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PHARMACEUTICAL AND MUTATIONAL INTERFERENCE WITH VIRULENCE OF *SALMONELLA ENTERICA*

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ABSTRACT

Within the species *Salmonella enterica* are a diverse range of bacteria that can cause illness in humans and many animals. Salmonellae are extremely versatile and can adapt to a variety of environments and hosts. Typhoid and paratyphoid fever, caused by human-restricted *S. enterica* serovars Typhi and Paratyphi are common in the developing world.

An increased resistance to the first line antibiotics has been recorded amongst salmonellae, which makes the basic and applied research aiming to farther the understanding of the disease and developing new regimes of treatment even more important.

We showed that salicylidene acylhydrazides are able to inhibit the activity of virulence-associated type III secretion systems SPI1 and SPI2 in *Salmonella enterica* serovar Typhimurium. The compounds strongly affected *Salmonella* pathogenicity island (SPI) 1 activity and also SPI2-mediated intracellular bacterial replication in murine macrophage-like cells. In addition, two of the compounds significantly inhibited bacterial motility and expression of extracellular flagellin. We also found that the proton pump inhibitor omeprazole had a bacteriostatic effect on intracellular replication of *S.* Typhimurium mediated by virulence-associated SPI2 T3SS.

We could demonstrate that *S.* Typhimurium rapidly acquires mutations in the putative transport protein SbmA, reducing the susceptibility of the bacteria to antimicrobial peptide PR-39 without an obvious fitness cost.

We report for the first time that expression of thioredoxin 1 in the facultative intracellular pathogen *S.* Typhimurium is induced at conditions that prevail during intracellular infection and that thioredoxin 1 is necessary for proper T3SS expression, protein secretion and virulence. Our findings define an entirely new functional niche for thioredoxin 1 and demonstrate a new level of inter-connection between core genome functions and horizontally-acquired virulence genes in bacteria.

LIST OF PUBLICATIONS

I. **Negrea, A**., Bjur, E., Ygberg, S.E., Elofsson, M., Wolf-Watz, H., and Rhen, M. (2007). Salicylidene acylhydrazides that affect type III protein secretion in Salmonella enterica serovar typhimurium. Antimicrob Agents Chemother *51*, 2867-2876.

II. Puiac, S., **Negrea, A**., Richter-Dahlfors, A., Plant, L., and Rhen, M. (2009). Omeprazole antagonizes virulence and inflammation in Salmonella enterica infected RAW264.7 cells. Antimicrob Agents Chemother. (Ahead of publication)

III. Pränting, M., **Negrea, A**., Rhen, M., and Andersson, D.I. (2008). Mechanism and fitness costs of PR-39 resistance in Salmonella enterica serovar Typhimurium LT2. Antimicrob Agents Chemother *52*, 2734-2741.

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Sun, S., **Negrea, A**., Rhen, M., and Andersson, D.I. (2009). Genetic analysis of colistin resistance in Salmonella typhimurium. Antimicrob Agents Chemother. (Ahead of publication)

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LIST OF ABBREVIATIONS

ATR	Acid tolerance response
CD18	Integrin, beta 2
Cfu	Colony forming unit
3D	Three-dimensional
DCs	Dendritic cells
IL-8	Interleukin-8
IL-6	Interleukin-6
LAMP1	Lysosomal-associated membrane protein 1
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
mLNs	Mesenteric lymph nodes
phox	Phagocyte NADPH oxidase
PPs	Peyer's patches
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
TNF-α	Tumor necrosis factor-alpha
SCV	*Salmonella* containing vacuole
SPI	*Salmonella* pathogenicity island
T3SS	Type III secretion system
Vi	Vi polysaccharide capsule antigen

1 INTRODUCTION

1.1 SALMONELLA

The genus *Salmonella* includes more than 2500 different serological variants (www.who.int/en) called serovars divided in two species: *Salmonella enterica* and *Salmonella bongori,* with the majority of the pathogenic strains belonging to *S. enterica*. The serovars are distinguished on the basis of antigens displayed on the surface of the bacteria and listed in the Kauffmann and White scheme (Popoff *et al.*, 2004, Sojka *et al.*, 1977). Salmonellae are extremely versatile and can adapt to a variety of environments and hosts. Many salmonellae are commensals that colonize the animals without causing disease. However, *S. enterica* serovars Typhi and Paratyphi are strictly human adapted and cause the invasive diseases typhoid and paratyphoid fever. Others generically named non-typhoidal *Salmonella* cause self-limiting gastroenteritis in humans.

1.1.1 Human typhoid and paratyphoid fever

It is estimated that *Salmonella* causes up to 1 billion infections every year. Typhoid fever is responsible for more than 20 million infections and accounts for approximately 200 000 to 600 000 deaths (Crump *et al.*, 2004, Merican, 1997). It is difficult to estimate the actual magnitude of salmonellae infections since many endemic areas lack facilities to precisely diagnose typhoid fever and because other febrile illnesses are very frequent. *S.* Typhi causes typhoid fever, a systemic infection in humans. Paratyphoid fever is caused by *S.* Paratyphi (A, B and C), a human-restricted serovar of *S. enterica*. The disease manifests similarly to typhoid fever and it is estimated that 25% of enteric fevers may be caused by *S.* Paratyphi.

These diseases are contracted by ingesting contaminated water or food, and since the humans are the only host the transmission occurs via the fecal-oral route. There is a very strong correlation between hygiene and the incidence of typhoid fever. Where hygiene is very good, such as in developed countries, *S.* Typhi infection is rare and typhoid fever is a disease of the returning traveller. In contrast, typhoid fever is

endemic in Africa and Asia and is very frequent in the Middle East. In these areas the incidence of the disease is low in the first years of life (0-4 years old) with a peak in school children and young people (5-20 years old) (Mahle & Levine, 1993). In the adult population the disease is less frequent, evidently due to immunity acquired from repeated episodes of typhoid fever.

Humans are the only known reservoir for *S.* Typhi with the infection dose ranging between 10^3 -10^9 bacteria. Strains that are positive for the Vi polysaccharide capsule antigen (Vi) are generally more virulent than strains than are Vi negative. Contamination occurs via the fecal-oral route and the bacteria have to survive with the gastric acid environment of the stomach in order to arrive in the small intestine where they adhere and invade the intestinal lymphoid follicles. One to three days after infection *Salmonella* can be recovered from the mesenteric lymph nodes, liver and spleen. Without antibiotic treatment secondary bacteraemia is initiated via spread of the organisms to the bone marrow and the gall bladder. Infection in the gall bladder tends to become chronic especially if gall bladder stones are present. Chronically infected individuals shed the bacteria in the feces and are an important reservoir for the disease.

Bacteraemia is accompanied by fever that often rises to 40 °C, diffuse abdominal pain, vomiting, anorexia and headache. Diarrhea is not a major symptom of typhoid fever. In up to 30 % of the cases rose coloured spots present on the lower chest and upper abdomen typically last couple of days but can be missed in patients with dark skin.

Bleeding in the gastrointestinal tract is one of the most common complications of typhoid fever. Bleeding occurs at the level of Peyer's patches and can be fatal if a large blood vessel is involved. There may be even intestinal perforations. *S.* Typhi can be detected in the feces of 10 % of the untreated patients for up to three months. Up to 3% of infections become chronic and *Salmonella* can be detected in the feces of these individuals for more than one year. Interestingly up to 25% of the chronic carriers do not present a prior history of typhoid fever. The infamous Typhoid Mary is an example of healthy carrier of typhoid fever that infected 47 people before she was sentenced to quarantine by the authorities.

Chronic carriers of *S.* Typhi have an increased risk of developing cancers in the biliary tract, pancreas, gall bladder and large bowel (Caygill *et al.*, 1994, Caygill *et al.*, 1995).

The number of the bacteria in the blood is generally low, with children having a higher bacterial blood count than adults. When bone marrow samples are cultured *Salmonella* can be detected in 80% of the patients (Hoffman *et al.*, 1986). Serological tests for

typhoid fever have been developed but the efficiency is hindered by the fact that one third of the patients do not mount a vigorous antibody response.
Typing of the clinical isolates is commonly performed using phage lysis patterns as an epidemiologic tool to discriminate between phage types (Demczuk *et al.*, 2003).

1.1.2 Non-typhoidal *Samonella* in humans

In humans non-typhoidal samonellosis is caused by a limited number of serovars such as *S. enterica* serovar Enteritidis, Dublin or Typhimurium. These food-born serovars are not human specific and are often spread as zoonoses. Human *Salmonella* gastroenteritis is accompanied by fever, vomiting, diarrhea, muscle and abdominal pain. The pathology of the gastroenteritis produced by the non-typhoidal *Salmonella* in humans is marked by the influx of a large number of neutrophils in the intestinal lumen. Most people recover without medical attention but some strains are more virulent and bacteria can be detected in the blood samples in 25% of infections with *S.* Dublin (Fang & Fierer, 1991).
The major risk for infection is via improper food production and catering, especially since *Salmonella* can grow on a large variety of substrates at temperatures ranging between 7 and 40 °C. Contact between raw food, especially chicken carcasses, and cooked food is estimated to contribute to more than 30% of the domestic acquired cases of salmonellosis. The major source of human contamination is via the presence of *Salmonella* in food animals particularly in eggs and poultry. *S.* Enteritidis can be present on the eggshell as a consequence of the infection in the reproductive tract of the laying hens. The bacteria can penetrate the shell and the membranes contaminating the egg content. The vaccination of chickens and laying hens has proven to drastically reduce the percentage of infected eggs. Meat and meat products can become contaminated with *Salmonella* as a result of fecal spillage during slaughtering.
Milk and milk products are also vehicles for *Salmonella* transmission, and most of the outbreaks are associated with consumption of raw, unpasteurized milk or cheeses.
The non-typhoidal *Samonella* infection dose in humans is variable ranging between 10^6 and 10^8 cfu and depends on the content of the meal, physiological state of the bacteria, level of gastric acidity and susceptibility of the host.

1.1.3 Antibiotic resistance

Before the introduction of antibiotics the fatality rate in typhoid fever was 10% and it is estimated that today the rate has decreased to less than 1% (Crump et al., 2004). Chloramphenicol was the first drug found to be effective in the treatment of typhoid fever (Woodward *et al.*, 1948). The resistance to antimicrobial agents appeared as a result of acquisition of mobile genetic elements that can harbour multiple genes that confer tolerance to different antibiotics. By 1970 chloramphenicol resistance was very well established and outbreaks of resistant *Salmonella* occurred simultaneously in different areas. During the typhoid outbreak in Mexico in 1972 over 10 000 cases were reported with 90% of the isolates being resistant to chloramphenicol and tetracycline. The resistance was linked to the presence of the IncH plasmid, carrying the chloramphenicol acetyl transferase gene, that is though to have been transferred from *E. coli*. Furthermore, multi drug-resistant (MDR) *S.* Typhi has emerged in many endemic areas where first line antibiotics like chloramphenicol, ampicillin and amoxicillin have been extensively used to treat typhoid fever.

Today fluoroquinolones and cefalosporins are the drugs used to treat MDR *Salmonella* but low level of resistance has been reported (Threlfall *et al.*, 2006) and the list of alternative drugs is very short. A high level of resistance to fluoroquinolones is described in non-typhoid *Salmonella* as a result of mutations acquired in the topoisomerase gene (Ling *et al.*, 2003) but the resistance was not transferred until now to *S.* Typhi.

Plasmid-encoded extended spectrum beta-lactamase is responsible for the appearance of cephalosporin resistance among *S. enterica* serovars (Shannon & French, 1998) with the potential of transferring the resistance to serovar Typhi.

To emerge victorious from the battle against microbes we must extend our understanding of the mechanisms of antibiotic resistance and continuously develop novel drugs and antimicrobial therapies.

A new approach is to interfere with the activity of the virulence factors which only pathogenic bacteria possess. In this way the selective pressure of such compounds is less strong and it is limited to the pathogenic bacteria, with the effect on the commensal flora reduced to minimum.

Such an approach has been applied to different bacteria like *Vibrio cholerae* and *E. coli,* where small-molecular-weight compounds have been shown to inhibit virulence regulation (Hung *et al.*, 2005, Gauthier *et al.*, 2005). In *S.* Typhimurium salicylidene

acylhydrazides inhibit the activity of type III secretion systems (T3SS) (Negrea *et al*., 2007).

Different derivatives of salicylidene acylhydrazide are active against type III secretion systems in other bacteria including *Yersinia pseudotuberculosis* (Kauppi *et al*., 2003a, Kauppi *et al*., 2003b), *Chlamydia trachomatis* (Muschiol *et al*., 2006) and *Shigella flexneri* (Veenendaal *et al*., 2009).

1.1.4 *Salmonella* in cattle

S. Typhimurium and Dublin are endemic in Europe and represent the majority of cases associated with salmonellosis in cattle (Sojka et al., 1977). The incidence of *Salmonella* infection in cattle in the U.K. decreased significantly from approximately 4000 cases in 1969 (Sojka et al., 1977) to approximately 500 cases in recent years. Like other *Salmonella* infections, transmission occurs via the fecal to oral route. Infected cattle excrete large numbers of *Salmonella* in the feces, contaminating the environment and promoting spread to other animals.

Salmonella infects both calves and adult animals. In young animals the disease appears in the first weeks of life and without treatment the calves die shortly after the onset of the disease. In adult animals the disease manifestations include severe diarrhea, fever, abortion and reduced milk production. Adult animals that survive the infection with *S.* Dublin often become carriers and shed the bacteria in their feces for the rest of their life.

In cattle, the bacteria adhere to the intestinal mucosa and invade the epithelial linings by inducing membrane ruffles that engulf the bacteria (Frost *et al.*, 1997).

1.1.5 *Salmonella* in pigs

Several serovars of *Salmonella* can cause infections in pigs with different outcomes. Septicaemia is the result of infection with host-restricted *S.* Choleraesuis. In contrast, promiscuous *S.* Typhimurium causes an enterocolitic type of disease especially in young animals. Watery diarrhea is the first symptom of the disease which can progress to the necrosis of the mucosal surface (Sojka et al., 1977, Watson *et al.*, 2000). The SPI1 type three secretion system contributes to the virulence of the bacteria in an experimental settings where pigs are challenged per orally with *S.* Choleraesuis.

1.1.6 *Salmonella* in birds

S. enterica serovar Gallinarum has a high mortality rate in farmed adult birds with transmission occurring by the fecal oral route. The disease is called fowl typhoid and the bacteria show a systemic dissemination. In contrast, *S.* Pullorum affects very young birds and manifests as diarrhea.
Selected strains of *S.* Typhimurium and *S.* Enteridis can also cause disease in young birds with a high mortality. *S.* Enteritidis can cause chronic infection in farmed chickens and is responsible for outbreaks in humans caused by consumption of uncooked eggs (Coyle *et al.*, 1988). Some serovars like Montevideo, Hadar and Kentucky can colonize the intestine of the farmed birds without symptoms, but can still cause gastroenteritis in humans (Roy *et al.*, 2001).
Pigeons can become infected with *S.* Typhimurium variant Copenhagen that seems to be a pigeon restricted variant (Pasmans *et al.*, 2003).

1.2 IN VIVO AND *IN VITRO* MODELS TO STUDY *SALMONELLA*

Salmonella infection can cause very different clinical manifestations like fever, bacteraemia, gastroenteritis and systemic spread of the bacteria to vital organs. The outcome is influenced by the virulence potential of different serovars, by environmental factors and by the host susceptibility to *Salmonella* diseases. In order to understand how and why *Salmonella* is such a successful pathogen, different infection models have proven very useful in deciphering the molecular mechanisms of the bacterial armamentarium. Different animal models have been developed to investigate the systemic and the intestinal phases of the disease.
Serovars of *Salmonella* that have a broad spectrum of hosts like *S.* Typhimurium tend to cause sub-clinical intestinal diseases in contrast to the host restricted serovars that cause more severe systemic diseases.
Most of our understanding of the molecular aspects of the *Salmonella* pathogenicity comes from studying the infection process in rodent models of salmonellosis.

1.2.1 Models for gastroenteritis

Mice are not suitable as animal models for studying human gastroenteritis because *S.* Typhimurium causes a systemic infection in mice that resembles the infection of *S.*

Typhi in humans. Instead the mouse model is highly suitable for study of typhoid diseases (Santos *et al.*, 2001).

The serovars that cause non-typhoid salmonellosis in humans are promiscuous in terms of host preference and are typically acquired by consumption of contaminated food or water. The bacteria that survive the acid barrier of the stomach can colonize the intestine and cause localized gastroenteritis. The disease manifests as diarrhea, fever and intestinal pain and is accompanied by a large influx of neutrophils in the lumen of the intestine.

Several *Salmonella* serovars can naturally infect cows, pigs and chickens, with the disease manifesting as enterocolitis. This serves as model system to study human non-typhoid salmonellosis. Infection of calves with serovar Typhimurium and serovar Dublin results in gastroenteritis that mirrors the disease in man and it is a valuable tool for studying the non-typhoidal *Samonella* infection (Wray & Sojka, 1978). The use of such animal models is hampered because infection experiments using large animals are expensive and subject to great variability between individuals since most of the animals are outbred.

The *S.* Typhimurium infection in mice is not suitable as an animal model for gastroenteritis because the hallmark of the human gastroenteritis, diarrhea, is not present in mice. In addition, the bacteria are capable of spreading systemically. The recently developed streptomycin pre-treated mouse model for human gastroenteritis (Barthel *et al.*, 2003), despite of the fact that it lacks important features of the natural disease, could be useful for illuminating host factors important for enteric salmonellosis by using different knock-out mice.

1.2.2 The mouse model for typhoid fever

S. Typhimurium infection in mice is used successfully as a model of *S.* Typhi infection in humans since the mice develop a typhoid-like disease with the bacteria spreading systemically without the presence of diarrhea. The fact that mice are a natural host for *S.* Typhimurium is an advantage of this model. *S.* Typhimurium infection in susceptible mouse lines like BALB/c has been used extensively as a model to study the pathogenesis of *S.* Typhi infection in humans and revealed many important virulence factors.

After per orally challenge of BALB/c mice the bacteria can be detected one day post infection in the Peyer's patches (PP) (Fig. 1).

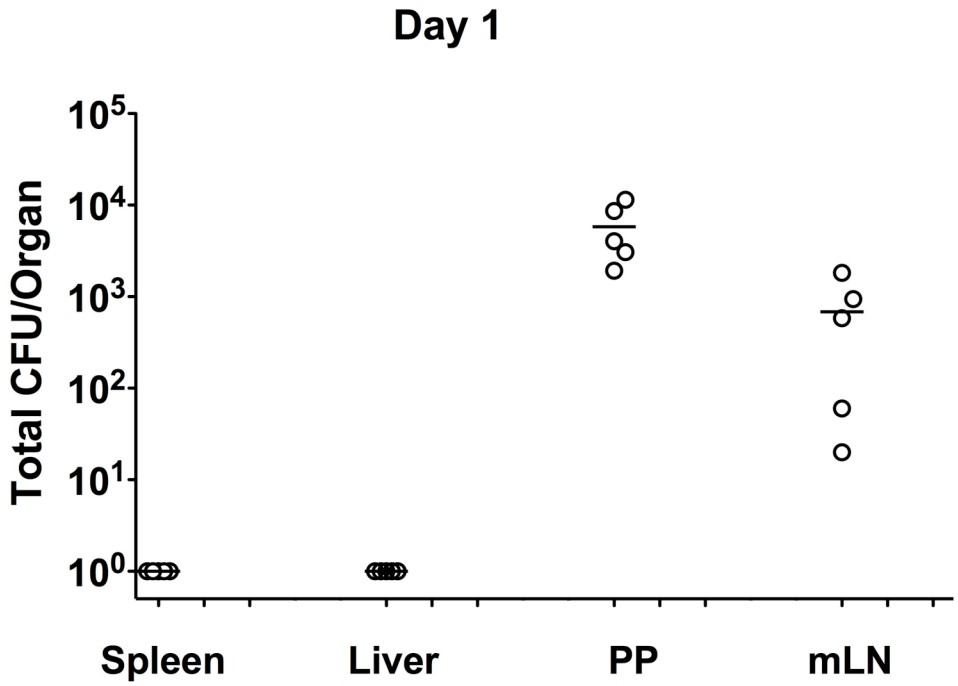

Figure 1. Visceral dissemination of *S.* Typhimurium in mice. BALB/c mice were challenged by the *per oral* route with wild type *S.* Typhimurium. The number of bacteria recovered from the mesenteric lymph nodes (mLN), Peyer´s patches (PP), liver and spleen were enumerated at day one post infection. Manuscript IV.

Furthermore, the bacteria can spread to the mesenteric lymph nodes (mLN), the liver and the spleen (Fig. 2). The bacteria multiply rapidly in the liver and spleen reaching 10^9-10^{10} bacteria per organ several days after infection, which results in hepatomegaly and splenomegaly.

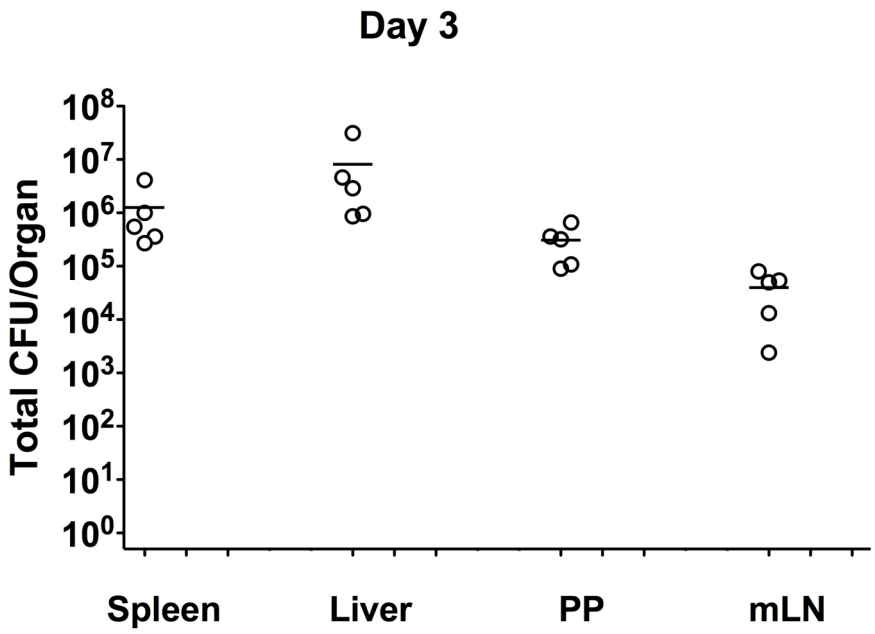

Figure 2. Visceral dissemination of *S.* Typhimurium in mice. BALB/c mice were challenged by the *per oral* route with wild type *S.* Typhimurium. The number of bacteria recovered from the mesenteric lymph nodes (mLN), Peyer´s patches (PP), liver and spleen were enumerated at day three post infection. Manuscript IV.

This animal model has proven to be very useful to study bacterial factors important in pathogenesis, like SPI1 and SPI2 Type III secretion systems (Hensel *et al.*, 1995). Type III secretion systems are supra-molecular machineries that enables Gram-negative bacteria to translocate effector proteins into eukaryotic cells and manipulate host cell processes for the benefit of the bacteria (Hansen-Wester & Hensel, 2001, Zhou & Galan, 2001).

One of the limitations of this model is that *S.* Typhimurium causes enteritis in humans rather than typhoid fever. In addition, some of the virulence genes present in *S.* Typhi are absent in *S.* Typhimurium and *vice versa* and therefore cannot be studied using this animal model.

Despite minor limitations, the murine model for typhoid fever have proven to be very useful for testing different live attenuated typhoid fever vaccine candidates.

1.2.3 Cell culture models

The use of cultured mammalian cells, especially epithelial and macrophage cell lines, has revealed many virulence factors responsible for *Salmonella* pathogenesis. Immortalized cultured cell lines have some limitations when it comes to addressing questions regarding progression of apoptosis, cell cycle control and the role of the immune system in controlling the infection. Some mammalian cell types like M cells are known to play an important role in the infection process but are difficult to maintain in culture.

The use of epithelial cell lines such as HeLa, Caco2, HT29 and MDCK facilitated the study of early events implicated in invasion of the intestinal linings and the replication of the bacteria inside epitheloid cells (Hautefort *et al.*, 2008). Within days after infection bacteria can be found localized inside macrophages in the liver and spleen of the infected animals. During this stage of the infection *S.* Typhimurium is a facultative intracellular pathogen capable of surviving and replicating inside membrane bound compartments termed *Salmonella* containing vacuole (SCV) (Richter-Dahlfors et al., 1997).

The use of macrophage-like cell lines: J774-A.1 and RAW264.7 have also been instrumental in deciphering the interplay between the host and the pathogen.

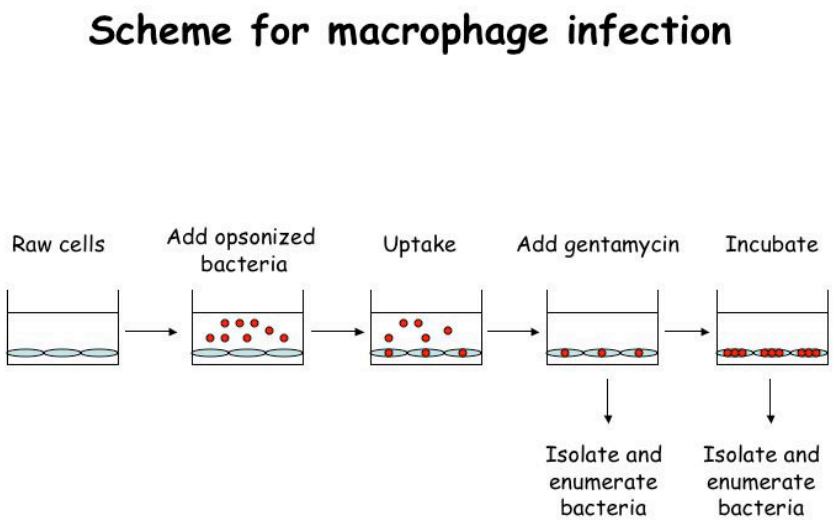

Figure 3. Scheme for the gentamycin protection assay. **(**Adapted from M. Clements)

1.3 *SALMONELLA* VIRULENCE FACTORS

During the course of infection *Salmonella* encounters different environments characterized by distinct properties such as nutrient availability, osmolarity, temperature, pH and different barriers of the host innate and adaptive immune system.
In order to be successful, pathogenic bacteria need to sense these characteristics of the surroundings and respond accordingly by gene expression programs that steer an adaptation response to the new host environment. This includes adaptation to new environments that lack nutrients, crossing host barriers, resistance to antimicrobial host defences and manipulation of host functions for the benefit of the pathogen.
Many of the genes necessary for virulence are clustered together in distinct regions on the bacterial chromosome called *Salmonella* Pathogenicity Islands (SPIs). Many SPIs differ from the rest of the genome in their guanine and cytosine content, indicating that these regions were acquired by horizontal gene transfer from other species.
SPIs are distinct DNA continuums characterized by genetic instability and are often associated with genes that code for tRNA.
Currently 14 pathogenicity islands have been identified in *Salmonella* species (SPI1-SPI14). The number and the content of the SPIs differs between different *Salmonella* serovars partly explaining the heterogeneity in host tropism and ability to reside in privileged niches (Hensel, 2004).

1.3.1 *Salmonella* Pathogenicity Island 1

SPI1 is a 40 kb island that was identified as a cluster of genes that allow *S.* Typhimurium to invade the intestinal barriers (Galan & Curtiss, 1989). SPI1 was the first island identified and it is present in all sub-species of *Salmonella*, suggesting that it was acquired early in the evolution of *Salmonella* species. SPI1 encodes for a prokaryotic type III protein secretion system (T3SS), via which a set of effector proteins are translocated from the bacteria into cytoplasm of the eukaryotic cells and regulatory gene systems for the expression of the island (Galan & Curtiss, 1989).
Type III secretion systems are supra-molecular apparatus composed of ~20 structural proteins assembled in a needle-like structure which spans the inner and outer bacterial membrane and projects from the bacterial surface. The computer generated 3D model

of the needle predicts that the structure is hollow inside and it is tempting to speculate that the effector proteins are secreted trough the needle (Galan & Wolf-Watz, 2006).
The SPI1 T3SS mediates the translocation of at least 13 effector proteins into host cells: AvrA, SipA, SipB, SipC, SipD, SlrP, SopA, SopB, SopD, SopE, SopE2, SptP and SspH1 (Zhou & Galan, 2001). The effector proteins contain a short signal sequence that targets them to the type III secretion system. Also, some of the effector proteins require the presence of chaperon proteins for their translocation (Galan & Wolf-Watz, 2006).
Salmonella can mediate its own uptake into non-phagocytic cells that compose the intestinal epithelium by utilizing SPI1 effector proteins to interfere with host cell actin polymerization. In this way they induce the formation of membrane ruffles that engulf the bacteria (Schlumberger & Hardt, 2006, Collazo & Galan, 1997). By concerted action, effector proteins interfere with the activation of Cdc42 and Rac1 to assist the membrane remodelling necessary for *Salmonella* mediated entry in to non-phagocytic eukaryotic cells (Gruenheid & Finlay, 2003). In the intestinal epithelium selected translocated SPI1 effector proteins induce profound inflammatory responses by activation of the transcription factor NF-kB, resulting in the production of proinflammatory cytokines (Hobbie *et al.*, 1997).

1.3.2 *Salmonella* Pathogenicity Island 2

SPI2 was identified as an island of approximately 40 kb associated with a tRNA gene, through a screening for mutants defective in virulence in a murine model for salmonellosis (Hensel *et al.*, 1995). The island codes for a second T3SS that is responsible for the secretion of selected effector proteins once the bacteria reside in the membrane bound compartment of the SCV. In addition, the SPI2 island encodes effector proteins, chaperones and the two-component regulatory system SsrA/SsrB. Many of the SPI2 effector proteins are encoded by genes localized outside the SPI2 island, which indicates separate acquisition events (Hansen-Wester *et al.*, 2002).

Deletion of some of the genes that encode for SPI2 effector proteins does not result in clear attenuation of the mutants in infection setups, probably reflecting redundant functions (Kuhle & Hensel, 2004).

SPI2 effector proteins create a vacuolar environment that sustains intracellular replication of *S. Typhimurium*. SPI2 effector proteins hijack the SCV from the endosomal degradation pathway by replacing early endosomal markers: early-

endosomal antigen 1 and transferrin receptor on the surface of the vacuole with late endosomal markers LAMP-1, LAMP-2 and LAMP-3 (Holden, 2002).

The function of SPI2 is essential for protection of the bacteria inside the SCV against the damage of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which represents mechanisms of adaptation to the intracellular environment of the phagocytic cells. The set of stimuli that activates the SPI2 apparatus for secretion is not completely deciphered, but the results using minimal media like MM5.8 and MES5.0 suggest that low pH, low concentration of Mg^{++} and phosphate are parameters that mirror the SPI2-inducing environmental cues prevailing in the intracellular vacuolar compartment where *Salmonella* resides and replicates (Beuzon *et al.*, 1999, Kox *et al.*, 2000).

1.3.3 Other SPIs

SPI3 is located near the *selC* locus in *S.* Typhimurium and serves as an integration point for horizontally acquired DNA (Blanc-Potard *et al.*, 1999). SPI3 is required for adaptation and survival of the bacteria in the nutrient-poor environment inside the SCV by encoding a high affinity magnesium acquisition system. Mutants deficient in SPI3 are impaired in the ability to proliferate in intracellular compartments and the ability to spread systemically (Blanc-Potard *et al*., 1999).

SPI4 was identified by comparing the genomes of *Escherichia coli* K-12 and *Salmonella* in the search for islands that are present in *Salmonella* but are absent from the genome of *E. coli* K-12 (Wong *et al.*, 1998). SPI4 codes for the components of a type I secretion system used by the bacteria to translocate proteins across the inner and outer membrane.

SPI5 is a small island located near the *serT* tRNA gene and has a mosaic structure of elements acquired independently. SPI5 genes code for effector proteins translocated by both SPI1 and SPI2 T3SSs. The island encodes the SopB SPI1 secreted effector protein contributing to the invasion of the epithelial cells and induction of inflammation in the bovine ileal loop model (Santos et al., 2001). Other effectors located in the SPI5 are expressed in coordination with SPI2 and secreted by the SPI2 T3SS (Knodler *et al.*, 2002).

As previously mentioned, there are more than 14 SPIs identified in different *Salmonella* species with various contributions to pathogenesis and a mosaic distribution. The continuous acquisition of big islands of foreign DNA by horizontal transfer is essential in host specificity exhibited by different *Salmonella* strains.

1.3.4 The virulence plasmid

Non-typhoidal *Salmonella* serovars contain a large plasmid that codes for virulence genes organised in the *spv* gene cluster (Gulig *et al.*, 1993, Gulig & Doyle, 1993). The functions of *spv* genes is not fully understood. SpvR is the regulator of the *spv* operon and *spvB* is a secreted mono-(ADP-ribosyl) transferase that targets mammalian actin (Tezcan-Merdol *et al.*, 2001, Rhen *et al.*, 1993). SpvC has phosphothreonine lyase activity on full-length phospho-Erk when translocated into infected macrophages (Mazurkiewicz *et al.*, 2008). The *spv* genes are activated when *Salmonella* resides inside the SCVs and are essential for the establishment of a systemic infection.

1.3.5 Power is nothing without control

PhoP/PhoQ is a two-component system widely distributed among Gram-negative pathogenic bacteria and used to regulate gene expression in response to environmental stimuli (Miller *et al.*, 1989). PhoQ is a sensor-kinase that is repressed by divalent cations and is activated in acidified phagolysosomes. It regulates genes important for intracellular survival, motility, invasion, and resistance to antimicrobial peptides. PhoP is responsible for regulating both, directly and indirectly, more than 200 genes, many of which are involved in virulence (Monsieurs *et al.*, 2005, Prost & Miller, 2008, Rhen & Dorman, 2005).

Mutants lacking the PhoP transcriptional regulator are derepressed for the expression of HilA, which in coordination with HilC, HilD and InvF are major regulators for the expression of SPI1 island (Bajaj *et al.*, 1995, Darwin & Miller, 1999). Being such a complex apparatus, the expression of SPI1 genes is tightly controlled by local and global regulatory systems that integrate the signals present in the environment, in order to allow the expression of the invasive genes at the right time.

HilA is a central transcriptional regulator that controls the expression of many genes in the SPI1 island, including HilC and HilD that act as modulators of HilA responses

(Lucas *et al.*, 2000). Global regulatory systems like the two component systems PhoP/PhoQ and BarA/SirA play an active role in the regulation of the island.

The SirA/BarA two-component system is directly and indirectly involved in the regulation of SPI1 and flagellar genes. In addition, SPI1 genes can be controlled at the level of mRNA stability. Mutants deficient in polynucleotide phosphorylase (PNPase) specifically accumulate mRNA for the genes located in the SPI1 and SPI2 pathogenicity islands (Clements *et al.*, 2002). Interestingly, the deregulation introduced by the loss of PNPase leads to a chronic carrier state when mice are infected per orally, creating an valuable animal model that resembles the chronic carriage phase of *S.* Typhi infection in humans (Clements et al., 2002, Rhen *et al.*, 2003).

In addition to PhoP/PhoQ, the expression of SPI2 genes is also regulated by the SPI2 encoded SsrA/SsrB two-component system (Cirillo *et al.*, 1998). The signals that are detected by the SsrA sensor are not precisely defined but it is clear that they prevail in the intracellular compartment where *Salmonella* resides and replicates.

OmpR/EnvZ is another two-component system implicated in the SPI1 and SPI2 gene activation by cooperating with the SsrA/SsrB system, allowing the bacteria to sense the surroundings and finely tune the response to the hostile intracellular environment of the macrophages (Lee *et al.*, 2000a, Garmendia *et al.*, 2003).

1.4 INNATE IMMUNE DEFENSES TO *SALMONELLA* INFECTION

Most infections with *Salmonella* occur by ingesting contaminated water and food. The first line of host defence that bacteria encounter is the low pH (1-2) in the stomach which constitutes an efficient barrier for many enteropathogenic bacteria. In response to moderate acidic stress *Salmonella* can mount an adaptive response termed the acid tolerance response (ATR) that involves the expression of acid shock proteins (Bearson *et al.*, 1998). The two-component system EnvZ/OmpR controlling the ATR can also sense the drop in pH in the phagosomal compartment inside macrophages and controls the expression of SPI2 island (Beuzon *et al.*, 2000, Beuzon *et al.*, 1999).

1.4.1 Antimicrobial peptides

The ability to resist to antimicrobial peptides constitutes an important virulence attribute of pathogenic bacteria (Peschel, 2002). The cathelicidins and defensins are

two important classes of antimicrobial peptides that limit the bacterial capacity to colonize epithelial surfaces. Cationic antimicrobial peptides (cAMP) are effective against *Salmonella* by disrupting the continuity of the negatively charged membrane (Hancock & Sahl, 2006). *Salmonella* can modify the structure of lipopolysaccharide (LPS), resulting in a more positively charged membrane that does not attract the positively charged antimicrobial peptides (Guo *et al.*, 1998, Guo *et al.*, 1997). In addition, under the control of PhoP/PhoQ, *Salmonella* expresses an outer membrane protease *pgtE* that can degrade antimicrobial peptides (Guina *et al.*, 2000).

1.4.2 Neutrophils

After passing additional barriers, like peristalsis, indigenous microflora, exfoliation of epithelial linings and the mucin layer, *Salmonella* finally faces its preferred target for invasion, the M cells. The low oxygen tension and osmolarity that characterize the intestinal environment constitute signals that trigger the expression and assembly of the SPI1 T3SS apparatus (Galan & Wolf-Watz, 2006). SPI1 effector proteins are translocated into the cytoplasm of the M cells and they mediate the formation of membrane ruffles that engulf the bacteria (Galan, 1996). The interaction of *Salmonella* with the epithelial linings leads to the production of proinflammatory molecules like tumor necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8) (MIP-2 in mice) leading to a massive recruitment of the neutrophils at the basolateral membrane of the epithelium. From here the neutrophils infiltrate to the intestinal lumen following a chemoattractant. By the time the bacteria are protected in the intracellular compartment, the neutrophils are chasing a false lure in the wrong place (Lee *et al.*, 2000b). This can be a profitable strategy for the *Salmonella* since the neutrophils are very bactericidal.

1.4.3 Dendritic cells, macrophages, oxidative and nitrosativ stress

Salmonella encounters the professional antigen presenting cells, dendritic cells (DCs), in the submucosal tissue and Peyer`s patches. These cells have a migratory behaviour and are used by *Salmonella* as dissemination vehicles from the intestinal tract to the bloodstream and the target organs, liver and spleen (Vazquez-Torres & Fang, 2000). Phagocytes that express the CD18 marker have also been shown to contribute to the dissemination of *Salmonella* to the liver and spleen after oral administration (Vazquez-

Torres *et al.*, 1999). Interestingly, *S.* Typhimurium is able to survive inside DCs in a modified vacuolar compartment lacking the lysosomal membrane glycoproteins (Garcia-Del Portillo *et al.*, 2000).

The ability to survive and replicate inside macrophages represents a vital trait of *Salmonella* during the *in vivo* and *in vitro* infection setups (Salcedo *et al.*, 2001, Shah *et al.*, 2005). Once inside the SCV *Salmonella* is capable of diverting the natural vacuolar maturation, by the coordinate action of the SPI2 effector proteins, creating a new compartment that does not interact with lysosomes and late endosomes (McCollister *et al.*, 2005).

The phagocyte NADPH oxidase (phox) catalyzes the reduction of molecular oxygen to superoxide, a precursor of reactive oxygen species like hydrogen peroxide, with potent antimicrobial activities (Vazquez-Torres & Fang, 2001). The importance of the reactive oxygen species is apparent in patients with chronic granulomatous disease which manifests by recurrent bacterial infections including *Salmonella* (Mouy *et al.*, 1989).

In mice nitric oxide synthases (NOS) and inducible nitric oxide synthases (iNOS) catalyze the production of nitric oxide (NO) with potent bacteriostatic activity against *Salmonella,* in a reaction that requires NADPH, oxygen and L-arginine (Vazquez-Torres *et al.*, 2000). iNOS is induced in response to bacterial LPS or in response to cytokines such as interferon γ (IFNγ) and TNF-α.

One of the biggest challenges that *Salmonella* faces when moving from an environmental to a pathogenic life-style is to adapt its redox potential in response to ever changing internal and external conditions. Severe oxidative stress generated by the production of ROI and RNI by the host cells can alter the redox status of the proteins that have cysteinyl side chains, generating sulfenic (P-SOH), sulfinic ($P\text{-}SO_2H$) and sulfonic ($P\text{-}SO_3H$) acids. The thiol group (-SH) is crucial for the formation of inter- and intramolecular disulfides (-S-S-) essential for protein folding, protein-protein interactions or for enzymatic activity (Berndt *et al.*, 2008). By diverting the SCV, *Salmonella* limits the action of oxygen independent and dependent antimicrobial mechanisms that phagocytic cells possess in order to control invading microorganisms. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by macrophages in response to *Salmonella* infection (Fig 4). To counterbalance oxidative stress *Salmonella* expresses a number of superoxide dismutases, catalases, hyperoxidases, reductase, radical scavengers and DNA repair mechanisms (De Groote *et al.*, 1997, De Groote *et al.*, 1996).

In many bacteria *trxA* mutants defective in expressing thioredoxin 1 (TrxA) are impaired in their response to oxidative stress and show increased sensitivity to hydrogen peroxide, establishing TrxA as an important oxido-protectant (Li *et al.*, 2003, Takemoto *et al.*, 1998, Comtois *et al.*, 2003). The Trx catalytically active site is composed by two cysteinyl residues, Cys-Gly-Pro-Cys, that serve to reduce the protein disulfide bonds (Holmgren, 1979). In addition, TrxA posses a protein chaperon function that is disconnected from cysteine interactions (Kumar *et al.*, 2004, Berndt et al., 2008, Kern *et al.*, 2003). In *Salmonella* TrxA is a housekeeping protein that strongly contributes to virulence in cultured macrophage-like cells and in mouse infection models (Bjur *et al.*, 2006).

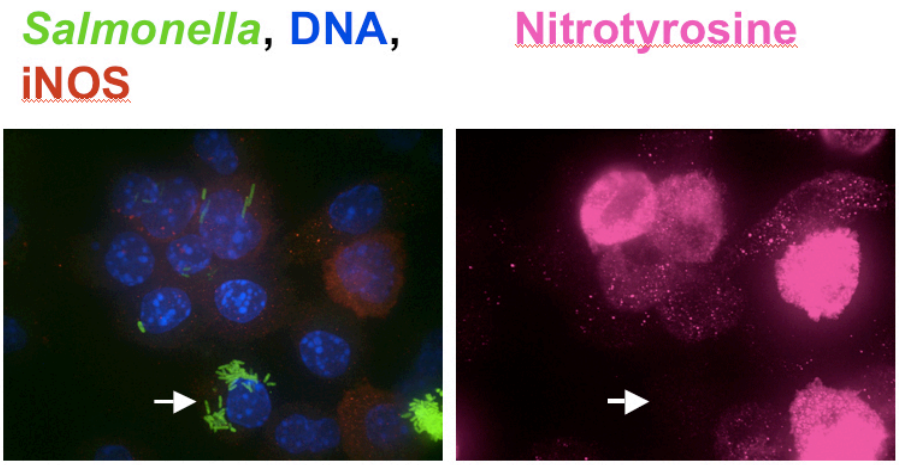

Figure 4. Immunofluorescence staining of intracellular GFP-tagged *S.* Typhimurium 14028 in RAW264.7 cells. The expression of iNOS and nitrotyrosine as an indicator of cell damage is shown at 16 hours post infection.

2 RESULTS AND DISCUSION

2.1 PAPER I

The discovery of antibiotics reshaped the world of pathogenic microbes and the danger that pathogenic bacteria posed to humans and livestock seemed to belong to the past. Soon after the introduction of antibiotics resistant bacteria have emerged rapidly and now antibiotic resistant bacteria are commonly reported in clinics.

A novel approach to discover new classes of antimicrobial agents is to search for inhibitors of selected virulence factors in order to disarm the bacteria (Hung et al., 2005, Gauthier et al., 2005). Conventional antibiotics have a broad range of action affecting also non-pathogenic bacteria that colonize the gastrointestinal tract, contributing in this way to the emergence of antibiotic resistance. In contrast, virulence blockers are predicted to affect only pathogenic bacteria by disarming their weapons and leaving the normal bacterial flora unaffected. Many small molecular compounds have been recently used to interfere with bacterial virulence factors without affecting viability, and have the potential to form a new class of antimicrobial agents used in clinics (Negrea et al., 2007, Muschiol et al., 2006, Gauthier et al., 2005).

T3SSs are used to export a variety of effector proteins into host cells and are an important virulence factor of many bacterial pathogens: *Chlamydia*, *E. coli*, *Pseudomonas*, *Salmonella*, *Shigella*, and *Yersinia*. T3SS is an attractive target for antimicrobial agents because it is an essential virulence factor as demonstrated by the inability of various mutants to cause disease. Salicylidene acylhydrazides were identified as inhibitors of T3SS in *Yersinia pseudotuberculosis* by screening a chemical library for the ability to block secretion of a tagged effector protein (Kauppi *et al*., 2003b, Nordfelth *et al.*, 2005).

In this study we report that salicylidene acylhydrazides strongly affect secretion of SPI1 effector proteins *in vivo*, and as a consequence SPI1 mediated invasion of epithelial cells. By using chromosomal transcriptional gene fusions we could demonstrate that the effect of salicylidene acylhydrazides resulted from the transcriptional inhibition of the SPI1 island at the level of the regulators HilA (Negrea et al., 2007), HilD and HilC (unpublished).

In *Salmonella* infected RAW 264.7 macrophage-like cells the compound INP0010 inhibited the replication of bacteria. This salicylidene acylhydrazide also had a drastic effect on the translocation of an epitope-tagged SPI2 effector protein when bacteria were grown in SPI2 inducing conditions.

Two of the compounds inhibited bacterial motility in soft agar plates. The expression of extracellular flagellin was shown to be decreased when bacteria were grown in the presence of compounds INP0404 and INP0405, explaining the defect in motility.

We can predict that pathogenic bacteria affected by the salicylidene acylhydrazides will attempt to develop resistance to this new class of antimicrobial agents, but at a very high cost of losing the functions of those virulence factors, becoming non-virulent bacteria. As salicylidene acylhydrazides have very little effect on bacterial growth we predict that they will pose low selective pressure on bacteria to fixate mutations. We can envision that salicylidene acylhydrazides could be used in clinics to combat resistant pathogenic bacteria that rely on the T3SS and related flagellar apparatus to caused disease. The list of susceptible bacteria could include: *Pseudomonas aeruginosa, Shigella spp.,* enteropathogenic *E. coli, Salmonella* serovars*, Yersinia spp., Chlamydia pneumoniae* and *Chlamydia trachomatis.*

2.2 PAPER II

Pathogenic bacteria that reside and replicate in the endosomal compartments of the host cells often rely on environmental signals like acidification of the vacuole, to induce the expression of virulence factors and cause disease (Merlen *et al.*, 2005, Miguel *et al.*, 2007, Rathman *et al.*, 1996). In this study we report that the proton pump inhibitor omeprazole can be used as a small-molecular compound to inhibit proliferation of *S.* Typhimurium in macrophage-like RAW264.7 cells. Omeprazole had bacteriostatic effects on intracellular *S.* Typhimurium in murine macrophage-like RAW264.7 cells during a 16 hours gentamicin protection assay. We could show that omeprazole lost its effects on intracellular bacterial replication when applied at later stages of the infection. Bafilomycin A1 posed a bactericidal effect on intracellular bacteria, in contrast to omeprazole that affected only the virulence-factor mediated replication of bacteria with no bactericidal effect on non-replicating bacteria. Neither proton pump inhibitors, omeprazole or bafilomycin A1, affected the capacity of *Salmonella* to replicate in culture medium or alter the minimal inhibitory concentration (MIC) to gentamicin.

Omeprazole-treated infected RAW264.7 cells were also impaired in the secretion of TNF-α, IL-6 and in iNOS expression, revealing that omeprazole suppressed inflammatory signaling.
We propose a novel property for omeprazole: the inhibition of SPI2-mediated bacterial replication even in the absence of NO production.

2.3 PAPER III

Antimicrobial peptides are defence weapons that are present in animals and plants, suggesting that they were developed early in the evolution of these groups of multicellular organisms (Zasloff, 2002). Resistance to antimicrobial peptides is surprisingly low when compared with conventional antibiotics, especially when it is consider that they were introduced long time ago and on a wide scale. *Salmonella* has developed a response mechanism to antimicrobial peptides that is regulated by PhoP/PhoQ system. The mechanism involves the PmrA/PmrB two-component system, which controls a number of genes that remodel the outer membrane by adding positively charged motifs ethanolamine and 4-aminoarabinose to the LPS (Gunn *et al.*, 2000).
In this study we have investigated the ability of *S.* Typhimurium to spontaneously develop resistance to the porcine antimicrobial peptide PR-39 by accumulation of mutations in the *sbmA* gene. PR-39 has a pronounced activity against *Salmonella* and it is proposed to have multiple intracellular targets rather than forming pores in the bacterial membrane (Agerberth *et al.*, 1991, Shi *et al.*, 1996).
To generate spontaneous mutations the bacteria were plated onto solid medium agar plates supplemented with PR-39, this approach generated 21 independent mutants that grew well on medium supplemented with PR-39. To identify the mutations, a P22 phage lysate grown on a mini-Tn*10* pool prepared from wild-type bacteria was used to generate tetracycline resistant mutant bacteria that lost the PR-39 resistance. DNA sequencing revealed that the mutations conferring resistance to PR-39 were located in the *sbmA* gene that is predicted to code a transporter protein with an ATP-binding-cassette. Different mutations in the *sbmA* gene, resulting in PR-39 resistance, did not affect *in vitro* or *in vivo* growth compared to the wild-type parental strain.
We also studied the fitness of the *sbmA* mutants in a mouse infection model. After 3 days of infection no differences in fitness could be observed between the mutants and

wild type bacteria. Interestingly one of the mutants, *sbmA* Q179Stop, showed a significant advantage in the competition with the wild-type strain.

These data demonstrated that *S.* Typhimurium can mutate in order to increase resistance to antimicrobial peptide the PR-39 and that the resistance does not come with a negative effect on fitness. We suggest, based on sequence similarity of *sbmA* in *S.* Typhimurium and *E. coli*, that the mechanism responsible for the resistance is the reduced uptake of the PR-39 peptide following inactivation of *sbmA*.

2.4 PAPER IV

One of the biggest challenges that *Salmonella* faces when moving from an environmental to a pathogenic life-style is to adapt its redox potential in response to ever changing internal and external conditions. Severe oxidative stress generated by the production of ROI and RNI by the host cells can alter the redox status of the proteins that have cysteinyl side chains, generating sulfenic (P-SOH), sulfinic ($P-SO_2H$) and sulfonic ($P-SO_3H$) acids. The thiol group (-SH) is crucial for the formation of inter- and intramolecular disulfides (-S-S-) essential for protein folding, protein-protein interactions or for enzymatic activity (Berndt *et al.*, 2008).

The Trx catalytically active site is composed by two cysteinyl residues, Cys-Gly-Pro-Cys, that serve to reduce the protein disulfide bonds (Holmgren, 1979). In many bacteria mutants of *trxA* are impaired in their response to oxidative stress and show increased sensitivity to hydrogen peroxide, establishing TrxA as an important oxido-protectant (Li *et al.*, 2003, Takemoto *et al.*, 1998, Comtois *et al.*, 2003). In addition, TrxA posses a protein chaperon function that is disconnected from cysteine interactions (Kumar *et al.*, 2004, Berndt et al., 2008, Kern *et al.*, 2003). In *Salmonella* thioredoxin 1 (TrxA) is a housekeeping protein that strongly contributes to virulence in cultured macrophage-like cells and in mouse infection models (Bjur *et al.*, 2006).

In paper IV we show that the previously described contribution of TrxA to the virulence of *S.* Typhimurium originates from its functional integration with the SPI2 T3SS under conditions that prevail in the intracellular vacuolar compartment of the host cells. We could demonstrate that *trxA* is co-induced with the SPI2 T3SS when bacteria are grown in a minimal medium mimicking the parameters characteristic for SCV, where *Salmonella* resides and replicates. These parameters include low pH, low concentration of Mg^{++} and phosphate. Furthermore, TrxA was required for proper induction of a SPI2 apparatus gene, *ssaG,* and for the secretion of the SseJ-2HA tagged

SPI2 effector protein. This led us to conclude that TrxA is strictly required for the functionality of SPI2.

TrxA dependant SPI2-mediated effector protein secretion could be demonstrated by fluorescence microscopy of *Salmonella* infected RAW264.7 or MDCK cells. To dissect the contribution of catalytic and non-catalytic TrxA to the intracellular replication in RAW264.7 cells, a *trxA* mutant was complemented with plasmid pFA3 resulting in a complete restoration of the intracellular replication. When complemented with the plasmid pFA8 that codes for a catalytically inactive TrxA we could detect a partial restoration of the replication in MDCK cells but not in RAW264.7 cells, suggesting different TrxA contributions to replication could be explained by the different intracellular environments predicted to occur in different cell types.

By probing the role of a *trxA* mutant, a SPI2 mutant and a double mutant SPI2/*trxA* to the intracellular replication we could demonstrate that SPI2 and TrxA contributed to the intracellular fitness of *S.* Typhimurium through a convergent pathway in cell lines commonly used for assaying *Salmonella* intracellular replication. However, more sensitive competition experiments in mice revealed that TrxA provided an additional fitness in a SPI2-deficient background and that SPI2 did not mediate an additional contribution to virulence in a TrxA-deficient background, allowing us to conclude that SPI2 is inactive in a trxA mutant *in vivo*.

The hypothesis that TrxA supports a disulphide-bond isomerization pathway could be discarded based on genetic evidence showing that *dsbC* mutants did not express a Δ*trxA* phenotype (Bjur et al., 2006). The periplasmic thioredoxin-related oxidoreductase DsbA has been implicated in T3SS activity and virulence of *S. Typhimurium* (Miki et al., 2004). In contrast to TrxA, DsbA affected the functionality of the SPI1 T3SS and the related fllagelar system. The defect observed in mice was a 10-fold reduction of the bacterial counts while the *trxA* mutant revealed a 1000-fold attenuation in an identical *in vitro* virulence assay (Fig 1 and 2).

The fact that we observed that, *S.* Typhimurium *trxA* mutants were not sensitized *in vitro* to nitrosative or oxidative stress prompted us to explore the connection between TrxA and virulence-associated SPI2 type III secretion system and led us to conclude that TrxA assists the induction of SPI2 under conditions defined by low pH and low concentrations of Mg^{++} and phosphate, or when the bacteria reside inside host cells.

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