Salmonella* Typhimurium with porcine macrophages. *In vitro*, SPI-1 seems to play a similar role in the interactions with porcine macrophages compared to murine macrophages. However, our studies demonstrated that SPI-1 effectors are not only involved in interactions of the bacteria with macrophages, but also have other functions, including the invasion in several other cell types. This implies that the contribution of SPI-1 related interactions with macrophages to *in vivo* persistence is not easy to determine.

As reviewed in the introduction of this thesis, data describing the importance of SPI-2 in the systemic phase of infection and the induction of a persistent infection in BALB/c mice are overwhelming. The importance of SPI-2 in the systemic phase of a *Salmonella* infection in food producing animals is less described. For host-restricted or host-adapted serotypes (Pullorum, Dublin, Choleraesuis) SPI-2 is a prerequisite for virulence and colonization in their respective hosts (Dunyak et al., 1997; Bispham et al., 2001; Jones et al., 2001; Wigley et al., 2002). However, for broad host range serotypes, such as *Salmonella* Typhimurium, the

role of SPI-2 in the pathogenesis of *Salmonella* infections in food producing animals is less straightforward (Tsolis et al., 1999; Zhao et al., 2002; Morgan et al., 2004). In accordance, our results suggest that SPI-2 of *Salmonella* Typhimurium may not contribute to persistence in pigs to the same extent as it does in the mouse typhoid model, since SPI-2 defective mutants were able to colonize the internal organs of pigs to a similar degree as their respective wild type strain and were able to persist until 28 days post inoculation.

Persistent *Salmonella* Typhimurium infections in pigs

Clinical salmonellosis is not a common problem in Belgian pig farms. *Salmonella* infections are mostly subclinical, but can develop into a lasting, but often unnoticed infection. This is called the carrier state. Several *Salmonella* virulence genes have been shown to play a role in persistency and long term shedding in laboratory mice (*shdA*, SPI-2). In the studies described in this thesis, the role of these genes in the development of a carrier state in pigs could not be confirmed.

Since the carrier state in pigs is difficult to detect, either by bacteriological or serological methods (Baggesen and Wegener, 1993; Nollet et al., 2005), these pigs can bias monitoring programmes. The mechanism of this concealed, but prolonged infection is not yet unravelled. However, the latest findings are changing our classical view of *Salmonella* as a fast growing intracellular pathogen and devastating bacterium. It has been suggested that *Salmonella* may reduce its own intracellular growth rate (Cano et al., 2001; Jantsch et al., 2003; Sheppard et al., 2003; Monack et al., 2004) and may actively decrease its impact on the infected tissues (Collier-Hyams et al., 2002; Haraga and Miller, 2003), almost as if it was a commensal. In addition, it was found recently that *Salmonella* is able to interfere with the antigen presentation and the development of acquired immunity (Mitchell et al., 2004; Qimron et al., 2004; Cheminay et al., 2005; Van der Velden et al., 2005; Alaniz et al., 2006; Luu et al., 2006). Even though the exact contribution of these mechanisms to the pathogenesis of the carrier state in pigs is not clear, it has been shown that serologically negative herds may still provide pigs that are bacteriologically positive in the gut and associated lymph nodes (Nollet et al., 2005). It has been suggested that these pigs were recently infected, so that the serological response was not fully developed at the time of sampling. However, if some *Salmonella* strains are truly able to actively decrease the immunological response, the current national monitoring programmes, which are based solely on serology, may show inadequate in these cases. It is clear that more research in this area is needed.

Future directions?

It is clear that still a lot of work has to be done to unravel the pathogenesis of *Salmonella* infections in pigs and a lot of questions arise from this work. Does *Salmonella* use specific virulence factors to colonize the tonsils and, if yes, which are those virulence factors? Which interactions ultimately result in a persistent infection and the carrier state? Which virulence genes are used by *Salmonella* to accomplish this? What is the contribution of the suppressed immunological response in the pathogenesis of *Salmonella* infections in swine and the development of a carrier state? Which cells are the target cells for persistency? Which mechanisms allow *Salmonella* to arise from its “hibernation” in carrier pigs during periods of stress and which sites/organs/cells are the sources of these high numbers of bacteria?

Next to these more fundamental questions, more detailed information about strategic control measures is needed. The models described in this thesis are useful to investigate the efficacy of these control measures in pigs.

Conclusions

Research on the pathogenesis of *Salmonella* infections in food producing animals remains an interesting and challenging topic. For relevant pathogenesis research, both host species and appropriate *Salmonella* strains should be chosen with care. SPI-1 driven invasion is crucial for intestinal, but not tonsillar colonization of pigs. The contribution of SPI-2 and *shdA* to the pathogenesis of persistent *Salmonella* infections in pigs is not as important as in the mouse typhoid model. It would be presumptuous, however, to exclude macrophages as possible sites of persistent colonization in pigs.

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SUMMARY

Human salmonellosis is frequently caused by an infection with *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium). Salmonellosis caused by this serovar is mainly associated with the consumption of pork. Pigs infected with *Salmonella* Typhimurium are generally subclinically colonized. These animals carry the bacterium in the tonsils, the intestines and the gut-associated lymphoid tissue (GALT). Such carriers are a major reservoir of *Salmonella* Typhimurium and pose an important threat to animal and human health. The mechanism by which *Salmonella* Typhimurium colonizes and persists in pigs is unknown. A thorough knowledge of the host-pathogen interactions should form the basis for the development and evaluation of efficient monitoring programmes and control measures.

Numerous *Salmonella* virulence genes have been described to play a role in the pathogenesis of salmonellosis in various animal species, especially in mice. These virulence genes are often situated on *Salmonella* Pathogenicity Islands (SPI). The Pathogenicity Islands are considered “quantum leaps” in bacterial evolution and were probably acquired by horizontal gene transfer. Typically, Pathogenicity Islands are present in the genome of pathogenic bacteria but absent in nonpathogenic strains of the same or related species. To date, 17 different SPI have been described in *Salmonella*. The most important and most characterized SPI are SPI-1 and SPI-2. Until recently, the role of SPI-1 was considered limited to the intestinal phase of infection and SPI-2 to the systemical phase of infection. Most of the SPI-related research was conducted with *Salmonella* Typhimurium in NRAMP$^{-/-}$ BALB/c mice.

The overall aim of this thesis was to get insights into the mechanisms by which *Salmonella* Typhimurium colonizes and persists in pigs. Therefore, the role of several *Salmonella* virulence genes in the pathogenesis of *Salmonella* Typhimurium infections in pigs was determined.

In the first chapter of this thesis, a *Salmonella* Typhimurium strain that was able to persistently infect pigs was selected. A porcine field strain and a standard laboratory strain of *Salmonella* Typhimurium were compared *in vitro* and *in vivo*. The standard laboratory strain

was more virulent in mice, while the porcine field strain was more effective in the induction of a persistent infection in pigs. The porcine field strain was chosen for further experiments.

In the second chapter, different *in vitro* and *in vivo* models to investigate the pathogenesis of *Salmonella* Typhimurium infections in pigs were evaluated. The invasive and proliferative characteristics of a *Salmonella* Typhimurium strain were assessed in both a non-polarized and a polarized porcine intestinal epithelial cell line. Neutrophils obtained from porcine blood were used to study the capacity of *Salmonella* to withstand killing by these phagocytes. The ability to induce an intestinal inflammatory response was investigated in an intestinal loop model. The systemic phase of infection was mimicked by studying the uptake and intracellular survival of *Salmonella* Typhimurium in porcine pulmonary alveolar macrophages and peripheral blood monocytes. Invasion in the polarized epithelial cell line was more pronounced and more reproducible compared to invasion in the non-polarized cell line. *Salmonella* Typhimurium was able to replicate quickly in macrophages, but not in monocytes. Neutrophils were able to rapidly kill *Salmonella* Typhimurium and migrated massively into the gut tissue and lumen in the porcine intestinal loop model. In an experimental oral infection model in pigs, it was shown that the course of the infection was dose dependent and that a uniform colonization status was achieved using at least 10^7 cfu. Days 5 and 28 post inoculation proved to be the optimal time points for assessment of colonization and persistency in pigs.

In the third chapter, the role of different *Salmonella* virulence genes in the pathogenesis of *Salmonella* Typhimurium infections in pigs was investigated.

In a first series of experiments, the role of the SPI-1 genes *hilA*, *sipA* and *sipB* in the interactions of *Salmonella* Typhimurium with the porcine gut and macrophages was investigated. HilA and SipB proved to be essential for the invasion of porcine macrophages and intestinal epithelial cells *in vitro*. A *sipA* mutant was impaired for invasion using a polarized epithelial cell line, but fully invasive in macrophages and a non-polarized epithelial cell line. In macrophages, SPI-1 was needed for the formation of spacious phagosomes. All SPI-1 mutants induced a significant decrease in influx of neutrophils in the porcine intestinal loop model compared with the wild type strain. Exposure of PAM to *Salmonella* Typhimurium induced the production of reactive oxygen species (ROS) and interleukin-8, but no differences were noticed between the induction mediated by the wild type strain and its

SPI-1 mutant strains. Both early and delayed cytotoxicity were seen in PAM, but only the early cytotoxicity was SPI-1 dependent. When pigs were orally inoculated with 10^8 colony forming units of both the wild type *Salmonella* Typhimurium strain and its isogenic *sipB*::kan mutant strain, the *sipB* mutant strain was significantly impaired to invade and colonize the intestines and the GALT, but not the tonsils.

In conclusion, SPI-1 mediated invasion is crucial for the colonization of the gut and for the influx of neutrophils towards the gut, but not for the colonization of the tonsils. SPI-1 also plays a role in the interactions of *Salmonella* Typhimurium with porcine macrophages, for example by inducing cell death.

In a second series of experiments, the interactions of a porcine field strain of *Salmonella* Typhimurium and a non-polar isogenic SPI-2 (*ΔssrA*) deletion mutant were compared in both *in vitro* and *in vivo* models. The *ssrA* mutant strain displayed decreased SPI-2 expression levels, showed a replication defect in mouse macrophages *in vitro* and was attenuated in a mouse model after oral inoculation. SPI-1 expression was not affected. Using green fluorescent protein expressing strains and flowcytometric analysis, the *ssrA* mutant strain was shown to be defective in intracellular replication in porcine macrophages. In an oral infection assay, piglets inoculated with the *ssrA* mutant strain followed a similar infection course as piglets infected with the wild type strain. At days 5 and 28 post inoculation, the animals of both groups were infected to the same extent in the gut and gut-associated lymphoid tissue, as well as in the internal organs.

These results demonstrate that a SPI-2 mutant strain of *Salmonella* Typhimurium is fully capable of colonizing pigs and is able to establish a long term persistent infection.

In a third series of experiments, the contribution of the fibronectin binding protein ShdA in the prolonged faecal shedding of *Salmonella* Typhimurium by pigs was investigated. Although the mechanism of prolonged faecal shedding is not yet clarified, this gene was identified as an important locus for intestinal colonization and persistence of *Salmonella* Typhimurium in mice. In these experiments, pigs were orally inoculated with a *Salmonella* Typhimurium wild type field strain or its isogenic *shdA* mutant strain. For the first few days after inoculation, the s*hdA* mutant strain was excreted in higher numbers in the faeces and more internal organs were infected. However, 2 to 4 weeks after inoculation, the s*hdA* mutant strain was excreted to a similar extent as the wild type strain. In a porcine intestinal loop

model, the wild type strain and *shdA* mutant strain did not show any differences in the induction of neutrophil influx into the intestinal wall and lumen.

In conclusion, although ShdA is important for long term shedding in mice, we could not confirm these findings in pigs.

The results presented in this thesis demonstrate that both host species and appropriate *Salmonella* strains should be chosen with care for relevant pathogenesis research. SPI-1 driven invasion is crucial for intestinal, but not tonsillar colonization of pigs. The contribution of SPI-2 and *shdA* to persistency of *Salmonella* in pigs is not of the same extent as in laboratory mice.

SAMENVATTING

Salmonellose bij de mens wordt vaak veroorzaakt door *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium). De ziekte, veroorzaakt door dit serotype, is voornamelijk geassocieerd met het eten van varkensvlees. Varkens die geïnfecteerd zijn met *Salmonella* Typhimurium zijn meestal subklinisch gekolonizeerd. Deze dieren zijn dragers van de kiem in hun tonsillen, hun darmen en de met de darmen geassocieerde lymfeknopen. Dragerdieren zijn een reservoir voor *Salmonella* Typhimurium en betekenen een gevaar voor de volksgezondheid. De mechanismen die *Salmonella* Typhimurium gebruikt om varkens te kolonizeren en om te persisteren in deze gastheer zijn niet gekend. Nochtans zou een grondige kennis van de kiem-gastheerinteracties de basis moeten vormen voor het ontwikkelen en de evaluatie van bewakingsprogramma's en bestrijdingsmaatregelen.

Er zijn reeds zeer veel *Salmonella* virulentiegenen beschreven die een rol spelen in het ontwikkelen van ziekte bij verschillende diersoorten, voornamelijk bij de muis. Deze virulentiegenen zijn vaak op *Salmonella* Pathogeniciteitseilanden gelegen (*Salmonella* Pathogenicity Islands; SPI). Deze eilanden van virulentiegenen zijn waarschijnlijk op een welbepaald tijdstip in het ontstaan van *Salmonella* min of meer in hun geheel verworven en zijn bijvoorbeeld afwezig in *Escherichia coli*, een nauw verwante bacteriesoort. Tot op vandaag zijn reeds 17 verschillende SPI beschreven in *Salmonella*. De belangrijkste en meest onderzochte zijn SPI-1 en SPI-2. SPI-1 is voornamelijk van belang voor de kolonizatie van de darmen en het ontstaan van diarree. SPI-2 is voornamelijk van belang voor het spreiden van de kiem in het lichaam van de gastheer en voor persistentie. Het belang van deze genen voor *Salmonella* Typhimurium infecties werd voornamelijk onderzocht in het BALB/c muismodel.

Het algemene doel van deze thesis was om inzicht te verkrijgen in de mechanismen die *Salmonella* Typhimurium gebruikt om varkens te kolonizeren en erin te persisteren. Hiervoor werd de rol van verschillende virulentiegenen onderzocht in de pathogenese van *Salmonella* Typhimurium infecties bij het varken.

In het eerste hoofdstuk van deze thesis werd een *Salmonella* Typhimurium stam geselecteerd die een persisterende infectie bij varkens kan veroorzaken. Hiervoor werd een veldstam, geïsoleerd uit een persistent geïnfecteerd varken, vergeleken met een standaard

laboratoriumstam die vaak wordt gebruikt voor onderzoek in muizen. De laboratoriumstam was virulenter in muizen, maar de varkensstam was efficiënter in het veroorzaken van een persisterende infectie bij varkens. Deze varkensstam werd gekozen om te gebruiken in verdere experimenten.

In het tweede hoofdstuk werden verschillende *in vitro* en *in vivo* modellen geëvalueerd om de pathogenese van *Salmonella* Typhimurium infecties bij varkens te onderzoeken. De invasieve capaciteiten van *Salmonella* in de darm werden nagebootst aan de hand van een invasiemodel met twee verschillende epitheliale intestinale varkenscellijnen. De intestinale fase van de infectie, waaronder het ontstaan van diarree, kon verder worden gekarakteriseerd aan de hand van een varkensdarmlusmodel en aan de hand van een zuivere cultuur van bloedneutrofielen van het varken. De systemische fase van de infectie werd nagebootst door gebruik te maken van modellen met monocyten en longmacrofagen die bij het varken werden geïsoleerd. De invasie in de gepolarizeerde epitheliale cellijn was meer uitgesproken en beter herhaalbaar in vergelijking met de invasie in de niet-gepolarizeerde epitheliale cellijn. *Salmonella* Typhimurium kon snel vermeerderen in macrofagen, maar niet in monocyten. Neutrofielen doodden *Salmonella* Typhimurium snel af en migreerden massaal naar het darmweefsel en het darmlumen in het varkensdarmlusmodel. In een experimenteel oraal infectiemodel bij varkens werd aangetoond dat het verloop van de infectie dosis afhankelijk was. Een uniforme kolonizatie van de varkens werd bekomen na inoculatie met ten minste 10^7 kve. Dag 5 en dag 28 na inoculatie waren de beste tijdstippen om respectievelijk de kolonizatie en de persistentie van de *Salmonella* Typhimurium stam in varkens te onderzoeken.

In het derde hoofdstuk van deze thesis werd de rol van verschillende virulentiegenen van *Salmonella* in de pathogenese van *Salmonella* Typhimurium infecties bij het varken onderzocht.

In een eerste reeks experimenten werden de interacties van de *Salmonella* Typhimurium veldstam en zijn isogene deletiemutanten in de SPI-1 genen *hilA*, *sipA* en *sipB* met de varkensdarm en met varkensmacrofagen onderzocht. HilA and SipB waren essentieel voor *Salmonella* Typhimurium om *in vitro* varkensdarmcellen en macrofagen te kunnen invaderen. De *sipA* mutant was volledig invasief in de niet gepolarizeerde epitheliale cellijn en in de macrofagen, maar was minder invasief dan de wild type veldstam wanneer met de

gepolarizeerde epitheliale cellijn werd gewerkt. In de macrofagen was SPI-1 tevens belangrijk om de zogenaamde 'spacious phagosomes' te vormen. Alle SPI-1 mutanten waren sterk verzwakt in hun vermogen om neutrofielen aan te trekken naar de darm en bijgevolg ook om diarree te veroorzaken. De interleukine-8 en zuurstofradicaalproductie door de macrofagen werden niet beïnvloed door een mutatie in SPI-1. Zowel vroege als late celdood werd gezien in de macrofagen, maar enkel de vroege celdood bleek SPI-1 afhankelijk. Wanneer varkens peroraal geïnfecteerd werden met een combinatie van de *Salmonella* Typhimurium veldstam enerzijds en de *sipB* deletiemutant anderzijds, bleek dat de mutantstam sterk verzwakt was in het invaderen en koloniseren van de darmen, maar niet in het kolonizeren van de tonsillen.

De SPI-1 afhankelijke invasie is bijgevolg van cruciaal belang voor de kolonizatie van de darmen en het onstaan van diarree, maar niet voor de kolonisatie van de tonsillen. SPI-1 speelt tevens een rol in de interacties van *Salmonella* Typhimurium met varkensmacrofagen, onder andere door het induceren van celdood.

In een tweede reeks experimenten werden de interacties van de wild type veldstam vergeleken met een deletiemutant in het *ssrA* gen, een belangrijke regulator van SPI-2. De *ssrA* mutant vertoonde verlaagde expressie van SPI-2 gerelateerde genen, was verzwakt voor vermeerdering in muizenmacrofagen *in vitro* en was tevens verzwakt in laboratorium muizen na orale inoculatie. Hoewel dit voor sommige SPI-2 mutanten beschreven wordt, werd er in de *ssrA* mutant geen significante daling van expressie van SPI-1 gecodeerde genen gezien. Aan de hand van flowcytometrische analyse van groen fluorescente *Salmonella* stammen, werd duidelijk dat de *ssrA* mutant ook verzwakt was in het overleven in varkensmacrofagen. In een oraal infectiemodel vertoonden biggen die geïnoculeerd waren met de *ssrA* mutantstam een gelijkaardig verlopende infectie in vergelijking met biggen die geïnoculeerd waren met de wild type veldstam. Zowel 5 als 28 dagen na inoculatie waren de dieren uit beide groepen in dezelfde mate geïnfecteerd in de inwendige organen.

Deze resultaten tonen aan dat een SPI-2 mutant van *Salmonella* Typhimurium nog steeds in staat is om aanleiding te geven tot een persisterende infectie in varkens.

In een derde reeks experimenten, werd het belang van het fibronectinebindend eiwit ShdA in het ontstaan van een persisterende infectie onderzocht. Alhoewel het mechanisme van een langdurige uitscheiding van *Salmonella* in de faeces nog niet is opgeklaard, werd dit gen bij muizen recent geïdentificeerd als een belangrijke factor in de intestinale kolonizatie en persistentie in muizen. Varkens werden peroraal geïnoculeerd met de wild type *Salmonella*

Typhimurium veldstam of met een isogene *shdA* deletie mutant. De eerste dagen na inoculatie scheidden de biggen die werden geïnoculeerd met de s*hdA* deletiemutant, meer bacteriën uit en vertoonden ze vaker diarree vergeleken met de dieren die met de wild type veldstam werden geïnoculeerd. Op lange termijn werden geen verschillen gezien in het verloop van de infectie of de uitscheiding van de kiem in de mest. In het varkensdarmlusmodel was de *shdA* mutant even efficiënt in het aantrekken van neutrofielen naar de darm in vergelijking met de wild type veldstam.

In deze experimenten werd aangetoond dat een *shdA* mutant van *Salmonella* Typhimurium in staat is om aanleiding te geven tot een persisterende infectie in varkens.

De resultaten van deze thesis tonen aan dat zowel de gastheermodellen als de *Salmonella* stammen met zorg gekozen moeten worden om relevant onderzoek te kunnen doen naar de pathogenese van *Salmonella* infecties. Tevens kan geconcludeerd worden dat SPI-1 afhankelijke invasie cruciaal is voor de kolonizatie van de darmen, maar niet van de tonsillen. De bijdrage van SPI-2 en *shdA* tot de persistentie van *Salmonella* Typhimurium in varkens is veel kleiner dan beschreven voor muizen.

CURRICULUM VITAE

Filip Boyen werd geboren op 18 mei 1978 in Tienen. Na het beëindigen van zijn studies algemeen secundair onderwijs, richting Latijn-Wiskunde aan het Onze-Lieve-Vrouwecollege in Tienen, begon hij in 1996 met zijn studies Diergeneeskunde aan de Universiteit Gent. In 2002 studeerde hij af als dierenarts, optie herkauwers, met grote onderscheiding.

Onmiddellijk daarna begon hij aan een doctoraatsonderzoek op de vakgroep Pathologie, Bacteriologie en Pluimveeziekten met als promotoren Prof. Dr. Frank Pasmans en Prof. Dr. Freddy Haesebrouck dat gefinancierd werd door het Bijzonder Onderzoeks Fonds van de UGent, getiteld: De rol van SPI1 en SPI2 bij de interacties van *Salmonella enterica* subsp. *enterica* serovar Typhimurium met intestinaal epitheel en macrofagen van het varken. Vanaf 1 januari 2003 werd deze beurs vervangen door een IWT specialisatiebeurs met als titel: De rol van virulentiefactoren van *Salmonella* Typhimurium bij de interacties van de kiem met enterocyten, neutrofielen en macrofagen van het varken. Deze beurs werd beëindigd op 31 december 2006. In deze tijdsspanne schreef hij de huidige doctoraatsthesis en behaalde hij met succes de doctoraatsopleiding.

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