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GENETIC ADAPTATION OF SALMONELLA ENTERICA TO PHAGOCYTIC CELLS

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Läppstiftet, en forskares bästa vän!

ABSTRACT

Salmonella enterica is a collection of closely related enteric bacteria that as a species is capable of infecting a wide variety of hosts. *S. enterica* serovar Typhimurium, (*S.* Typhimurium), causes a systemic disease in mice which in many aspects resembles human typhoid fever caused by *S. enterica* sv. Typhi. Both these infections are characterized by extensive bacterial growth in the macrophages of the reticuloendothelial system. The pathogenicity of *S.* Typhimurium has been correlated with its ability to survive and grow in macrophages, so we set out to define the parameters that govern intracellular replication of *S.* Typhimurium and how alterations in such parameters affect virulence. To accomplish this, we used two very different approaches. The first approach involved the development and use of a method that allowed RNA extraction from bacteria that had been growing inside eukaryotic cells, and subsequent analysis of gene expression applying microarray technology. This provided a detailed description of the dramatic changes in transcriptional activity that accompanies bacterial transition from an extracellular to an intracellular location. The second approach involved selecting for *S.* Typhimurium mutants that had increased growth yields in macrophage-like cells. These selection experiments showed that growth advantage mutants indeed could be isolated from phagocytic cells. Phenotypically, the mutations in these bacteria were associated with a modulation in the expression of existing virulence functions, such as the lipopolysaccharide and secreted virulence proteins. Furthermore, we found that most of the mutants that had gained growth advantage, had done so by limiting the host cells production of antimicrobial nitric oxide. Microarray analysis was then used to probe the gene expression profile of one host-adapted strain. This analysis revealed that virulence gene expression was affected. Selected virulence genes were "relaxed" for their expression in this host-adapted strain when grown *in vitro*, whereas others showed an amplified expression during intracellular growth. Host-adaptation could thus be viewed as an exaggeration of the normal adaptation to an intracellular environment. These changes in the ability to grow inside host cells were also associated with changes in the virulence potential and/or pathogenesis. Whereas some host-adapted mutants were attenuated for virulence, others had decreased lethality, and one mutant was able to cause chronic infection, thus reflecting additional aspects of salmonellosis. These data suggest that *S.* Typhimurium can indeed alter its virulence potential, and that the spectra of changes that mediate such shifts are broad. However, even if the ability to grow in macrophages was required for the normal pathogenesis, increased growth in macrophages did not necessarily mediate increased virulence, suggesting that adaptation is coupled to trade-offs. In conclusion, the virulence level of *S.* Typhimurium is well adjusted, and intentional modifications can identify important aspects of this key host-pathogen interaction.

LIST OF PUBLICATIONS

I

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II

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III

Mark O. Clements, Sofia Eriksson, Arthur Thompson, Sacha Lucchini, Jay C. Hinton, Staffan Normark, and Mikael Rhen. (2002) Polynucleotide Phosphorylase is a Global Regulator of Virulence and Persistency in *Salmonella enterica. Proc Natl Acad Sci USA.* **99**(13):8784-8789.

IV

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CONTENTS

LIST OF ABBREVIATIONS

ATR	acid tolerance response
CD	cluster of differentiation
CDS	coding sequences
CFU	colony forming units
Cl_2MDP	dichlorormethylene diphosphonate
DFI	differential fluorescence induction
FUN	function unknown
GAM	growth advantage mutant
GFP	green fluorescent protein
GSNO	S-nitrosoglutathione
IFN-γ	interferon gamma
IFN-γR	interferon gamma receptor
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
ip.	intraperitoneal
IRF-1	interferon regulatory factor one
iv.	intravenous
IVET	*in vivo* expression technology
LAMP-1	lysosomal membrane glycoprotein
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MC	mast cell
M-6-PR	mannose-6-phosphate receptor
MHC-II	major histocompatibility complex two
NF–κB	nuclear factor kappa B
NK cell	natural killer cell
NO	nitric oxide
NOS	nitric oxide synthase
Nramp	natural resistance associated macrophage protein
phox	NADPH phagocyte oxidase
PNPase	polynucleotide phosphorylase
po.	per oral
ROS	reactive oxygen species
RNI	reactive nitrogen intermediates
SCV	*Salmonella* containing vacuole
sc.	subcutaneous
SDS	sodium dodecyl sulphate
SPI	*Salmonella* pathogenicity island
STM	signature tagged mutagenesis
sv.	serovar(s)
TNF-α	tumor necrosis factor alfa
WHO	world health organisation
wt	wild type

1 INTRODUCTION TO SALMONELLOSIS

The genus *Salmonellae* can be divided into two species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* can be divided into subspecies, and further into serovars (sv.). As a genus *Salmonellae* are responsible for diseases that clinically range from mild gastroenteritis to severe systemic disease and death. Several of the approximately 2500 sv. are promiscuous in their choice of host organism, and this is reflected in the broad range of organisms that can be infected by *Salmonellae*, such as reptiles, birds, and mammals[1]. The disease caused by a specific strain is not identical in each host, and some will not even experience any symptoms, but merely function as carriers of the infection[1]. In this thesis I will focus on two aspects of systemic salmonellosis, the acute and the chronic carrier states of infection.

1.1 Epidemiology & human disease

Most serovars that are pathogenic to mammals belong to *Salmonella enterica* subspecies I. This group includes a number of serovars that mainly cause acute gastroenteritis in man, commonly sv. Typhimurium and sv. Enteritidis[2,3]. The World Health Organisation (WHO) estimates the number of diarrhoeal disease cases caused by *Salmonella* reaches 1.3 billion annually[2,3]. Subspecies I also includes sv. Typhi, which gives rise to a severe systemic disease called typhoid fever. While typhoid fever is rare in Sweden, in other parts of the world (mainly Asia and Africa), it is still very prevalent; WHO estimates 16.6 million cases annually, with around 600 000 concomitant deaths [2,3].

S. Typhi is entirely human specific and no other host reservoir has been found[4]. Clinically, fever is the first symptom of *S.* Typhi infection, followed by headache, abdominal pain, myalgia and fatigue[4]. The infectious dose, as well as the incubation time varies substantially [4,5]. The main source of infection is contaminated water, and infection is acquired via the faecal-oral route[3]. A portion of the ingested bacteria survive the low pH of the stomach, and migrate to the distal ileum where they replicate and colonize[4]. This is followed by adherence to the intestinal epithelium, and without necessarily causing tissue damage translocation, across the intestinal wall, leading to a primary bacteraemia[4]. Bacteria are then enriched in the liver and spleen as a result of the increased phagocytic capacity of the reticuloendothelial system during infection[5]. *S.* Typhi can be isolated during the disease, with a high frequency, from blood and bone marrow in infected patients[6].

Knowledge of the role of individual immune cells in controlling human typhoid fever, as well as the molecular events involved is limited. However, experimental as well as epidemiological evidence points toward a central role for the macrophage [7-11]. The fact that *S.* Typhi replicates in human phagocytic cells, but not in murine phagocytic cells, opens the possibility that macrophages could function as an *in vivo* replication niche for *Salmonella,* and implies that macrophages could be partially responsible for the human tropism of *S.* Typhi[8-11]. Disease transmission is obtained by secretion of *S.* Typhi in the faeces and urine [6]. In about 3-5% of the people that develop acute typhoid fever, a chronic carrier state follows[12]. In these individuals, the bacteria reside in the biliary tree, without causing noticeable disease[4]. Large amounts of bacteria may then be secreted in the faeces, which augments bacterial spread[5].

1.2 The mouse model for typhoid fever

The disease that *S. enterica* sv. Typhimurium causes in mice is very similar to that of *S.* Typhi in man. This observation lead to the design of a mouse model for systemic salmonellosis by Philip Carter and Frank Collins. These models included both iv. and ip., as well as po. challenge[13-16]. This model, sometimes with modifications, is still used extensively by the "*Salmonella* community".

If given in large enough doses *S.* Typhimurium has the capacity to cause lethal infection in mice. The LD_{50} varies with the strain of *S.* Typhimurium, the infection model, the mouse strain as well as the gender and age of the mice. Different lines of S. Typhimurium are intrinsically more or less able to cause disease, ranging from the low virulent LT2-strain to the highly virulent SR-11[17,18]. Susceptibility also varies with age and mouse strain; $Nramp^{-/-}$ are more susceptible than $Nramp^{+/+}$ [19,20]. As expected, susceptibility varies with the route of infection. More bacteria are needed to cause disease using po. than using ip. or iv. challenge[17] (personal observations).

1.2.1 Infection & pathology

Upon po. bacterial challenge, the inoculum first reaches the stomach where the decrease in pH inevitably leads to some bacterial killing; upon intragastric challenge of *S. enterica* sv. Enteritidis only 1% survive the first hour post infection[14]. The surviving bacteria will rapidly move to the small intestine, and invade specialised so-called M-cells of the Peyer´s patches [21]. Bacteria can be found in ileal Peyer´s patches as early as 6h post infection, but colonization is more evident at 72h

post infection[14]. This colonization includes infection of, and replication within, phagocytic cells underlying the M-cells [14,22]. Early during colonization there is an influx of neutrophils into the Peyer´s patch dome, and if this influx is reduced, significantly more bacteria are required to cause disease [23].

It has been postulated that *Salmonella* can be transferred from the intestine to the bloodstream within CD18-expressing phagocytic cells [24-26]. Severely attenuated mutants that are prevented from entry via Peyer´s patches could hence use this route to cross the intestinal wall. Such mutants can be found within CD18-expressing cells in peripheral blood. Accordingly, this route of entry is prevented in $CD18^{-/-}$ mice[24]. Vazquez-Torres *et al.* also showed that upon bacterial entry via $CD18^{+}$ cells the host fails to mount a mucosal IgA response despite that the systemic IgG response is normal[24]. Since po. infection with *Salmonella* normally triggers a mucosal IgA response [24], these data strongly argue that most *Salmonella* enter via Peyer´s patches and not by transport by tissue macrophages/dendritic cells (DC).

After crossing the epithelium the bacteria will spread through the mesenteric lymph nodes and the blood stream[15,24], leading to a transient bacteraemia, to finally reach the liver and spleen. During this stage, bacteria can be found in the tissue macrophages, in which replication is evident[27,28]. At least in the spleen this bacterial proliferation, is accompanied by a reduction of $NK1.1^{+}$ cells, T cells, and B cells, and an increase in neutrophils and macrophages[29]. These alterations are followed by, possibly associated with, splenic granuloma formation. Granulomas are cell clusters that form as a localized response. In the case of salmonellosis, the granulomas mainly consist of macrophages and the formation of these clusters appear critical for controlling the infection[30, 31].

If mice are given a dose of bacteria that cannot be controlled by the immune system, they succumb to infection when the bacterial load reaches approximately 10^{8} [31]. Conversely, if the infection is limited and finally cleared, the mice become immune to subsequent challenge [31].

In an ip. infection, the bacteria are injected into the peritoneal cavity to circumvent the invasive intestinal phase. The peritoneal fluid contains monocytes, macrophages, NK cells (natural killer cells), complement factors, and antibodies. In addition, neutrophils are recruited within 3h post injection as a response to LPS in the peritoneal cavity[32]. The presence of complement factors will lead to C3b deposition on the bacterial surface, which greatly increases phagocytosis efficacy[33]. Various *Salmonella* strains differ in the degree of complement activation/deposition, and O-antigenic variants that deposit more/quicker

complement are rapidly killed in the peritoneal cavity[34-37]. In the case of a secondary infection, antibodies may play a role in bacterial clearance[31]. Compared to the time schedule observed for a po. infection, bacteria injected ip. reach the liver and spleen much faster, and significant numbers are already evident 24h post infection[38]. There are a number of bacterial mutants that are attenuated when mice are infected po., but not upon ip. infection. These include mutants deficient in specific components that are required for invasion of the epithelial barrier[39].

Salmonella can also be injected iv., which allows direct access to the bloodstream, avoiding both invasion of the epithelium and translocation to the lymph nodes. Bacteria will rapidly accumulate mostly in the liver and spleen, and are already in an intracellular location 2h post injection. This was shown by the failure of an antibiotic that is not taken up by eukaryotic cells, injected 2h post infection, to rescue mice from salmonellosis [40]. The growth within the liver and spleen then follows the principles described above.

A limited number of experiments have used a subcutaneous (sc.) challenge. This route has been particularly used to study the rapid extravasion of neutrophils in response to *Salmonella* infection[41].

1.3 T cells & B cells in salmonellosis

Salmonella infection, clearance and immunity involve all arms of the immune system, and as indicated above, include several types of local cellular, and systemic responses. The role of all the different types of lymphocytes in salmonellosis is still not complete, but important conclusions can be drawn. T cells, in particular the $CD4^+$ subset but also the $CD8^+$ subset, are required for control/ clearance of *Salmonella* infection[42]. Nude mice and MHC-II deficient mice both fail to clear an infection with an attenuated *Salmonella* strain (Δ*aroA*) and develop a type of chronic infection[43a,43b]. Furthermore, the ability to clear the *Salmonella* infection seemed to correlate with the ability of isolated spleen cells from these mice to respond with IFN-γ production to antigen stimulation[43a]. Which of the T cell functions that makes the strongest contribution to bacterial clearance remains unknown.

Using mice with strongly reduced B cell functions ($Ig\mu^{-/-}$) results in a lack of control of virulent *Salmonella* both in primary (po.) and secondary scenarios (po./iv.)[44]. However, B cell deficient mice still retain the ability to clear an infection with an attenuated *Salmonella* strain (Δ*aroA*)[44]. Similar results have been obtained with other B cell deficient mice [31]. The role of antibodies in the protection against a second

challenge is subject to discussion and varies from system to system. Antibodies do seem to play a role during early infection, but the degree of protection varies with the inherent susceptibility of the mouse strain[31,42,45a,45b].

1.4 Phagocytic cells

The most important function of the phagocytic cell, from an immunological perspective, is to internalise and degrade particulate material. Bacterial binding and uptake, as well as host signalling are complex processes, which are mediated by a variety of surface receptors, often in combination. For example, bacteria that are opsonised with antibodies can be taken up via Fc-receptors, whereas bacteria that have bound complement can bind complement receptors. There are also receptors that recognise and bind conserved bacterial motifs, with or without adaptor molecules. This group includes the mannose receptor, the scavenger receptors, CD14, and the Toll-like receptors. The specific role of these receptors in bacterial uptake varies[46,47].

The newly formed vacuole containing the ingested particle will then develop during a multiple-step process referred to as vacuole maturation, and fuse with other types of vacuoles to form a phagolysosome[48]. This compartment, which is very anti-microbial, is characterized by low pH and the presence of acid hydrolases, free radicals and antibacterial peptides[47]. Particle degradation is in some cases followed by antigen presentation to lymphocytes. Additional roles of phagocytic cells include cytokine and chemokine production.

There are in principal four groups of myeloid immune cells: monocytes/macrophages, DC, mast cells (MC) and polymorphonuclear granulocytes. These types of cell contribute to varying degrees to the functions of phagocytic cells described above. Monocytes/macrophages can be subdivided into circulating monocytes and different types of macrophages. Tissue macrophages are found in many organs, and of most interest in the context of systemic salmonellosis are the resident Kupffer cells of the liver and the resident splenic macrophages. DC are antigen-presenting cells that reside along the surface linings of the body and in secondary lymphoid organs. *Salmonella* has been found within the DC in the Peyer´s patches of the small intestine[49]. However, there are no data that would support bacterial proliferation within DC *in vitro*[50-52]. MC are phagocytic and antigen presenting cells that are found for example on mucosal surfaces. MC can phagocytose opsonised bacteria, and upon such stimulation become activated. This leads to release of chemoattractants and proinflammatory

mediators, followed by recruitment of inflammatory cells such as neutrophils and influx of serum components. MC can also ingest bacteria that are not opsonised, which may lead a defective response, converting MC into bacterial reservoirs. Complement opsonised *Salmonella* can be phagocytosed by MC efficiently *in vitro*, and this leads to antigen presentation to T cells[53-55]. Polymorphonuclear granulocytes are short-lived circulating cells that are recruited upon infection. They can functionally be divided into neutrophils, basophils and eosinophils, of which neutrophils are most abundant, making up 90% of circulating granulocytes.

1.4.1 Function of macrophages during salmonellosis

The central role of macrophages for *Salmonella* proliferation, while evident today was not always so. There were early reports that questioned whether *Salmonella* was an intracellular parasite at all, and suggested that the intracellular location of *Salmonella* in cell culture experiments was merely an artefact[56]. Collective evidence now suggests that during the replicative phase of the murine infection *Salmonella* is mainly found intracellularly and that macrophages represent a central growth niche[57]. Strong contributions to these conclusions derive from the direct observation of *Salmonella* inside macrophages *in vivo* [27,28,58], and from data demonstrating that mutants that cannot replicate inside cultured macrophages are avirulent[59,60,61]. This view of course neither excludes that *Salmonella* can localize inside neutrophils or DC during infection, nor does it neglect the importance of other cell types for controlling salmonellosis.

Besides microscopical analysis, several other approaches have been designed to address the roles of macrophages during various stages of murine salmonellosis. In the sections below, I will try to emphasize a few aspects that I consider relevant. It follows that the answer you get is subject to the model you use, which again is chosen according to the question. However, essential information can be gathered from the results obtained when infecting various knockout-mice or mice depleted for certain cells and/or soluble factors and /or receptors with *Salmonella*.

Injecting mice with silica reduce the phagocytic capacity of splenic macrophages [62]. When such mice were infected ip. with *Salmonella*, bacterial counts in the spleen were increased, leading to the conclusion that macrophages were important in controlling bacterial numbers[38]. An alternative interpretation is that silica treatment aids bacterial replication by increasing the amounts of macrophages in the RES. Similar results to those of O´Brien *et al.*were obtained by Gulig *et*

al. In these experiments two types of depletion were tested: silica or dichloromethylene diphosphonate liposomes (Cl_2MDP). Cl_2MDP selectively accumulates in macrophages, which leads to their death. Infecting these mice po. with high doses of *Salmonella* lead to the conclusion that in the absence of macrophages *Salmonella* can reside in other cells, but that the growth/survival pattern observed differed from that in a wt mouse[63].

A somewhat different result was obtained in experiments in which mice were depleted for their macrophages with Cl_2MDP, and infected iv. with a low dose of *S.* Typhimuirum. In these mice bacterial yields were reduced, and the mice were hence more resistant to *Salmonella* infection. In addition, less organ damage was evident, most likely due to reduced cytokine and free radical production. The clearance of a strain that cannot replicate in macrophages was not affected[57]. As compared to the previous experiments by O´Brien *et al.* and Gulig *et al.,* very efficient macrophage depletion was achieved in the experiments by Wijburg *et al.* [38.57.63]. These results collectively suggest that the macrophage is an important growth niche for *S.* Typhimurium in wt mice, but that in the absence of macrophages wt bacteria may utilise other host cells.

The ability of macrophages to limit the proliferation of an intracellular parasite usually correlates with the activation state of the macrophage. Hence a parallel approach to understanding macrophage function is to study *Salmonella* infection in mice that lack components involved in macrophage activation. Such activation can in most cases be achieved *in vitro* by stimulation with for example bacterial LPS, but efficient responses *in vivo* involve tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), and interferon-γ (IFN-γ). A series of *in vivo* experiments emphasize that TNF-α, IL-12 and IFN-γ are essential for control of bacterial proliferation[30,31,64-71]. Histopathology has revealed massive infiltration of mononuclear cells in the spleens of TNF-α depleted and IL-12 depleted mice, accompanied by reduced macrophage granuloma formation [30.31.68. 70]. Anti-IL-12 antibody treated mice also displayed little signs of illness, despite high bacterial counts. This is most likely due to a reduced transcription of macrophage activators/effectors (IFN-γ and inducible nitric oxide synthase)[30]. As expected, these unstimulated mononuclear cells appeared less efficient in controlling the intracellular bacterial replication[30]. Administrating IFN-γ to the infected IL-12 depleted mice, reversed this scenario, indicating that the main role of IL-12 was to induce IFN-γ,[30]. These data are supported by the observations that IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice are much more

susceptible to po. infection with *Salmonella*[43a,65]. Furthermore, mice deficient in IL-4 are more resistant to *Salmonella* infection, and show little signs of organ damage, this is most likely due to altered cytokine balance upon infection of such mice [72].

So which cell type/types produces the IFN-γ? The initial literature states that early IFN-γ is most likely produced by NK cells[73]. However, mice depleted of their NK cell populations and infected iv. or ip. with *Salmonella* had significantly less bacteria recovered from the spleen[74]. More recent data suggest that the macrophages and the infiltrating neutrophils of the spleen are responsible for most of the early IFN-γ production[29]. Late IFN-γ is produced by $CD4^+$ T cells[71].

In summary, early IFN-γ, produced by macrophages and neutrophils holds a key position when it comes to controlling a *Salmonella*-infection, but at the cost of tissue damage and toxicity. Furthermore, the formation of macrophage granulomas appears critical for eradicating the infection, possibly involving T cell produced IFN-γ.

1.4.2 Function of neutrophils during salmonellosis

The complex *in vivo* role of neutrophils in murine salmonellosis has been elucidated in direct and indirect ways. First, if resistant mice are made neutropenic they become very susceptible to *Salmonella* infection. When such mice are infected iv. there is no initial bacterial killing, and bacteria can be recovered from various organs, including the lungs, the kidneys, and the brain early during the infection [75]. These results are somewhat contradictory to those of Vassiloyanakopoulos *et al.*, which indicate that the effects of neutrophil activities are not apparent until after the bacteria have reached the liver and spleen. Furthermore, the extent to which neutrophils contribute to bacterial clearance in this system varied with the inherent susceptibility of the mouse strain and the bacterial strains[40]. That the effects of treating mice with agents that make them neutropenic vary was confirmed by the report by Gulig *et al.* where mice infected po. with *S.* Typhimurium were in some experiments more susceptible to *Salmonella,* whereas in other experiments they were not [63].

More information regarding the role of neutrophils in salmonellosis can be gained from knockout mice that have an altered phenotype when it comes to neutrophil responses. For example, when *Salmonella* is injected ip. into mice lacking lipopolysaccharide binding protein (LBP), the chemotactic signals that guides the neutrophils into the peritoneal cavity are absent, and neutrophil infiltration becomes delayed. This results in reduced bacterial killing,

which renders the mice very susceptible to salmonellosis[32]. Whereas the LBP$^{-/-}$ mice have an increased susceptibility to ip. infections, there are also examples of mice with disturbances in the neutrophil response but that show increased resistance to infection. For example, caspase 1$^{-/-}$ mice are more resistant to po. *Salmonella* infection. Experiments revealed that the infiltration of neutrophils into the Peyer´s patches of caspase 1$^{-/-}$ mice was disturbed, and that this lead to a hampered infection that failed to disseminate efficiently [23]. Furthermore, a key role in this infiltration was ascribed to the caspase 1-mediated activation of interleukin-1β (IL-1β) which functioned as a chemoattractant [76,77].

It is also interesting to note that although caspase 1 also mediates interleukin-18 (IL-18) activation, caspase 1$^{-/-}$ mice did not show altered susceptibility upon ip. challenge[23]. These results are somewhat contradictory to the data obtained when neutralizing IL-18 in mice infected iv. with *Salmonella*. In these mice the disease was exacerbated in an IFN-γ-dependent way [78].

In summary, neutrophils participate in bacterial clearance and/or dissemination of *Salmonella in vivo*. However, based on the *in vitro* bacterial killing abilities of neutrophils[40], it is unlikely that they represent a significant growth niche for *Salmonella*.

1.4.3 Defence mechanisms of the phagocytic cell

Phagocytic cells represent an important aspect of defence against invading microorganisms, and their activity is achieved by a concerted action of anti-microbial strategies, which can be divided into free radical-based and non-free radical-based defence effectors. The first group involves the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). The second group refers to the production of antibacterial peptides and in the case of *Salmonella*, which do not escape from the phagolysosome, also to mechanisms associated with this compartment.

1.4.3.1 Free radicals & *Salmonella*

The expression of radical-based defences by phagocytic cells is absolutely central to resistance against *Salmonella*[79]. In the macrophage, practically all the ROS and RNI products originate from the production of NO and superoxide, and in mice lacking NO production and/or superoxide production, *Salmonella* infection is tremendously escalated [80-82]. In addition, several groups have shown that *Salmonella* express proteins that functionally interfere with the radical-producing enzymes and the ROI and RNI products[83-86]. Free radicals are also

important in human salmonellosis. While people lacking NO production have not been described, people that cannot produce superoxide are more prone to *Salmonella* infections[87].

NO is produced by nitric oxide synthases (NOS) through the enzymatic oxidation of L-arginine, yielding citrulline and NO, and involving the intermediate N-hydroxyarginine[88]. There are three isoforms of NOS, but the enzyme relevant during infections is mostly inducible NOS (iNOS). Microbial components such as LPS, with or without host-derived IFN-γ and TNF-α, induce iNOS expression *in vitro* from several cell types. For example, host NO production can be detected around 8h post infection from *Salmonella*-infected J774-A1. macrophage-like cells[89]. Since NO is equally toxic to both prokayotic and eukaryotic cells[90], it is obviously important to strictly regulate iNOS activity. This is achieved both through a spatial regulation[86], and a feedback inhibition[91,92]. At the transcriptional level, induction of iNOS expression is dependent on at least the regulators nuclear factor kappa B (NF-kB), interferon regulatory factor 1 (IRF-1) [93,94]. In order to catalyse NO formation, iNOS needs to be dimerized, which requires the presence of several cofactors as well as the substrate L-arginine[94,95]. To ensure sufficient amounts of L-arginine, both the cell synthesis and import of L-arginine are concomitantly induced with iNOS[96,97]. Additionally, the iNOS protein can be post-translationally modified, which stabilizes the protein, and enables it to re-localize to small intracellular vesicles[98]. It is likely that iNOS vesicles can fuse with a phagosome, and upon such fusion, induce radical production[86]. The short half-life of radicals ensures that the host cell is spared whilst targeting the phagosomes.

Superoxide is produced by NADPH phagocyte oxidase (phox). The induction of murine phox activity is rapid, and is actually an assembly of the existing parts, which includes gp91, p22, cytochrome b_{558}, p40, p67 and Rac1[84, 99]. The subsequent superoxide production occurs less than 1h post infection. This bacterial activation of phox involves phosphorylation of selected components, leading to assembly and membrane localization[99]. As in the case of iNOS, phox is believed to localize to the phagosome upon activation [85,100].

Both NO and superoxide are labile, reactive compounds, which decompose within, and in close vicinity of, *Salmonella*-infected macrophages[82]. The majority of the NO molecules are oxidized to nitrate, but a substantial part is also metabolised to nitrite. Similarly, the most abundant ROS metabolite is hydrogen peroxide, whereas superoxide is only the second most abundant[82]. The relatively low amounts of superoxide and NO in these cells may be a

reflection of the consumption of superoxide by NO for the production of peroxynitrite [99]. The effect of exposure to these free radical derivatives is nitrosylation and oxidation of not only bacterial, but also host proteins, lipids and nucleic acids[84,90]. The productions of ROS and RNI have synergistic effects. For example, peroxynitrite is more toxic than NO and superoxide alone [84]. Similarly, NO can probably enhance the toxicity of hydrogen peroxide, by reducing Fe^{3+} to Fe^{2+}, and thus providing substrate for the Haber-Weiss reaction[84].

To combat these free radical defences *Salmonella* is equipped with not only systems that will limit the fusion of iNOS and phox with the phagolysosome, but also with radical scavengers (glutathione, homocysteine), repair systems (RecBCD), and enzymes that can convert these harmful molecules into less toxic ones (catalases, superoxide dismutases, alkyl hydroperoxide reductase, glutathione reductase) [83,85,86,100-105]. Many of these functions are required for virulence in wt mice.

1.4.3.2 Antibacterial peptides & *Salmonella*

As a group, antibacterial peptides are broad spectral antimicrobial substances present on the body surfaces, in blood, as well as in several types of cells. Murine macrophages produce antibacterial peptides/ proteins, some of which are active against *Salmonella* [106,107]. The mechanism of antibacterial action in most cases involves electrostatic attraction, and subsequent disruption of the membrane integrity of the target cell. *Salmonella* can mount a relatively high resistance to antibacterial peptides and cationic antibiotics and the described mechanisms for resistance so far involves either an alteration in the LPS structure or an induction of proteases. LPS-mediated resistance is achieved by adding functional groups such as 4-aminoarabinose to the LPS, or differential acylation of the LPS, whereas protease induction can be represented by expression of the outer membrane protease PgtE[108-118]. Most of these alterations are regulated directly or indirectly by the phoP/Q virulence regulator[117,119,120]. It has been very difficult to assess the role of individual systems in resistance to antimicrobial peptides *in vivo*, since most of the bacterial mutations that mediate resistance also confer other phenotypes; some LPS mutants are taken up differently into phagocytic cells [89,121](personal observations) these mutants may also reside in a different compartment intracellularly [122]. Furthermore, there is very little data on *Salmonella* infection in mice that lack certain antimicrobial peptides, and the immunomodulatory effects that antimicrobial peptides have on

eukaryotic cells, such as macrophages, would complicate such analysis further [123].

1.4.3.3 The *Salmonella*-containing vacuole

Salmonella can either actively invade (discussed below) or be passively ingested by the phagocytic cell, yet there are data to suggest that the route of entry does not seem to alter the final localization of intracellular bacteria[124,125]. Phagocytic ingestion of *Salmonella* could be mediated by several surface receptors. The situation *in vivo* is likely to involve several of these, but experimental evidence, points to a central role for complement-opsonisation. In the presence of complement factors, C3b will be deposited on the bacterial surface and macrophages can then readily ingest the bacteria[34,35]. Selected mutants of *Salmonella* that are severely attenuated *in vivo*, reveal a growth defect in macrophage cell cultures but only if opsonised with complement[61,126,127]. These data suggest that the route of bacterial entry does, after all, play a role in virulence.

In contrast to *Listeria monocytogenes* that replicates in the host cell cytoplasm, wt *Salmonella* remains in a membrane-bound compartment intracellularly. This vacuole neither matures along the normal phagosome-phagolysosome pathway, nor reflects any of the intermediates. Rather, it seems that this type of vacuole, called the *Salmonella* containing vacuole (SCV), is entirely *Salmonella*-specific[127-131]. The formation of the SCV is not a passive process[132-135]. In contrast, the process is bacteria-driven and includes the translocation of several bacterial proteins into the host cell cytoplasm, and, as during invasion, re-modulation of the host cell actin [136-140]. The SCV is not totally separated from the normal endocytic trafficking, but the accessibility of trackers to the SCV compartment is limited[141]. In molecular terms the SCV is characterized by the presence of the lysosomal membrane glycoproteins (LAMP-1), and in some experimental set ups the mannose-6-phosphate receptor (M-6-PR), and lysosomal hydrolytic enzymes[127,129,130]. Notably, *Salmonella* (via SopE) retains the early marker Rab5, which is most likely an attempt to avoid/delay lysosome fusion[142,143]. Expression of constitutively active Rab5 alone is enough to escalate bacterial intracellular growth[144].

Location within the SCV is a prerequisite for replication, as mutational inactivation of the SPI2 regulated *sifA* gene causes disintegration of the SCV, and aborted bacterial replication[127]. The SifA protein is required for the formation of the extensions of the SCV membrane referred to as sifs. As for the

composition of the intravacuolar environment, not much is known [131]. The pH in the SCV of J774-A.1 cells is estimated to decrease from 5.5 to 4.9 between 2h and 6h post infection, making the SCV a moderately acidic compartment but not nearly as acidic as a classical phagolysosome[145]. This pH is evidently favorable, since neutralization reduces bacterial growth yields in macrophage cell cultures[146].

Recent data also demonstrate that *Salmonella* can actively exclude host defence molecules such as RNI and ROS from the SCV [85,86,100]. In summary, the SCV formation is a bacteria-directed event, and the SCV represents a less hostile environment than the phagolysosome, evidently allowing at least some bacterial populations to replicate [147].

1.5 Genes, gene regulation & virulence

The *S.* Typhimurium LT2 genome consists of 4597 coding sequences (CDS), and 4489 of these are contained on the bacterial chromosome. *S.* Typhimurium also harbours a unique, cryptic, virulence-associated plasmid (pSLT), which encodes the remaining 108 CDS[148].

A comparison of the *S.* Typhimurium LT2 and the *Escherichia coli* K12 genomes, revealed that at least 71% of the CDS in the *S.* Typhimurium genome have homologues in *E. coli*, and therefore most likely represent house-keeping functions. Furthermore, 22% of the CDS on the *S.* Typhimurium genome are sequences that constitute big or small clusters of virulence genes referred to as "pathogenicity islands", fimbrial operons, potential virulence factors, or prophages. Most of these are believed to be horizontally transferred, and some have a role in virulence[148-150]. Despite intense work by many groups, about 20% of the *Salmonella* CDS still await functional characterisation.

1.5.1 Virulence genes

The term virulence gene is often used to describe genes that are needed for virulence in one or several of the mouse models, sometimes for bacterial survival and growth in macrophages *in vitro*, and sometimes to describe genes needed for assumed virulence functions. Virulence genes can be schematically divided into groups. A first distinction can be made between genes that are directly involved in virulence and genes that are house-keeping genes but that are somehow needed for virulence. The *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2) constitute true virulence genes, as do toxins, but one can also include many bacterial adhesins, and some of the systems aimed at combating host defence. In the sections below I will

give a few examples of different types of virulence genes.

The two large pathogenicity islands of *Salmonella*, SPI1 and SPI2, each code for a complete type III secretion machinery with several regulatory components and a number of effector proteins [39]. These secretion systems, also called "contact-dependent secretion machineries", resemble a molecular syringe/needle structure [151] and function to inject macromolecules into the eukaryotic cell [39]. From an evolutionary perspective these systems are believed to be related to the flagellar hook, and homologues of SPI1 exist among other Gram bacteria [152,153]. SPI1 and SPI2 are not genetically linked, and are located at 63 and 31 centisomes, respectively, on the *Salmonella* chromosome[39]. The first biological role of SPI1 emanated from the ability of *Salmonella* to invade non-phagocytic cells *in vitro*, and bacteria that had mutations in SPI1 became non-invasive [154-157]. It was suggested that SPI1 was particularly required for passage from the intestinal lumen to the basolateral side of the epithelium [158]. Indeed, mutants that had decreased SPI1 expression were attenuated upon po. challenge but retained virulence upon ip. challenge [159,160]. SPI2 mutants, on the other hand, are attenuated using all routes of infection. This attenuation derives from the intracellular replication deficiency of these mutants [61,127]. The SPI2 genes are induced for expression inside eukaryotic host cells and function to orchestrate the SCV formation [39,61,134,161,127,162-164].

Salmonella is not particularly prodigious in its production of exotoxins, although, enterotoxin activities have been reported [165,166]. In addition, *Salmonella* express factors that can be regarded as toxins. These include some of the effector proteins translocated by the type III secretion systems [39]. Another example of toxin-like activities are the *spv* genes. These genes are not expressed in rich bacterial growth media, but are induced for expression inside eukaryotic host cells[163,167-169]. The individual roles of all the *spvRABCD* genes are not known, but SpvB function to inhibit actin polymerisation. This inhibition relates to the ability of SpvB to ADP-ribosylate G-actin[170]. Thus SpvB is a mono-(ADP-ribosyl)transferase and in this respect it resembles many classical bacterial toxins. Mutational deletion of the *spvB* gene results in decreased virulence[171].

Endotoxin, or LPS, is an important virulence determinant. LPS has a dual function in that in addition to being pro-inflammatory, also forms a protective outer leaflet on the bacterial surface, mediating resistance to harmful compounds, and through the expression of the O-antigen shields the bacteria from the complement membrane attack complex. Many LPS mutants are attenuated for virulence, or give rise to

an altered infection pathogenesis [89,111,114,172].

Adherence to the intestinal wall is required for efficient invasive disease [173]. *S.* Typhimurium LT2 has 12 fimbrial operons [148] that collectively contribute to its ability to adhere, grow and cause disease [174]. The individual role, of these fimbriae in virulence, has been difficult to assess due to fimbrial redundancy, but also partially because many of these are not expressed under ordinary laboratory conditions .

As described above, phagocytic cells express antibacterial factors that function to limit bacterial survival and replication. As a response to these insults, *Salmonella* express proteins that counteract the effect of these systems. Examples include the superoxide dismutases (SodA, SodB, SodCI, SodCII), and the SOS response, some of which are needed for survival in cultured macrophages or mice[83,102-104,175,176]. The SOD enzymes catalyse the conversion of superoxide to hydrogen peroxide, and virulence of SOD mutants can be increased by blocking ROI or RNI production[83]. It is likely that SOD can reduce the antimicrobial effect of NO by quenching the substances needed for production of toxic peroxynitrite. The SOS and recombination mutants are also more sensitive to radical stress[104]. Other examples include mutations in the *htrA* gene (shock protein), or its regulatory component RpoE. Deletion of either of these genes, also renders *Salmonella* more sensitive to oxidative stress, hence leading to attenuation[177,178]. Interestingly, the RpoE mutant is virulent in mice lacking the oxidative burst[179].

Additional house-keeping functions required for virulence include metabolic enzymes and regulatory proteins that participate in virulence gene expression. Deletion of genes that are required for the synthesis of a compound that is not available intracellularly causes attenuation. The growth of such mutants can sometimes be partially restored by adding the compound that is missing. The bona-fide example is the *aroA*-mutation; such mutants have disturbances in the synthesis of chorismatic acid, and thus cannot make p-aminobenzoic acid and 2,3-dihydroxybensoate, which are absent from mouse tissue, and are the most likely cause of the attenuation[180]. The interpretation of these results was challenged by the observation that such mutants can grow in IFN-γ knockout mice [65], and in T-cell deficient mice[43a]. Nonetheless, *aroA*-mutants, as well as *aroC*- and *aroD*-, mutants are severely attenuated in a wt mouse, and are widely used as both tools and vaccine carriers[181]. Similar arguments hold for the *pur*-mutants which are defective for purine synthesis[181].

Among the house-keeping-virulence regulators are the *cya* and *crp* genes, which code for adenylate cyclase

and cAMP receptor protein, respectively. These genes are normally involved in catabolite repression, but deletion of one or both components of the *cya-crp* system also affects virulence gene regulation[182]. However, the reason for the severe attenuation of these mutants remains unknown[183].

1.5.2 Regulation of virulence gene expression

Many important responses to environmental change in *Salmonella* are achieved at the level of gene transcription. Expression of genes can be either up-, or downregulated through interplay between different transcription factors, the level of DNA supercoiling and presence of "histone-like" DNA-binding proteins, with the aim of properly adjusting the bacterial physiology to new demands [184-186]. Gene regulatory proteins also have a central role in *Salmonella* virulence gene regulation. In particular, alternative sigma factors and two component regulatory systems seem to translate starvation and milieu changes into suitable gene responses [117,162,187,188]. Equally important to induction of a response is a prompt termination. This can be achieved either by a ceased induction, or with an active binding/induction of a repressor component. Despite the apparently important role, not much is known regarding post-transcriptional regulatory events in virulence regulation. However, post-transcriptional regulation of the virulence-associated sigma factor RpoS [189] and the identification of small regulatory RNA molecules in virulence regulation [190] suggest that this neglected part deserves much attention in the future.

1.5.2.1 Methods for studying gene expression

The manner through which regulatory spider-webs have been untangled mainly includes two methods: (I) the study of gene promoter activity using promoter fusions, and/or (II) deletion of genes and analysis of the concomitant phenotype. Many such studies have relied on recombinant plasmids in which the promoter of interest was fused to a reporter gene, and its activity was measured either from *in vitro* bacterial cultures, of from intracellular bacteria in cell lysates/whole eukaryotic cells [191]. The expression of bacterial genes under *in vivo* conditions has been largely focused on genes that are upregulated for expression upon entry into eukaryotic cells[192-195]. In recent years, increasing numbers of studies have been based on light-emitting green fluorescent protein (GFP) -gene-, or promoter-fusions. The GPF-fusions have the advantage of allowing measurement of the activity in individual bacteria[191].

A more recent approach to measure gene expression is to use microarrays. This enables measurement of changes in gene expression at a given time-point on a whole genome scale. In most set-ups, PCR products or oligonucleotides corresponding to the organisms CDS, are spotted on, and cross-linked to, glass slides. The sample RNA or DNA is then fluorescently labelled and hybridised to the arrays. After hybridisation, slides are scanned and fluorescent spot intensities are quantified. The data obtained can then be analysed to give information as to the relative amounts of an RNA or DNA species[196a]. This method offers large quantity gene expression analysis, but has so far not been much used to study *Salmonella* gene expression[196b]. It has however been used to study the macrophage gene expression response to *Salmonella* infection[196c].

1.5.2.2 Themes in virulence gene regulation

Most virulence genes are not transcribed when *Salmonella* is grown in standard laboratory media, but are induced upon transition to a host or host-like environment[162,163,169,197]. There are virulence-associated genes, which are expressed during selected growth phases *in vitro*, however[89]. By using examples selected from the literature of virulence gene regulation, I will try to highlight the strategies used for sensing and regulation, and will discuss a few common nominators. There are of course many more genes/gene clusters that are involved in virulence, and some of these are mentioned in other sections of this thesis.

The regulation of SPI1 expression has been extensively studied, and will be used as an example to illustrate some aspects of virulence gene regulation is *Salmonella*. The genes encoded on SPI1 strongly regulated and both induction and repression of specific genes have been described. Expression of SPI1 responds to a multitude of environmental cues including osmolarity, oxygen tension, availability of inorganic phosphate and pH[153,198,199]. *In vitro*, genes on SPI1 are expressed when the bacteria approaches the stationary phase of growth in rich medium[89]. The first SPI1 regulatory protein to be discovered was the SPI1-encoded OmpR/ToxR-like transcription regulator HilA[155]. HilA is an activator that is required for efficient SPI1 expression[200]. Additional activators of SPI1 expression include *invF*, *hilC* and *hilD* also encoded within SPI1[201,202]. One likely regulatory cascade is that environmental signals are sensed by BarA and EnvZ, inducing activation of SirA and OmpR which, directly or indirectly activates *hilA*[199,203]. *hilA* activation can be mediated/achieved by several components, including FliZ, HilC and HilD[199,202]. HilA then activates downstream targets including

invF, and InvF induces expression of a second group of SPII genes. The system can then be turned off by a second two-component system, the PhoP/Q system, and possibly also by the PhoB/PhoR system[199,204,205].

In conclusion, the basic on and off switches of SPI1 are governed by two-component regulatory systems, sensing the extracellular conditions, and regulators coded within the cluster itself to ensure specificity. This type of regulation is common for virulence regulons.

As described in 1.5.1, one role of SPI1 is to invade non-phagocytic cells. However, more recent data suggest that the *in vivo* scenario is more complex. Deletion of the entire SPI1 has a strikingly different virulence phenotype (po./streptomycin) as compared to deletion of only the regulator *hilA*, suggesting that there are additional regulatory functions on SPI1. Deletion of *hilA* leads to a reduced colonizing ability, but even if the mean time of death was increased, the oral LD_{50} was not changed[206]. In this scenario, the disease is still lethal but delayed, possibly due to an altered immune response. These data concur with the observation that *invA* mutants replicate less in HeLa cells, implying that SPI1 also contribute to growth[207]. Surprisingly, when the entire SPI1 island is deleted the bacteria can grow to higher loads without causing lethal disease[206]. This maybe linked to the described roles of SPI1 in induction of apoptosis, and for transepithelial migration of neutrophils[208,209]. These data suggest that in addition to mediating invasion, SPI1 also functions both to aid intracellular replication, as well as to trigger an appropriate immune defence.

These *in vivo* data suggest that there are bacterial regulatory elements that operate *in vivo*, of which the identity and the biological function are not yet recognized. In order to unravel the complete regulatory networks, new tools that operates *in vivo* or under *in vivo*-like conditions must be developed.

Growing bacteria under *in vitro* conditions that mimic the intravacuolar environment induces yet another set of virulence-associated genes. This so-called "within host-like environment" consists of a minimal medium with low pH, low phosphate and low Mg^{2+} concentration [162,169,197,210,211]. There are two examples in this group of genes that have been more extensively studied than others. The first is SPI2 [162,197] and the second example are the pSLT encoded *spv* genes[169,171,211]. The induction of SPI2 involves the two component system EnvZ/OmpR, which activates SsrA/B (encoded within SPI2), this activation is possibly modulated by PhoP/Q[60,127,188,212]. The induction of *spv* is mainly dependent on PhoP/Q, the alternative sigma factor RpoS, and the SpvR regulator itself [169,117]. Even if the inducing conditions

for SPI2 and *spv* are different from the SPI1 inducing conditions, the regulatory structure is very similar. SPI2 and *spv* virulence regulation has "hijacked" house-keeping regulatory systems (PhoP/Q, OmpR/EnvZ, and RpoS), and added specificity via internal regulatory components (SsrA/B and SpvR).

1.5.2.3 Increasing the complexity of gene expression

One function of the PhoP/Q system functions is to turn off SPI1[204,205] but there are no known repressors that would function to actively turn SPI2 off. In this context, it is interesting to note that SPI1 and SPI2 are not expressed independently of each other. There is a significant cross-talk, that is not associated with the common regulator PhoP/Q, and mutations have been described in SPI2 that reduce the ability of such strains to express SPI[126]. The regulatory component that is responsible for this co-regulation has yet to be identified.

Furthermore, the SPI1 and SPI2 secretion machineries also function to translocate proteins encoded outside the island itself, including proteins encoded within other SPIs, and outside the "SPI-collection"[39]. Expression of these genes is generally co-regulated with the corresponding island. In this context the divergent expression of SPI5 is a good example. One half of SPI5 is induced by "SPI1 conditions", whereas the other is induced by "SPI2 conditions"[139]. Similarly, the expression of SPI4, is *hilA* dependent, and always follows the expression of SPI1[213].

To illustrate the importance of virulence regulation, there are regulatory mutants that have comparable detrimental effects on virulence as a mutation in an effector gene. A good example of this is that a constitutively active version of the PhoP virulence regulator is completely avirulent[214]. There are also examples of situations when a supposedly global alteration in gene expression results in decreased virulence. For example, *dam*- mutants in which the DNA adenine methylation is absent, have altered gene expression, as well as deficiencies in mismatch repair and replication, and are strongly attenuated for virulence [215-218]. A similar argumentation holds for mutants that are affected for the expression of nucleoid-associated proteins [160,219].

In conclusion, *Salmonella* virulence genes have been acquired sequentially as blocks or as individual genes. The acquired blocks have included regulatory elements, but some regulatory events are dependent on house-keeping functions. More recently acquired virulence genes, or single genes, exploit regulatory pathways/ machineries used/ expressed by other virulence genes. How this tremendous co-ordinated expression of horizontally

transferred blocks has been accomplished from an evolutionary perspective remains a conundrum.

1.5.2.4 Selection for *in vivo* gene expression

In the mid-90s, several groups in parallel invented a variety of methods that allowed for the selection of genes that were specifically induced within, or required for, growth within host cells or mice. These approaches, referred to as *in vivo*-expression technology (IVET), signature-tagged mutagenesis (STM) and differential fluorescence induction (DFI) represented a new concept in research on bacterial pathogenesis[192-195]. IVET and DFI identify genes that are substantially induced within host cells, without telling whether they are needed for virulence. STM directly probes the role of a particular gene/genes in virulence, without taking into account its expression. None of these three methods quantitate the expression levels, or can identify genes that are selectively downregulated during intracellular replication. Alternative molecular methods, including differential display and various types of subtractive hybridisation, can identify such genes, but these have been sparsely used for bacteria. Even if these techniques have revealed relatively few targets and missed some of the obvious ones, most importantly STM has lead to the identification of SPI2[193].

1.5.3 Altered virulence & avirulence

Different isolates of *Salmonellae* may exhibit quite different virulence levels in isolated tests. This may reflect different contents of virulence genes among the different isolates, different alleles of given virulence, or a different expression patterns of virulence genes. An interesting speculation in this context is whether *Salmonella* also contain "avirulence genes" to tune their level of pathogenicity.

The term avirulence is extensively used in the field of plant pathogens. When a plant is infected by a pathogen carrying a "correct *avr*-allele" the plant is resistant to the infection. Mechanistically, this is achieved by type III secretion-mediated injection of bacterial effector proteins into the plant cell, which causes the cells to induce a so-called hypersensitive response. This response includes vigorous free radical production that leads to localized necrosis, and limitation of the infection[220]. The defence that plant cells mount upon infection is quite similar to that of macrophages, and homologues of the plant *avr* genes have been identified in other pathogens. The *Salmonella avrA* protein is secreted via the SPI1secretion machinery[221], and was recently described to inhibit NF-κB activation and induction of

proinflammatory cytokines[222]. So, in contrast to in the plant case, *Salmonella avr* act to limit immune reaction.

However, if avirulence genes are described, as genes that upon disruption give rise to mutants that are less immuno-stimulatory, analogous to in plants, this opens the possibility to assign other avirulence functions in *Salmonella*. Hence by analogy the composition of the *Salmonella* wt LPS is an avirulence function. There are specific modifications of the LPS that are less proinflammatory; for example $PhoP^C$ mutants display altered decoration of the lipid A, resulting in decreased TNF-α production from adherent monocytes[114]. Also, selected deacylation of lipid A (Δ*waaM*) results in decreased expression of TNF-α, interleukin-1 (IL-1) and NO from a macrophage cell line, and in a strain that is less lethal in relation to colony forming units (CFU)[172]. There are also situations when deletion of a whole pathogenicity island gives rise to mutants that can grow to a higher yield[206], thus confusing the concept of virulence. As described above, a reduced inflammatory response often corresponds with the ability of *Salmonella* to grow to a higher yield, without causing lethal disease, possibly aiding bacterial spread.

In parallel, interesting results have been obtained using strains that are not efficiently cleared by the immune system. Thus mice may contain mutant bacteria in their organs even at a time point when wt bacteria are cleared. These mutants also have a higher LD_{50}. One such strain lacks the Clp-XP ATP-dependent protease, and these mutants grow much more slowly and persist longer than the wt *in vivo*[223]. Sukupolvi *et al.* described another Typhimurium mutant that causes "persistent" infections. This mutant was initially identified as defective in AgfA adhesive fiber expression, and to cause persistent infections in mice. The fact that these mice also secreted mutant bacteria in their faeces for prolonged periods of time suggested that these mutants were actively replicating in chronically infecting mice[17]. If avirulence could be used in the sense of "causing less disease", which could give rise to prolonged colonization, this would in fact increase the likelihood of transmission.

An alternative definition of an avirulence gene would be a gene that upon deletion gives rise to a mutant that is more lethal than the parental wt. The gene *pcgL*, encoding a periplasmic D-Ala-D-Ala dipeptidase, would be classified as an avirulence gene using both definitions. PcgL mutants have both a lower LD_{50} and grows to higher yields than the wt[224].

In summary, the data connected to altered virulence in *Salmonella* suggest that avirulence can be grouped in classes. *Salmonella* indeed has genes that upon mutation reduce the

inflammatory response, enabling growth to a higher yield, with implications such as increased spread. There are also attenuating mutations that give rise to a persistent infection, possibly increasing spread, and hence could be viewed as gain-of-function mutations. In addition, there are mutants that become more lethal. These data suggest that the virulence level of *Salmonella* can be increased by single gene alterations.

Finally, what are the reasons for this plasticity in virulence level? A simplified theoretical model states that two opposing forces determine the level of virulence. If transmission from one host to another is not rate-limiting, virulence increases; conversely if transmission is rate-limiting, virulence decreases to allow for longer colonisation and increased likelihood of spread to a new host[225]. This implies that there are situations in which it is beneficial to be able to adjust the virulence level to external conditions.

2 AIMS OF INVESTIGATION

To (I) define the genes involved in the intrinsic adaptation of *S.* Typhimurium to growth inside host phagocytic cells, to (II) describe mutants with increased such adaptation, and finally to (III) analyse how this adaptation affects pathogenicity.

3. RESULTS

3.1 *Salmonella* gene expression *in vivo* (Article I)

Microarray technology has provided a method that enables measurement of bacterial gene expression on the whole genome scale. However, this method requires µg amounts of good quality RNA. This has not been technically possible to achieve from intracellular bacteria, which has hampered the use of microarrays for measuring bacterial intracellular gene expression. Article I describes the development of a method to isolate RNA from bacteria that have been growing inside host macrophage-like cells, and the subsequent application of this method to describe the temporal whole genome expression profile of *S.* Typhimurium inside J774-A.1 cells.

In these experiments J774-A.1 cells were infected with *S.* Typhimuirum SL1344, and bacterial RNA was extracted at 4h, 8h, and 12h post infection. This RNA was then labelled and analysed on a whole genome *Salmonella* microarray. In these experiments we noted a total of 919 genes that had more than a 2-fold change in expression upon entry into the eukaryotic cell (Fig. 1). Furthermore, this analysis described new ways through which an intracellular pathogen has adapted to an intracellular life style, and such data could provide clues as to how other intracellular pathogens have adapted to survival in different milieus.

3.1.1 RNA extraction, microarray technology & analysis

The single most important step in the isolation of total RNA from intracellular bacteria was the creation of a protocol that allowed for the simultaneous disruption of the eukaryotic, but not the prokaryotic cell, combined with the immensely important stabilisation of the bacterial RNA. This was achieved by combining a bacterial RNA stabilising solution with a detergent (Fig. 1, article I).

The second important issue was the use of genomic DNA as a reference channel in each microarray experiment. The strength of the RNA signal could hence be expressed as a ratio to the signal detected with the DNA. This allowed for comparison both between time-points and several different environments.

We used complement-opsonised bacteria grown statically in complete cell culture medium as a reference for probing relative induction, or repression in gene expression.

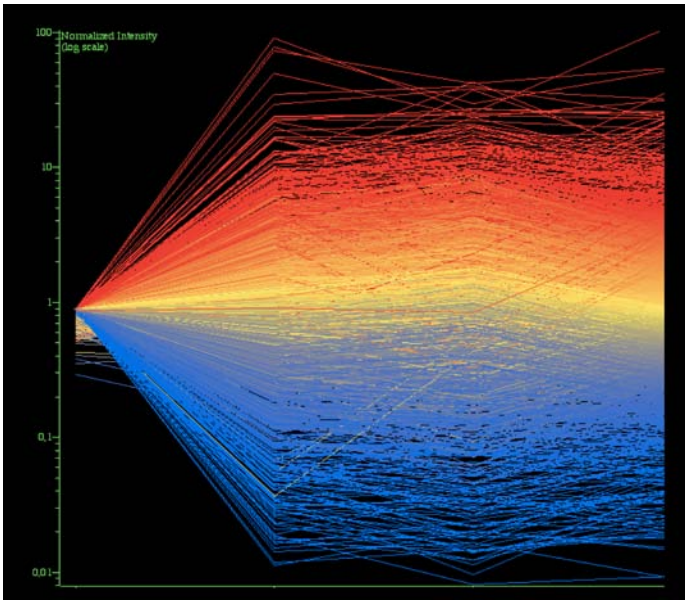

Figure 1. The pattern of *Salmonella* genes expressed in the SCV as a function of time. The expression profile of the control sample (extracellular) and post-infection samples (4h, 8h, 12h) are shown. Expression in the three conditions are normalised to the control sample. The genes that are up-regulated are shown in red, and the genes that are down-regulated are shown in blue. Genes that do not change in expression are indicated in yellow.

3.1.2 Lessons from *in vivo* *Salmonella* gene expression

As discussed in the introduction, bacteria respond to changes in their milieu, and take measures to adapt their gene expression accordingly. Consequently, by analysing the gene expression profiles of intracellular bacteria we should be able to deduce information about the SCV milieu. The altered gene expression profiles indicated that the SCV in J774-A.1 is aerobic, with moderately low pH and it contains low concentrations of Mg^{2+}, inorganic phosphate, and Fe^{3+}, but high concentrations of K^{+} and Fe^{2+}. The data also indicated that uronic acids might be an important source of carbon for intracellular bacteria. The degree of changes in gene expression detected also suggested that the SCV milieu is considerably different from the extracellular environment for the bacteria.

Expression of the SPI genes, pSLT genes and other virulence-associated genes, offered both confirmatory results as well as some surprises. As expected, SPI1 was repressed, and SPI2 and *spv* were induced intracellularly. We could also identify genes that were downregulated intracellularly but that are known to be required for virulence in the mouse model.

We could follow the induction of selected macrophage antibacterial effectors by considering the induction bacterial defence systems, stress responses, as well as the SOS system. The gene expression data confirmed the presence of oxidative, and nitrosative stress in the SCV, and estimated the pH to be fairly acidic.

A great part of the CDS that were regulated upon entry into J774-A.1 cells were genes of unknown function (FUN genes), representing an enormous pool of "science to be". Interestingly, there was an over-representation of downregulated genes amongst this pool of FUN genes. This could reflect the fact that most experimental set-ups that analyse virulence gene expression select for induced genes. In any case, the ability to downregulate certain genes may well be of equal importance to obtain virulence as the ability to induce a response.

Finally, the results obtained with two different lines of *S.* Typhimurium (SL1344 and SR-11) gave strikingly similar results, making it likely that the data are also applicable for other *S.* Typhimurium (article I + IV).

3.1.3 Implications & applications of *in vivo* microarrays

The picture that emerged from the *in vivo* expression analysis was that *Salmonella* is actually not that unhappy in the SCV of J774-A.1 cells. It is likely that the bacteria would be in greater distress in a more aggressive cell type.

Yet, in these pioneering experiments we chose to use the macrophage-like cell line J774-A.1. The advantage comes from the fact that J774-A.1 are less efficient in limiting bacterial replication compared to peritoneal macrophages [226]. This enabled extraction of sufficient amounts of bacteria, and hence RNA, to perform the expression analysis. Furthermore, J774-A.1 still retains many of the properties of peritoneal macrophages, for example being equally efficient in bacterial killing [226], and thus represents a relevant model system.

Direct applications from article I could be the creation of novel expression systems using the identified *in vivo* induced promoters. Moreover, knowledge of intracellular gene expression, especially of the FUN genes, could indicate new targets for the design of antibacterial compounds. It also allows for the design of appropriate media that would represent the *in vivo* environment *in vitro*. This knowledge could be used to study virulence gene expression in a "correct" environment.

The development of "*in vivo* array technology" opens new possibilities to study gene expression and regulation in an *in vivo* milieu. This could be achieved by analysing relative differences in RNA contents in regulatory mutants grown inside host cells (article IV). In parallel, one could also envision a scenario in which the wt bacterial response to a particular substance/cell type/modified cell could be probed in an *in vivo* situation.

3.2 Isolation & characterisation of macrophage-adapted mutants (Article II)

Bacteria can alter their genome content through the acquisition of genes, clusters of genes, or whole plasmids through transformation, transduction, or transposition. New DNA may give bacteria a competitive advantage over fellow bacteria, for example by increasing their growth in a certain environment, or by offering growth in a totally new niche. A parallel strategy for growth adaptation, at least theoretically, would be to modulate the existing pool of genes through translocations, inversion or through the introduction of mutations. In article II we tested this hypothesis by isolating mutants that had increased growth yields under *in vivo* conditions that did not allow import of foreign bacterial DNA.

3.2.1 Selection for, & characterisation of, fast growth mutants

Selection was based on the concept that increased spread of a pathogen leads to increased virulence. If virulence equals replication efficiency within a host,

then artificial support of spread would drive the balance towards increased replication. To test this hypothesis, we performed a selection based on growth competition between individual bacteria in macrophage-like J774-A.1 cells. However, the *spv* intracellular growth genes have a marked cytotoxic effect on eukaryotic cells [170]. Thus prolonged growth in cell cultures is expected to select for mutants defective in *spv* expression rather than in mutants with an increased intracellular replication rate. To circumvent this problem we chose a *S.* Typhimurium strain of line LT2 (TT16729) for the selection. Line LT2 carries a defective *rpoS* gene that does not allow for full induction of *spv* expression, yet the LT2 bacteria retain both the ability to grow in cultured cells and to cause disease in mice[18].

The experiment was initiated by treating strain TT16729 with diethyl sulphate, and the level of mutagensis was calculated so that we would introduce approximately one mutation per bacterial genome. Because enrichment of single mutants from a large initial population would require more rounds of replication than could be provided by a single macrophage, the selection was carried out using a cycling procedure (Fig. 2, article II). This procedure involved repeated isolation of bacteria and subsequent re-infection of J774-A.1 cells. After 15 to 20 cycles the final cell lysates were plated on agar. Single colonies were isolated and tested for a possible increase in intracellular replication. This was done by infecting J774-A.1 cells with a 50:50 ratio of the original parental strain and a potential mutant, and by repeating the cycling procedure for three rounds (Fig. 3A, article II). Mutants that could outgrow the parental strain in J774-A.1 cells were called GAM for growth advantage mutant, and were further investigated.

To evaluate whether the GAMs isolated represented separate mutants or siblings, all mutants were subjected to a panel of phenotypic tests. These included tests for serum tolerance, acid tolerance, starvation responses, replication during aerobic, microaerophilic, and anaerobic growth in various liquid media and plates at various temperatures, LPS analysis, secreted protein profiling, outer membrane profiling, invasion and growth in non phagocytic cells, uptake and growth in phagocytic cells . Finally, the virulence level was probed by infecting mice ip. with a 50:50 ratio of the original parental strain and the GAM, and following the change in ratio as a function of time during a replicative infection (Fig. 3B, article II).

These combined screens identified many alterations in bacterial virulence-associated phenotypes, which included alterations in LPS structure and in the profiles of proteins secreted by invasion-competent bacteria (Fig. 8, article II). The screens also enabled the

classification of the mutants into nine tentative groups.

3.2.2.1 Macrophage-associated phenotypes

We next set out to define the mechanism for increased bacterial growth yields in J774-A.1 cells. Macrophages respond to intracellular *Salmonella* with a strong NO production, and as inhibition of this response results in increased bacterial loads (Fig. 1A and 1B, article II), we tested whether NO was involved in the GAM phenotypes. Inhibition of NO production in J774-A.1 cells using the competitive inhibitor N-monomethyl-L-arginine (NMMA) resulted in a massive increase in the bacterial growth yields, indicating an important role for NO in restricting the bacterial yields in J774-A.1 cells. Since *Salmonella* encode for systems that render the bacteria more resistant to NO, one could imagine that some GAMs might exhibit higher resistance to NO than the wt. This turned out to be unlikely, since the GAMs were equally sensitive to S-nitrosoglutathione (GSNO) *in vitro*. In contrast, we found that five out of the nine GAMs isolated inhibited production of NO from J774-A.1 cells (Fig. 4, article II). A detailed analysis suggested that the mechanism of inhibition acted at the level of enzymatic iNOS activity, since all mutants induced iNOS transcription, and iNOS protein was detected in at least wt amounts (Fig. 5B, 5C and 6, article II). Furthermore, blocking protein synthesis in intracellular bacteria by addition of chloramphenicol to infected cells lead to a restored NO production in all cases (Fig. 7, Article II). These data suggested that inhibition of host NO production represents a central strategy to achieve higher growth yields in J774-A.1 cells.

From the bacterial point of view this inhibition can mechanistically be achieved in many different ways. Recent data suggest that wt *Salmonella* can reduce the exposure to NO by limiting iNOS fusion with the phagolysosome with the aid of SPI2[86]. It is therefore interesting to speculate that the GAM mechanisms may involve exaggerated versions of the wt activities. This hypothesis is supported by the observed alterations of SPI-expression in selected GAMs (Fig. 8B, Article II).

3.2.3 Discussion & conclusions of macrophage adaptation

Transposon mutagenesis has been used to isolate *Salmonella* mutants with increased growth yields in eukaryotic cells. This screen used a virulent *S.* Typhimurium strain, and consequently resulted in inactivation *spv* gene expression [227]. We used chemical mutagenesis instead. While this choice complicates mapping, it has the

advantage over transposon mutagenesis in that it increases the probability of isolating "gain-of-function" mutations. Indeed, many of the GAMs isolated revealed phenotypes that would be consistent with activation/derepression of a cryptic repressed trait. An unexpected finding was the result that none of the GAMs had increased virulence in the mouse model (Fig. 3B, article II). We did identify one class that could grow to higher yields without increasing lethality. This is analogous to the situation in mice that lack certain cytokines, or with SPI1/LPS mutants that are less inflammatory. Hence this group could represent host-adaptation-mutants as they showed increased bacterial loads without any increased severity of the disease. Nonetheless, most GAMs had reduced growth by several orders of magnitude compared to the wt strain in the *in vivo* competitions (Fig. 3B, article II). In conclusion, mutants that cannot replicate in macrophages are avirulent, but mutants that replicate better in macrophages are not necessarily classified as being more virulent.

These data indicated that it is indeed possible to select for mutants that grow faster during a selected phase of the infection, but that such alterations can be detrimental during other phases of the infection. This suggests that an increased intracellular replication might be associated with fitness trade-offs. Indeed, as discussed previously many GAMs had decreased abilities to mount stress responses, to tolerate serum, or to replicate at reduced temperatures. Such defects may also play a role during survival in the environment. It is possible that these mutants represent snap-shots of how the *Salmonella* genome is expressed during selected phases of the infection cycle. Indeed, microarray analysis of the selected mutants has revealed traces of the type of regulation evident during intracellular proliferation in J774-A.1 cells.

3.3 Altered gene regulation & chronic salmonellosis (Articles III & IV)

A mutant that had the ability to cause not only an acute but also a chronic carrier state in the mouse model next drew our attention. Interestingly, this mutant could replicate faster in murine macrophage-like J774-A.1 cells, and was hence a GAM. We determined that the responsible mutation localised in a gene that encoded a house-keeping enzyme involved in RNA degradation. Analysis of the RNA expression profile of this mutant revealed a tilted effect in that primarily transcripts encoding SPI genes, *spv* genes, prophage genes and motility-associated genes were affected. (articles III and IV).

3.3.1 Identification of the bacterial mutation that mediates chronic infection

An attempt to identify the mutation responsible for the chronic infection isolated a *yafK*::Tn*10*d-cam insertion that associated with the known phenotypes of the mutant. Sequencing of the region flanking the insertion from both wt and mutant revealed a single base change within a CDS having 97 % identity with the *E. coli pnp* gene encoding polynucleotide phosphorylase (PNPase). PNPase is a 3´ to 5´ exonuclease with polyadenylation activity and at least in *E. coli* is a subunit of the RNA degradosome [228,229]. The mutation in the chronic infection strain altered codon 614 from GAA to TAA, causing truncation of the PNPase protein from 734 aa to 613 aa. This truncated PNPase lacked exonuclease activity (Fig. 1, article III).

PNPase in *E. coli* is associated with the ability to mount a cold shock response, and mutants lacking PNPase grow slowly at low temperatures[229]. We determined that this was also true for the *S.* Typhimurium *pnp* mutant. No growth defect was observed at 37°C or 39°C.

To ensure that this mutation was sufficient to confer the observed phenotypes, an identical mutation in *pnp* was constructed by allelic replacement in the SR-11 wt MC1, giving rise to MC71. MC71 is identical in all tested aspects to the original isolated mutant MC2.

3.3.2 Effect on bacterial gene expression *in vitro*

Since PNPase is a house-keeping enzyme, we expected the effects of the truncation to be global. Whole genome DNA microarrays thus represented a excellent tool to study alterations of the RNA pools in MC1 and MC71. However, we did not know which stage of the infection that the lack PNPase activity would be important for the ability of MC71 to cause chronic infection. We therefore set out to probe the RNA profiles of bacteria in various environments. We grew the wt and the PNPase mutant in three different media: in Luria Broth (LB), in complete cell culture medium (RPMI) and in a medium that mimicked the intracellular environment (MM5.8).

We registered 109 genes that had altered RNA contents in the PNPase mutant grown in LB. Most of the upregulated genes belonged to SPI1 and SPI2, or represented genes linked to SPI. The scenario for bacteria grown in RPMI was similar, with altered regulation of around 200 genes being detected. The upregulated genes included SPI1, SPI2, SPI4, SPI5, and genes on integrated prophages. In contrast, most of the expression of flagellar and motility components were drastically reduced in the *pnp*-mutant.

The picture was somewhat different in MM5.8. Under these conditions we detected altered regulation of around 50 genes. The prophages were still relatively more expressed in MC71, and the flagella and motility were downregulated. We also detected a relative increase in *spv* transcripts in MC71, along with increased expression of shock response genes (Fig. 2).

In conclusion, the tropism of PNPase changed with the growth conditions, but the genes that were upregulate mostly represented those horizontally acquired, whereas those downregulated belonged to motility genes.

3.3.3 Replication *in vivo*

The data obtained from the microarray analysis suggested that MC71 was affected for traits involved in bacteria-host interactions. This hypothesis was tested by measuring replication within phagocytic cells and mice. In both these environments MC71 replicated faster than MC1, despite unaltered yields (Fig. 4, article III). This indicated that PNPase could act to suppress bacterial replication *in vivo*.

3.3.4 Effect on bacterial gene expression *in vivo*

Even if there was not a causal link between the observed increased replication in macrophages and mice, and the establishment of a persistent infection, such an association remained an attractive hypothesis. We therefore probed the mRNA profiles obtained from bacteria grown inside J774-A.1 cells. This analysis further narrowed the spectrum of genes that were affected in MC71. We recorded increased levels of *spv*, *rtc*, *entC* and STM2236 mRNA, and decreased levels of a putative regulator, and a handful of motility components (Fig. 2). Furthermore, we found that the up regulation of *spvABC*, *entC*, and STM2236 mRNA in MC71 was dependent on SpvR *in vivo* (Fig. 2, article IV). Since the *spv* genes were previously associated with the ability of *S.* Typhimurium to replicate in macrophages and mice, these were selected for further analysis.

3.3.5 *spv* expression in MC71

In these experiments we used nothern blot analysis to define the amounts, as well as the half-life of individual *spv* mRNA directly from the virulence plasmid. For detection, we designed probes identifying either of the two main *spv* transcripts. We did not find any *spvR* mRNA from MC1, but we did detect *spvR* mRNA in MC71, even at several time points after the blockage of RNA synthesis (Fig. 5, article IV). The *spvA* probe detected two main bands, *A1* and *A2*, from MC1 and MC71 (Fig. 5, article IV). The amounts of *spvA1* in MC71 were increased compared to in

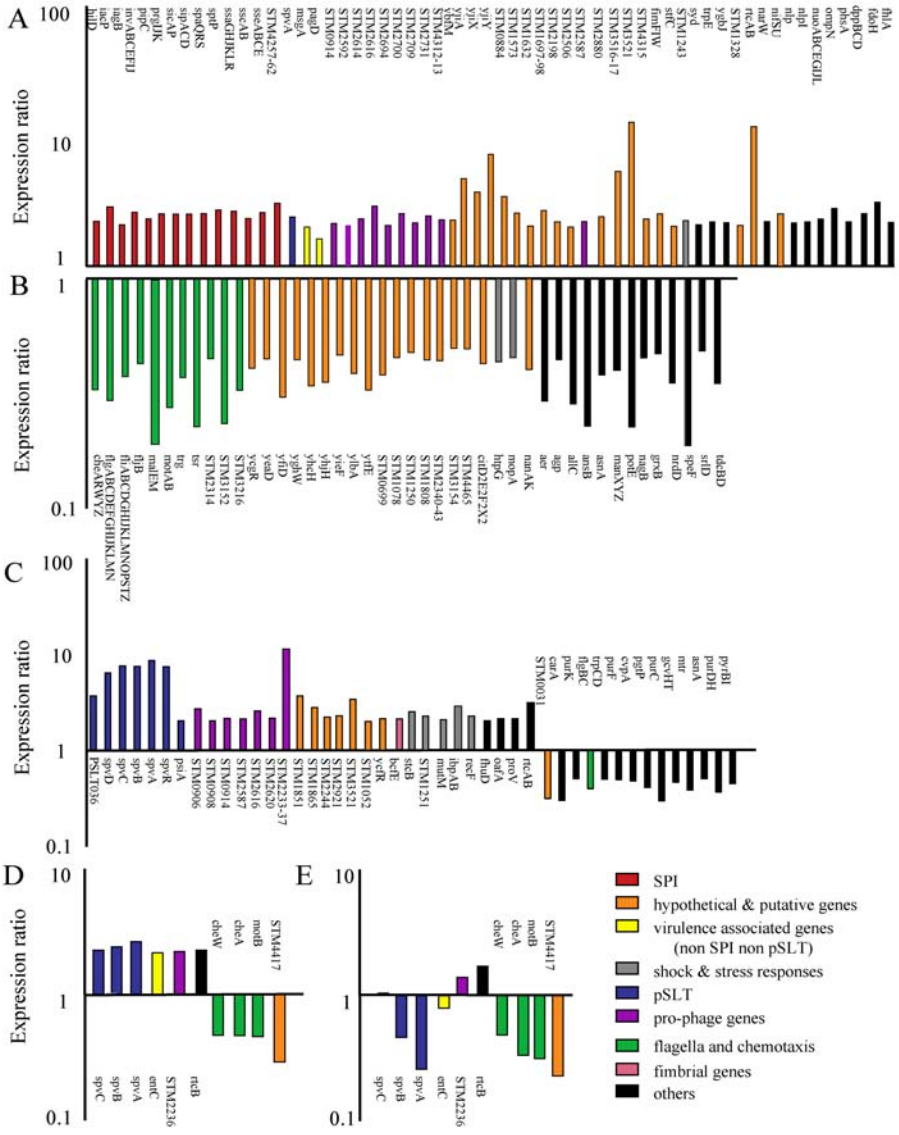

Figure 2: Subtractive gene expression patterns of *S.* Typhimurium wt MC1 and *pnp* mutant MC71 *in vitro* and *in vivo*. Bacteria were grown in serum-supplemented cell culture medium (RPMI) (A and B), in low pH minimal medium (MM5.8) (C), or inside J774-A.1 macrophage-like cells (D and E), where after bacterial RNA was extracted and the amounts of individual mRNA species were quantified using microarray technology. The expression profiles obtained from MC1 and MC71 mutant bacteria were subtracted and the results are displayed on a logarithmic scale as the ratio between the MC71 and MC1 (MC71/MC1) are shown in A-D. E displays the subtracted profile obtained when comparing MC71*spvR* ::Km and MC1 wt in J774-A.1 cells (MC71 *spvR*::Km / MC1 wt). The results are given as the means of quadruplicate values. The figure only includes values that differed more than two-fold and that showed a P-value less than 0.005.

MC1, but the half-life of this messenger was longer in MC1 (Fig. 5, article IV). *spvA2* was also more abundant in MC71 (Fig. 5, article IV). These data show that the *pnp* mutation is associated with higher mRNA contents of transcripts initiated from both *spvR* and *spvA* promoters, and that the degradation of at least the *spvA* mRNA is affected in MC71.

3.3.6 Summary of chronic salmonellosis

The role of PNPase during cold shock is to degrade *csp* mRNA, achieving a transient expression of Csps. These Csps are essential for dealing with the halts in transcription and translation upon such temperature shifts, but resumed growth requires degradation [228,229].

The data presented in articles III and IV expands the role of PNPase in gene regulation to include not only gene regulation during cold shock but also virulence regulation. Based on the data presented in article I, entry into a phagocytic cell can be viewed as an *in vivo* shock. We can thus assign PNPase the role of functioning as the timer in plastic responses to the *in vivo* shock.

Apart from the results directly associated with infection, the array data had information to offer regarding the activity of PNPase in various environments. These data suggested that it is not only the expression level of a particular transcript that determines if it will be degraded by PNPase, but that the specificity of PNPase is dependent on the growth conditions (Figs. 1, 2 and 6, article IV).

3.3.7 The challenge ahead, solving the riddle of chronic salmonellosis

A most difficult question still remains to be answered. Which of the functions that are upregulated, or downregulated, in the PNPase mutant are responsible for the chronic infection? This question is going to be very difficult to answer since the development of an acute infection is a prerequisite for the development of a chronic carrier state. Any mutation that affects virulence would terminate the infection and hence the chronic stage as well.

An alternative approach would be to define genes that are not needed for acute infection, but that are particularly important for the chronic stages. Such "chronicity genes" have been defined in *Mycobacterium tuberculosis,* and in *Brucellae*[230]. Are there similar gene functions in *Salmonella,* and do they play a role during the murine infection with MC71?

The ability of MC71 to actively replicate without being cleared in apparently immuno-competent mice is fascinating [17]. We still do not know how this is achieved, but interesting ideas can be derived from mice that

lack proper T cell responses; such mice fail to clear a *Salmonella* infection. One way through which the chronic infection could be achieved could be though a reduced late IFN-γ production by T cells. This would lead to an alteration of the macrophage activation state, possibly disturbing granuloma formation. Another question that arises is whether these chronic infections can be reactivated to acute infections. This scenario is evident in mice chronically infected with *M. tuberculosis*, given a NO inhibitor [230]. Could the outcome of a chronic infection with MC71 be altered by adding or deleting IFN-γ or IL-12, or by blocking NO production?

Finally, human carriers of *S.* Typhi are at a greater risk of developing cancer in the hepatobiliary tract [231,232]. In such carriers, *S.* Typhi can be found in the gallbladder [6], something which is also true in our mouse model. Therefore the ultimate question is if chronically infected mice can also be used as a model to study this aspect of human persistent *S.* Typhi.

4 Concluding remarks

The main methodological advances presented in this thesis involved the development of a method for measuring the bacterial gene expression profile on a whole genome scale for bacteria, and the selection for mutants that were adapted for the intracellular milieu. The transcriptometric microarray analyses illustrated a monumental adjustment in gene expression that accompanied bacterial transition from an extracellular to an intracellular environment. Evidently, this illustrates the bacterial dedication for adapting its molecular architecture and physiology to the new prevailing environment. In this respect, it appears remarkable that it is possible to isolate mutants that are better adapted to the intracellular niche. Interestingly, increased intracellular fitness often associated with altered expression of defined virulence traits, and with the ability to reduce the macrophage antibacterial effector NO. In part, the results also uncoupled intracellular growth from virulence, in that most of the macrophage-adapted mutants were attenuated for virulence in mice, implicating the existence of fitness trade-offs between these two environments.

The availability to probe bacterial gene expression growing inside host cells will enable us to define the exact expression profiles of macrophage adapted mutants, and allow us to pinpoint regulatory alterations and regulatory cascades involved in bacterial host adaptation. Of particular interest was the *pnp* mutant. This mutant behaved as a typical growth advantage mutant in that it showed increased replication rate inside host cells, but at the cost of an slightly increased LD_{50} po., and a reduced capacity to grow at low temperature. Interestingly, however, this mutant had gained the ability to cause chronic infection. Transcriptometric analyses revealed alterations in the expression of selected virulence genes *in vitro*, and a close to specific up-regulation of the *spv* intracellular growth genes for *pnp* mutant bacteria growing inside host cells. Thus, in one respect the chronicity mutation could be viewed as a gain-of-function mutation since it would allow for a prolonged period of spread. However, the increase in effective infectious dose, and the inability to complete a cold shock response, would cause significant drawbacks for the mutants so the net outcome is dubious in terms of fitness.

One interpretation of the findings presented here would be that *Salmonella* possess several different ways of fine-tuning virulence. The purpose of this would be to optimally confront the different challenges encountered during the complex infection, and possibly to allow the bacteria to establish a persistent carrier state instead of just causing a temporary

acute infection. Such traits may usually be cryptic, or expressed very temporarily, and exposed as a result of a mutational interference.

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6 REFERENCES

1. Selander, R. K., Li, J. & Nelson, K. in Escherichia coli *and* Salmonella, *cellular and molecular biology* (eds. Neidhardt, F. C. *et al.*) chapter 147 (ASM press, Washington DC, 1996).
2. Pang, T., Bhutta, Z. A., Finlay, B. B. & Altwegg, M. Typhoid fever and other salmonellosis: a continuing challenge. *Trends Microbiol* **3**, 253-5 (1995).
3. Pang, T., Levine, M. M., Ivanoff, B., Wain, J. & Finlay, B. B. Typhoid fever-important issues still remain. *Trends Microbiol* **6**, 131-3 (1998).
4. Hornick, R. B. *et al.* Typhoid fever: pathogenesis and immunologic control. *N Engl J Med* **283**, 686-691 (1970).
5. Hornick, R. B. *et al.* Typhoid fever: pathogenesis and immunologic control. *N Engl J Med* **283** , 739-746 (1970).
6. Gilman, R. H., Terminel, M., Levine, M. M., Hernandez-Mendoza, P. & Hornick, R. B. Relative efficacy of blood, urine, rectal swab, bone-marrow, and rose-spot cultures for recovery of *Salmonella typhi* in typhoid fever. *Lancet* **1**, 1211-3 (1975).
7. Ottenhoff, T. H. *et al.* Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. *Nat Genet* **32**, 97-105 (2002).
8. Vladoianu, I. R., Chang, H. R. & Pechere, J. C. Expression of host resistance to *Salmonella typhi* and *Salmonella typhimurium*: bacterial survival within macrophages of murine and human origin. *Microb Pathog* **8**, 83-90 (1990).
9. Pascopella, L. *et al.* Host restriction phenotypes of *Salmonella typhi* and *Salmonella gallinarum*. *Infect Immun* **63**, 4329-35 (1995).
10. Ishibashi, Y. & Arai, T. A possible mechanism for host-specific pathogenesis of *Salmonella* serovars. *Microb Pathog* **21**, 435-46 (1996).
11. Schwan, W. R., Huang, X. Z., Hu, L. & Kopecko, D. J. Differential bacterial survival, replication, and apoptosis-inducing ability of *Salmonella* serovars within human and murine macrophages. *Infect Immun* **68**, 1005-13 (2000).
12. Anderson, G. W., Hamblen, A. D. & Smith, H. M. Typhoid carriers: A study of their disease producing potentialities over a series of years as indicated by a study of cases. *Am J Public Health* **26**, 396-405 (1936).
13. Carter, P. B. & Collins, F. M. Growth of typhoid and paratyphoid bacilli in intravenously infected mice. *Infect Immun* **10**, 816-822 (1974).
14. Carter, P. B. Spread of enteric fever bacilli from the intestinal lumen. 182-187.

15. Carter, P. B. & Collins, F. M. The route of enteric infection in normal mice. *J Exp Med* **139**, 1189-203 (1974).
16. Carter, P. B. & Collins, F. M. Assessment of typhoid vaccines by using the intraperitoneal route of challenge. *Infect Immun* **17**, 555-60 (1977).
17. Sukupolvi, S., Edelstein, A., Rhen, M., Normark, S. J. & Pfeifer, J. D. Development of a murine model of chronic *Salmonella* infection. *Infect Immun* **65**, 838-42 (1997).
18. Wilmes-Riesenberg, M. R., Foster, J. W. & Curtiss, R., 3rd. An altered rpoS allele contributes to the avirulence of *Salmonella typhimurium* LT2. *Infect Immun* **65**, 203-10 (1997).
19. Emmerling, P., Hof, H. & Finger, H. Age-related defense against infection with intracellular pathogens. *Gerontology* **25**, 327-36 (1979).
20. Gruenheid, S. & Gros, P. Genetic susceptibility to intracellular infections: Nramp1, macrophage function and divalent cations transport. *Curr Opin Microbiol* **3**, 43-8 (2000).
21. Jensen, V. B., Harty, J. T. & Jones, B. D. Interactions of the invasive pathogens *Salmonella typhimurium, Listeria monocytogenes,* and *Shigella flexneri* with M-cells and Murine Peyer´s Patches. *Infect Immun* **66**, 3758-3766 (1998).
22. Jones, B. D., Ghori, N. & Falkow, S. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J Exp Med* **180**, 15-23 (1994).
23. Monack, D. M. *et al. Salmonella* exploits caspase-1 to colonize Peyer's patches in a murine typhoid model. *J Exp Med* **192**, 249-58 (2000).
24. Vazquez-Torres, A. *et al.* Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature* **401**, 804-8 (1999).
25. Rescigno, M. *et al.* Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* **2**, 361-7 (2001).
26. Rescigno, M., Rotta, G., Valzasina, B. & Ricciardi-Castagnoli, P. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology* **204**, 572-81 (2001).
27. Salcedo, S. P., Noursadeghi, M., Cohen, J. & Holden, D. W. Intracellular replication of *Salmonella typhimurium* strains in specific subsets of splenic macrophages *in vivo*. *Cell Microbiol* **3**, 587-97 (2001).

28. Richter-Dahlfors, A., Buchan, A. M. & Finlay, B. B. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. *J Exp Med* **186**, 569-80 (1997).
29. Kirby, A. C., Yrlid, U. & Wick, M. J. The innate immune response differs in primary and secondary salmonella infection. *J Immunol* **169**, 4450-9 (2002).
30. Mastroeni, P. *et al.* Interleukin-12 is required for control of the growth of attenuated aromatic-compound-dependent salmonellae in BALB/c mice: role of gamma interferon and macrophage activation. *Infect Immun* **66**, 4767-76 (1998).
31. Mastroeni, P. Immunity to systemic *Salmonella* infections. *Curr Mol Med* **2**, 393-406 (2002).
32. Fierer, J., Swancutt, M. A., Heumann, D. & Golenbock, D. The role of lipopolysaccharide binding protein in resistance to *Salmonella* infections in mice. *J Immunol* **168**, 6396-403 (2002).
33. Liang-Takasaki, C. J., Mäkelä, P. H. & Leive, L. Phagocytosis of bacteria by macrophages: changing the carbohydrate of lipopolysaccharide alters interaction with complement and macrophages. *J Immunol* **128**, 1229-35 (1982).
34. Saxen, H., Reima, I. & Mäkelä, P. H. Alternative complement pathway activation by *Salmonella* O polysaccharide as a virulence determinant in the mouse. *Microb Pathog* **2**, 15-28 (1987).
35. Mäkelä, P. H., Hovi, M., Saxen, H., Valtonen, M. & Valtonen, V. *Salmonella*, complement and mouse macrophages. *Immunol Lett* **19**, 217-22 (1988).
36. Mäkelä, P. H., Hovi, M., Saxen, H., Muotiala, A. & Rhen, M. *Role of LPS in the pathogenesis of salmonellosis* (eds. Nowotny, A., Spitznagel, J. J. & Ziegler, E. J.) (Elsevier Science publishers, 1990).
37. Mäkele, P. H. *et al.* Salmonella *as an invasive enteric pathogen* (eds. Wadström, T., Mäkele, P. H., Svennerholm, A.-M. & Wolf-Watz, H.) (Plenum press, London, 1991).
38. O'Brien, A. D., Scher, I. & Formal, S. B. Effect of silica on the innate resistance of inbred mice to *Salmonella typhimurium* infection. *Infect Immun* **25**, 513-20 (1979).
39. Hansen-Wester, I. & Hensel, M. *Salmonella* pathogenicity islands encoding type III secretion systems. *Microbes Infect* **3**, 549-59 (2001).

40. Vassiloyanakopoulos, A. P., Okamoto, S. & Fierer, J. The crucial role of polymorphonuclear leucocytes in resistance to *Salmonella dublin* infections in genetically susceptible and resistant mice. *Proc Natl Acad Sci U S A* **95**, 7676-7681 (1998).

41. Coates, N. J. & McColl, S. R. Production of chemokines *in vivo* in response to microbial stimulation. *J Immunol* **166**, 5176-82 (2001).

42. Mittrucker, H. W. & Kaufmann, S. H. Immune response to infection with *Salmonella typhimurium* in mice. *J Leukoc Biol* **67**, 457-63 (2000).

43a. Hess, J., Ladel, C., Miko, D. & Kaufmann, S. H. *Salmonella typhimurium aroA-* infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. *J Immunol* **156**, 3321-6 (1996).

43b. Sinha, K., Mastroeni, P., Harrison, J., de Hormaeche, R. D. & Hormaeche, C. E. *Salmonella typhimurium aroA, htrA*, and *aroD htrA* mutants cause progressive infections in athymic (nu/nu) BALB/c mice. *Infect Immun* **65**, 1566-9 (1997).

44. Mittrucker, H. W., Raupach, B., Kohler, A. & Kaufmann, S. H. Cutting edge: role of B lymphocytes in protective immunity against *Salmonella typhimurium* infection. *J Immunol* **164**, 1648-52 (2000).

45a. Mastroeni, P., Villarreal-Ramos, B. & Hormaeche, C. E. Adoptive transfer of immunity to oral challenge with virulent *Salmonellae* in innately susceptible BALB/c mice requires both immune serum and T cells. *Infect Immun* **61**, 3981-4 (1993).

45b. Eisenstein, T. K., Killar, L. M. & Sultzer, B. M. Immunity to infection with *Salmonella typhimurium*: mouse-strain differences in vaccine- and serum-mediated protection. *J Infect Dis* **150**, 425-35 (1984).

46. Medzhitov, R. Toll-like receptors and innate immunity. *Nature Reviews Immunology* **1**, 135-145 (2001).

47. Aderem, A. & Underhill, D. M. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* **17**, 593-623 (1999).

48. Garin, J. *et al.* The phagosome proteome: insight into phagosome functions. *J Cell Biol* **152**, 165-80 (2001).

49. Hopkins, S. A., Niedergang, F., Corthesy-Theulaz, I. E. & Kraehenbuhl, J. P. A recombinant *Salmonella typhimurium* vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. *Cell Microbiol* **2**, 59-68 (2000).

50. Svensson, M., Johansson, C. & Wick, M. J. *Salmonella enterica* serovar Typhimurium-induced maturation of bone marrow-derived dendritic cells. *Infect Immun* **68**, 6311-20 (2000).
51. Garcia-Del Portillo, F., Jungnitz, H., Rohde, M. & Guzman, C. A. Interaction of *Salmonella enterica* serotype Typhimurium with dendritic cells is defined by targeting to compartments lacking lysosomal membrane glycoproteins. *Infect Immun* **68**, 2985-91 (2000).
52. Marriott, I., Hammond, T. G., Thomas, E. K. & Bost, K. L. *Salmonella* efficiently enter and survive within cultured CD11c+ dendritic cells initiating cytokine expression. *Eur J Immunol* **29**, 1107-15 (1999).
53. Abraham, S. N. & Malaviya, R., Mast cells in infection and immunity. *Infect Immun* **65,** 3501-3508 (1997).
54. Féger, F., Varadaradjalou, S., Gao, Z., Abraham, S. N., & Arock, M. The role of mast cells in the host defence and their subversion by bacterial pathogens. *Trends Immunol* **23,** 151-158 (2002).
55. Sher, A., Hein, A., Moser, G., & Caulfield JP. Complement receptors promote the phagocytosis of bacteria by rat peritoneal mast cells. *Lab Invest.* **41,** 490-9(1979).
56. Hsu, H. S. Pathogenesis and immunity in murine salmonellosis. *Microbiol Rev* **53**, 390-409 (1989).
57. Wijburg, O. L., Simmons, C. P., van Rooijen, N. & Strugnell, R. A. Dual role for macrophages *in vivo* in pathogenesis and control of murine *Salmonella enterica* var. Typhimurium infections. *Eur J Immunol* **30**, 944-53 (2000).
58. Nnalue, N. A., Shnyra, A., Hultenby, K. & Lindberg, A. A. *Salmonella* choleraesuis and *Salmonella typhimurium* associated with liver cells after intravenous inoculation of rats are localized mainly in Kupffer cells and multiply intracellularly. *Infect Immun* **60**, 2758-68 (1992).
59. Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci U S A* **83**, 5189-93 (1986).
60. Shea, J. E., Hensel, M., Gleeson, C. & Holden, D. W. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* **93**, 2593-7 (1996).
61. Hensel, M. *et al.* Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol Microbiol* **30**, 163-74 (1998).

62. Levy, M. H. & Wheelock, E. F. Effects of intravenous silica on immune and non-immune functions of the murine host. *J Immunol* **115**, 41-8 (1975).
63. Gulig, P. A., Doyle, T. J., Hughes, J. A. & Matsui, H. Analysis of host cells associated with the Spv-mediated increased intracellular growth rate of *Salmonella typhimurium* in mice. *Infect Immun* **66**, 2471-85 (1998).
64. Mastroeni, P. *et al.* Serum TNF alpha in mouse typhoid and enhancement of a *Salmonella* infection by anti-TNF alpha antibodies. *Microb Pathog* **11**, 33-38 (1991).
65. Bao, S., Beagley, K. W., France, M. P., Shen, J. & Husband, A. J. Interferon-gamma plays a critical role in intestinal immunity against *Salmonella typhimurium* infection. *Immunology* **99**, 464-72 (2000).
66. Mastroeni, P., Villarreal, B., Demarco de Hormaeche, R. & Hormaeche, C. E. Serum TNF alpha inhibitor in mouse typhoid. *Microb Pathog* **12**, 343-9 (1992).
67. Mastroeni, P., Villarreal-Ramos, B. & Hormaeche, C. E. Effect of late administration of anti-TNF alpha antibodies on a *Salmonella* infection in the mouse model. *Microb Pathog* **14**, 473-80 (1993).
68. Mastroeni, P., Skepper, J. N. & Hormaeche, C. E. Effect of anti-tumor necrosis factor alpha antibodies on histopathology of primary *Salmonella* infections. *Infect Immun* **63**, 3674-82 (1995).
69. Muotiala, A. thesis. Cellular defence mechanisms in experimental murine salmonellosis: specific immunity and role of IFN-γ. (Helsinki, 1993)
70. Mastroeni, P., Harrison, J. A., Chabalgoity, J. A. & Hormaeche, C. E. Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. *Infect Immun* **64**, 189-96 (1996).
71. John, B. *et al.* Role of IL-12-independent and IL-12-dependent pathways in regulating generation of the IFN-gamma component of T cell responses to *Salmonella typhimurium*. *J Immunol* **169**, 2545-52 (2002).
72. Everest, P. *et al. Salmonella typhimurium* infections in mice deficient in interleukin-4 production: role of IL-4 in infection-associated pathology. *J Immunol* **159**, 1820-7 (1997).
73. Schwacha, M. G., Meissler, J. J., Jr. & Eisenstein, T. K. *Salmonella typhimurium* infection in mice induces nitric oxide-mediated immunosuppression through a natural killer cell-dependent pathway. *Infect Immun* **66**, 5862-6 (1998).

74. Smith, R. A., Brzezicki, M. J., Griggs, N. & Mahrer, S. The role of natural killer cells in experimental murine salmonellosis. *Nat Immun Cell Growth Regul* **8**, 331-7 (1989).

75. Conlan, J. W. Critical roles of neutrophils in host defence against experimental systemic infections of mice by *Listeria monocytogenes*, *Salmonella typhimurium* and *Yersinia enterocolitica*. *Infect Immun* **65**, 630-635 (1997).

76. Monack, D. M., Detweiler, C. S. & Falkow, S. *Salmonella* pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1. *Cell Microbiol* **3**, 825-37 (2001).

77. Monack, D. M., Navarre, W. W. & Falkow, S. *Salmonella*-induced macrophage death: the role of caspase-1 in death and inflammation. *Microbes Infect* **3**, 1201-12 (2001).

78. Mastroeni, P. *et al.* Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent *Salmonella typhimurium*. *Infect Immun* **67**, 478-83 (1999).

79. Shiloh, M. U. & Nathan, C. F. Reactive nitrogen intermediates and the pathogenesis of *Salmonella* and mycobacteria. *Curr Opin Microbiol* **3**, 35-42 (2000).

80. Shiloh, M. U. *et al.* Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* **10**, 29-38 (1999).

81. Mastroeni, P. *et al.* Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. *J Exp Med* **192**, 237-48 (2000).

82. Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H. & Fang, F. C. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. *J Exp Med* **192**, 227-36 (2000).

83. De Groote, M. A. *et al.* Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc Natl Acad Sci U S A* **94**, 13997-4001 (1997).

84. Fang, F. C. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* **99**, 2818-25 (1997).

85. Vazquez-Torres, A. *et al.* *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* **287**, 1655-8 (2000).

86. Chakravortty, D., Hansen-Wester, I. & Hensel, M. *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. *J Exp Med* **195**, 1155-66 (2002).
87. Mouy, R., Fischer, A., Vilmer, E., Seger, R. & Griscelli, C. Incidence, severity, and prevention of infections in chronic granulomatous disease. *J Pediatr* **114**, 555-60 (1989).
88. Campos, K. L., Giovanelli, J. & Kaufman, S. Characteristics of the nitric oxide synthase-catalyzed conversion of arginine to N-hydroxyarginine, the first oxygenation step in the enzymic synthesis of nitric oxide. *J Biol Chem* **270**, 1721-8 (1995).
89. Eriksson, S. *et al. Salmonella typhimurium* mutants that downregulate phagocyte nitric oxide production. *Cell Microbiol* **2**, 239-50 (2000).
90. Stamler, J. S. Redox Signalling:nitrosylation and related target interactions of nitric oxide. *Cell* **78** , 931-936 (1994).
91. Colasanti, M. *et al.* Induction of nitric oxide synthase mRNA expression. Suppression by exogenous nitric oxide. *J Biol Chem* **270**, 26731-3 (1995).
92. Hinz , B., Brune ,K., Pahl, A. Nitric oxide inhibits inducible nitric oxide synthase mRNA expression in RAW 264.7 macrophages. *Biochem Biophys Res Commun.* **271**,353-7(2000).
93. Martin E, Nathan C, Xie QW.Role of interferon regulatory factor 1 in induction of nitric oxide synthase.*J Exp Med.* **180**,977-984(1994).
94. Nathan, C. & Xie, Q. W. Nitric oxide synthases: roles, tolls, and controls. *Cell* **78**, 915-918 (1994).
95. Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F. & Nathan, C. F. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A* **88**, 7773-7 (1991).
96. Nussler, A. K., Billiar, T. R., Liu, Z. Z. & Morris, S. M., Jr. Coinduction of nitric oxide synthase and argininosuccinate synthetase in a murine macrophage cell line. Implications for regulation of nitric oxide production. *J Biol Chem* **269**, 1257-61 (1994).
97. Wileman, S. M., Mann, G. E. & Baydoun, A. R. Induction of L-arginine transport and nitric oxide synthase in vascular smooth muscle cells: synergistic actions of pro-inflammatory cytokines and bacterial lipopolysaccharide. *Br J Pharmacol* **116**, 3243-50 (1995).

98. Vodovotz, Y., Russell, D., Xie, Q. W., Bogdan, C. & Nathan, C. Vesicle membrane association of nitric oxide synthase in primary mouse macrophages. *J Immunol* **154**, 2914-25 (1995).

99. Vazquez-Torres, A. & Fang, F. Oxygen-dependent anti-*Salmonella* activity of macrophages. *Trends Microbiol* **9**, 29-33 (2001).

100. Gallois, A., Klein, J. R., Allen, L. A., Jones, B. D. & Nauseef, W. M. *Salmonella* pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane. *J Immunol* **166**, 5741-8 (2001).

101. De Groote, M. A., Testerman, T., Xu, Y., Stauffer, G. & Fang, F. Homocysteine antagonism of nitric oxide-related cytostasis in *Salmonella typhimurium*. *Science* **272**, 414-417 (1996).

102. Tsolis, R. M., Baumler, A. J. & Heffron, F. Role of *Salmonella typhimurium* Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages. *Infect Immun* **63**, 1739-44 (1995).

103. Sly, L. M., Guiney, D. & Reiner, N. E. *Salmonella enterica* serovar Typhimurium periplasmic superoxide dismutases SodCI and SODCII are required for protection against the phagocyte oxidative burst. *Infect Immun* **70**, 5312-5315 (2002).

104. Buchmeier, N. A., Lipps, C. J., So, M. Y. & Heffron, F. Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. *Mol Microbiol* **7**, 933-6 (1993).

105. Buchmeier, N. *et al.* DNA repair is more important than catalase for *Salmonella* virulence. *J Clin Invest* **95**, 924-925 (1995).

106. Hiemstra, P. S., van den Barselaar, M. T., Roest, M., Nibbering, P. H. & van Furth, R. Ubiquicidin, a novel murine microbicidal protein present in the cytosolic fraction of macrophages. *J Leukoc Biol* **66**, 423-8 (1999).

107. Hiemstra, P. S. *et al.* Antimicrobial proteins of murine macrophages. *Infect Immun* **61**, 3038-46 (1993).

108. Vaara, M. *et al.* Characterization of the lipopolysaccharide from the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*. *FEBS Lett* **129**, 145-9 (1981).

109. Guo, L., *et al.* Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell.* **95**,189-98 (1998).

110. Shafer, W. M., Casey, S. G. & Spitznagel, J. K. Lipid A and resistance of *Salmonella typhimurium* to antimicrobial granule proteins of human neutrophil granulocytes. *Infect Immun* **43**, 834-8 (1984).
111. Yethon, J. A. *et al. Salmonella enterica* serovar typhimurium waaP mutants show increased susceptibility to polymyxin and loss of virulence In vivo. *Infect Immun* **68**, 4485-91 (2000).
112. Deiwick, J. *et al.* Mutations in *Salmonella* pathogenicity island 2 (SPI2) genes affecting transcription of SPI1 genes and resistance to antimicrobial agents. *J Bacteriol* **180**, 4775-80 (1998).
113. Kox, L. F., Wosten, M. M. & Groisman, E. A. A small protein that mediates the activation of a two-component system by another two-component system. *Embo J* **19**, 1861-72 (2000).
114. Guo, L. *et al.* Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science* **276**, 250-3 (1997).
115. Helander, I. M., Kilpelainen, I. & Vaara, M. Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: a 31P-NMR study. *Mol Microbiol* **11**, 481-7 (1994).
116. Tamayo, R., Ryan, S. S., McCoy, A. J. & Gunn, J. S. Identification and Genetic Characterization of PmrA-Regulated Genes and Genes Involved in Polymyxin B Resistance in *Salmonella enterica* Serovar Typhimurium. *Infect Immun* **70**, 6770-8 (2002).
117. Groisman, E. A. The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* **183**, 1835-42 (2001).
118. Brodsky, I. E., Ernst, R. K., Miller, S. I. & Falkow, S. *mig-14* is a *Salmonella* gene that plays a role in bacterial resistance to antimicrobial peptides. *J Bacteriol* **184**, 3203-13 (2002).
119. Fields, P. I., Groisman, E. A. & Heffron, F. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243**, 1059-62 (1989).
120. Groisman, E. A., Parra-Lopez, C., Salcedo, M., Lipps, C. J. & Heffron, F. Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. *Proc Natl Acad Sci U S A* **89**, 11939-43 (1992).

121. Euteneuer, B., Storkel, S. & Loos, M. Differences in attachment and phagocytosis of *Salmonella minnesota* strains (S form, Re mutant) by mouse peritoneal macrophages: participation of endogenous C1q and bacterial surface components (LPS, porins). *Curr Top Microbiol Immunol* **121**, 85-97 (1985).
122. Gibbons, H. S., Lin, S., Cotter, R. J. & Raetz, C. R. Oxygen requirement for the biosynthesis of the S-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, A new Fe2+/alpha-ketoglutarate-dependent dioxygenase homologue. *J Biol Chem* **275**, 32940-9 (2000).
123. Scott, M. G., Rosenberger, C. M., Gold, M. R., Finlay, B. B. & Hancock, R. E. An alpha-helical cationic antimicrobial peptide selectively modulates macrophage responses to lipopolysaccharide and directly alters macrophage gene expression. *J Immunol* **165**, 3358-65 (2000).
124. Rathman, M., Barker, L. P. & Falkow, S. The unique trafficking pattern of *Salmonella typhimurium*-containing phagosomes in murine macrophages is independent of the mechanism of bacterial entry. *Infect Immun* **65**, 1475-85 (1997).
125. Alpuche-Aranda, C. M., Racoosin, E. L., Swanson, J. A. & Miller, S. I. *Salmonella* stimulate macrophage macropinocytosis and persist within spacious phagosomes. *J Exp Med* **179**, 601-8 (1994).
126. Hensel, M. *et al.* Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* Pathogenicity Island 2. *Mol Microbiol* **24**, 155-67 (1997).
127. Beuzon, C. R. *et al. Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *Embo J* **19**, 3235-49 (2000).
128. Buchmeier, N. A. & Heffron, F. Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. *Infect Immun* **59**, 2232-8 (1991).
129. Holden, D. W. Trafficking of the *Salmonella* vacuole in macrophages. *Traffic* **3**, 161-9 (2002).
130. Mills, S. D. & Finlay, B. B. Isolation and characterization of *Salmonella typhimurium* and *Yersinia pseudotuberculosis*-containing phagosomes from infected mouse macrophages: *Y. pseudotuberculosis* traffics to terminal lysosomes where they are degraded. *Eur J Cell Biol* **77**, 35-47 (1998).
131. Garcia-del Portillo, F. *Salmonella* intracellular proliferation: where, when and how? *Microbes Infect* **3**, 1305-11 (2001).

132. Abshire, K. Z. & Neidhardt, F. C. Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. *J Bacteriol* **175**, 3734-43 (1993).
133. Burns-Keliher, L., Nickerson, C. A., Morrow, B. J. & Curtiss, R., 3rd. Cell-specific proteins synthesized by *Salmonella typhimurium*. *Infect Immun* **66**, 856-61 (1998).
134. Ruiz-Albert, J. *et al.* Complementary activities of SseJ and SifA regulate dynamics of the *Salmonella typhimurium* vacuolar membrane. *Mol Microbiol* **44**, 645-61 (2002).
135. Garvis, S. G., Beuzon, C. R. & Holden, D. W. A role for the PhoP/Q regulon in inhibition of fusion between lysosomes and *Salmonella*-containing vacuoles in macrophages. *Cell Microbiol* **3**, 731-44 (2001).
136. Meresse, S. *et al.* Remodelling of the actin cytoskeleton is essential for replication of intravacuolar *Salmonella*. *Cell Microbiol* **3**, 567-77 (2001).
137. Brumell, J. H., Goosney, D. L. & Finlay, B. B. SifA, a Type III Secreted Effector of *Salmonella typhimurium*, Directs *Salmonella*-Induced Filament (Sif) Formation Along Microtubules. *Traffic* **3**, 407-15 (2002).
138. Hansen-Wester, I., Stecher, B. & Hensel, M. Type III secretion of *Salmonella enterica* serovar Typhimurium translocated effectors and SseFG. *Infect Immun* **70**, 1403-9 (2002).
139. Knodler, L. A. *et al. Salmonella* effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems. *Mol Microbiol* **43**, 1089-103 (2002).
140. Miao, E. A. *et al. Salmonella typhimurium* leucin rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. *Mol Microbiol* **34**, 850-864 (1999).
141. Garcia-del Portillo, F. & Finlay, B. B. Targeting of *Salmonella typhimurium* to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors. *J Cell Biol* **129**, 81-97 (1995).
142. Hashim, S., Mukherjee, K., Raje, M., Basu, S. K. & Mukhopadhyay, A. Live *Salmonella* modulate expression of Rab proteins to persist in a specialized compartment and escape transport to lysosomes. *J Biol Chem* **275**, 16281-8 (2000).

143. Mukherjee, K., Parashuraman, S., Raje, M. & Mukhopadhyay, A. SopE acts as an Rab5-specific nucleotide exchange factor and recruits non-prenylated Rab5 on *Salmonella*-containing phagosomes to promote fusion with early endosomes. *J Biol Chem* **276**, 23607-15 (2001).
144. Baldeon, M. E., Ceresa, B. P. & Casanova, J. E. Expression of constitutively active Rab5 uncouples maturation of the *Salmonella*-containing vacuole from intracellular replication. *Cell Microbiol* **3**, 473-86 (2001).
145. Alpuche Aranda, C. M., Swanson, J. A., Loomis, W. P. & Miller, S. I. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. *Proc Natl Acad Sci U S A* **89**, 10079-83 (1992).
146. Rathman, M., Sjaastad, M. D. & Falkow, S. Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. *Infect Immun* **64**, 2765-73 (1996).
147. Abshire, K. Z. & Neidhardt, F. C. Growth rate paradox of *Salmonella typhimurium* within host macrophages. *J Bacteriol* **175**, 3744-8 (1993).
148. McClelland, M. *et al.* Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**, 852-6 (2001).
149. Lucas, R. L. & Lee, C. A. Unravelling the mysteries of virulence gene regulation in *Salmonella typhimurium*. *Mol Microbiol* **36**, 1024-33 (2000).
150. Marcus, S. L., Brumell, J. H., Pfeifer, C. G. & Finlay, B. B. *Salmonella* pathogenicity islands: big virulence in small packages. *Microbes Infect* **2**, 145-56 (2000).
151. Kubori, T. *et al.* Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* **280**, 602-5 (1998).
152. Hueck, C. J. *et al. Salmonella typhimurium* secreted invasion determinants are homologous to *Shigella* Ipa proteins. *Mol Microbiol* **18**, 479-90 (1995).
153. Galan, J. E. Molecular genetic bases of *Salmonella* entry into host cells. *Mol Microbiol* **20**, 263-71 (1996).
154. Betts, J. & Finlay, B. B. Identification of *Salmonella typhimurium* invasiveness loci. *Can J Microbiol* **38**, 852-7 (1992).
155. Lee, A. C., Jones, B. D. & Falkow, S. Identification of *Salmonella Typhimurium* invasion locus by selection for hyperinvasive mutants. *Proc Natl Acad Sci U S A.* **1**,1847-51 (1992).

156. Ginocchio, C., Pace, J. & Galan, J. E. Identification and molecular characterization of a *Salmonella typhimurium* gene involved in triggering the internalization of *Salmonellae* into cultured epithelial cells. *Proc Natl Acad Sci U S A* **89**, 5976-80 (1992).

157. Ginocchio, C. C., Olmsted, S. B., Wells, C. L. & Galan, J. E. Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium*. *Cell* **76**, 717-24 (1994).

158. Penheiter, K. L., Mathur, N., Giles, D., Fahlen, T. & Jones, B. D. Non-invasive *Salmonella typhimurium* mutants are avirulent because of an inability to enter and destroy M cells of ileal Peyer's patches. *Mol Microbiol* **24**, 697-709 (1997).

159. Jones, B. D. & Falkow, S. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. *Infect Immun* **62**, 3745-52 (1994).

160. Wilson, R. L. *et al.* Fis, a DNA nucleoid-associated protein, is involved in *Salmonella typhimurium* SPI-1 invasion gene expression. *Mol Microbiol* **39**, 79-88 (2001).

161. Cirillo, D. M., Valdivia, R. H., Monack, D. M. & Falkow, S. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol Microbiol* **30**, 175-88 (1998).

162. Deiwick, J., Nikolaus, T., Erdogan, S. & Hensel, M. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. *Mol Microbiol* **31**, 1759-73 (1999).

163. Heithoff, D. M. *et al.* Coordinate intracellular expression of *Salmonella* genes induced during infection. *J Bacteriol* **181**, 799-807 (1999).

164. Fang, F. & Vazquez-Torres, A. *Salmonella* selectively stops traffic. *Trends Microbiol* **10**, 391-2 (2002).

165. Rahman, H. & Sharma, V. D. Immunogenicity and antigenic relationship of *Salmonella* enterotoxin with other enterotoxins. *Zentralbl Bakteriol* **282**, 416-26 (1995).

166. Zhang, S. *et al.* The *Salmonella enterica* serotype typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves. *Infect Immun* **70**, 3843-55 (2002).

167. Rhen, M., Riikonen, P. & Taira, S. Expression of *Salmonella typhimurium* virulence plasmid genes in cultured macrophages. *Infect Agents Dis* **2**, 285-7 (1993).

168. Fierer, J. *et al.* Expression of the *Salmonella* virulence plasmid gene *spvB* in cultured macrophages and nonphagocytic cells. *Infect Immun* **61**, 5231-6 (1993).
169. Wilson, J. A., Doyle, T. J. & Gulig, P. A. Exponential-phase expression of spvA of the *Salmonella typhimurium* virulence plasmid: induction in intracellular salts medium and intracellularly in mice and cultured mammalian cells. *Microbiology* **143** , 3827-39 (1997).
170. Tezcan-Merdol, D. *et al.* Actin is ADP-ribosylated by the *Salmonella enterica* virulence-associated protein SpvB. *Mol Microbiol* **39**, 606-19 (2001).
171. Gulig, P. A. *et al.* Molecular analysis of spv virulence genes of the *Salmonella* virulence plasmids. *Mol Microbiol* **7**, 825-30 (1993).
172. Khan, S. A. *et al.* A lethal role for lipid A in *Salmonella* infections. *Mol Microbiol* **29**, 571-9 (1998).
173. Baumler, A. J., Tsolis, R. M. & Heffron, F. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. *Infect Immun* **64**, 1862-5 (1996).
174. van der Velden, A. W., Baumler, A. J., Tsolis, R. M. & Heffron, F. Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice. *Infect Immun* **66**, 2803-8 (1998).
175. Fang, F. C. *et al.* Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. *Proc Natl Acad Sci U S A* **96**, 7502-7 (1999).
176. Farrant, J. L. *et al.* Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. *Mol Microbiol* **25**, 785-96 (1997).
177. Humphreys, S., Stevenson, A., Bacon, A., Weinhardt, A. B. & Roberts, M. The alternative sigma factor, sigmaE, is critically important for the virulence of *Salmonella typhimurium*. *Infect Immun* **67**, 1560-8 (1999).
178. Johnson, K. *et al.* The role of a stress-response protein in *Salmonella typhimurium* virulence. *Mol Microbiol* **5**, 401-7 (1991).
179. Testerman, T. L. *et al.* The alternative sigma factor sigmaE controls antioxidant defences required for *Salmonella* virulence and stationary-phase survival. *Mol Microbiol* **43**, 771-82 (2002).
180. Hoiseth, S. K. & Stocker, B. A. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238-9 (1981).

181. Cardenas, L. & Clements, J. D. Oral immunization using live attenuated *Salmonella* spp. as carriers of foreign antigens. *Clin Microbiol Rew* **5**, 328-342 (1992).
182. O'Byrne, C. P. & Dorman, C. J. The spv virulence operon of *Salmonella typhimurium* LT2 is regulated negatively by the cyclic AMP (cAMP)-cAMP receptor protein system. *J Bacteriol* **176**, 905-12 (1994).
183. Curtiss, R., 3rd & Kelly, S. M. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infect Immun* **55**, 3035-43 (1987).
184. Clements, M., Eriksson, S., Tezcan-Merdol, D., Hinton, J. C. & Rhen, M. Virulence gene regulation in *Salmonella enterica*. *Ann Med* **33**, 178-85 (2001).
185. Azam, T. A. & Ishihama, A. Twelve species of the nucleoid-associated protein from *Escherichia coli*. Sequence recognition specificity and DNA binding affinity. *J Biol Chem* **274**, 33105-13 (1999).
186. Marshall, D. G., Bowe, F., Hale, C., Dougan, G. & Dorman, C. J. DNA topology and adaptation of *Salmonella typhimurium* to an intracellular environment. *Philos Trans R Soc Lond B Biol Sci* **355**, 565-74 (2000).
187. Lee, I. S., Lin, J., Hall, H. K., Bearson, B. & Foster, J. W. The stationary-phase sigma factor sigma S (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. *Mol Microbiol* **17**, 155-67 (1995).
188. Lee, A. K., Detweiler, C. S. & Falkow, S. OmpR regulates the two-component system SsrA-SsrB in *Salmonella* pathogenicity island 2. *J Bacteriol* **182**, 771-81 (2000).
189. Cunning, C., Brown, L. & Elliott, T. Promoter substitution and deletion analysis of upstream region required for *rpoS* translational regulation. *J Bacteriol* **180**, 4564-70 (1998).
190. Julio, S. M., Heithoff, D. M. & Mahan, M. J. *ssrA* (tmRNA) plays a role in *Salmonella enterica* serovar Typhimurium pathogenesis. *J Bacteriol* **182**, 1558-63 (2000).
191. Hautefort, I. & Hinton, J. C. Measurement of bacterial gene expression *in vivo*. *Philos Trans R Soc Lond B Biol Sci* **355**, 601-11 (2000).
192. Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 686-8 (1993).
193. Hensel, M. *et al.* Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**, 400-3 (1995).

194. Valdivia, R. H. & Falkow, S. Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* **277**, 2007-11 (1997).

195. Pfeifer, C. G., Marcus, S. L., Steele-Mortimer, O., Knodler, L. A. & Finlay, B. B. *Salmonella typhimurium* virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. *Infect Immun* **67**, 5690-8 (1999).

196a. Lucchini, S., Thompson, A. & Hinton, J. C. Microarrays for microbiologists. *Microbiology* **147**, 1403-14 (2001).

196b. Wilson, J.W., *et al.* Microarray analysis identifies *Salmonella* genes belonging to the low-shear modeled microgravity regulon.*Proc Natl Acad Sci U S A.* **99**,13807-13812 (2002).

196c. Rosenberger, C. M., Scott, M.G., Gold, M. R., Hancock, R. E., & Finlay, B. B. *Salmonella typhimurium* infection and lipopolysaccharide stimulation induce similar changes in macrophage gene expression. *J Immunol* **164**, 5894-5904 (2000).

197. Beuzon, C. R., Banks, G., Deiwick, J., Hensel, M. & Holden, D. W. pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella typhimurium*. *Mol Microbiol* **33**, 806-16 (1999).

198. Bajaj, V., Lucas, R. L., Hwang, C. & Lee, C. A. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. *Mol Microbiol* **22**, 703-14 (1996).

199. Lucas, R. L. *et al.* Multiple factors regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **182**, 1872-1882 (2000).

200. Bajaj, V., Hwang, C. & Lee, A. C. *hilA* is a novel ompR/toxR family member that activates the expression of *Salmonella typhimurium* invasion genes. *Mol Microbiol* **18**, 715-727 (1995).

201. Kaniga, K., Bossio, J. C. & Galan, J. E. The *Salmonella typhimurium* invasion genes invF and invG encode homologues of the AraC and PulD family of proteins. *Mol Microbiol* **13**, 555-68 (1994).

202. Lucas, R. L. & Lee, C. A. Roles of hilC and hilD in regulation of hilA expression in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **183**, 2733-45 (2001).

203. Johnston, C., Pegues, D. A., Hueck, C. J., Lee, A. C. & Miller, S. I. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. *Mol Microbiol* **22**, 715-727 (1996).

204. Behlau, I. & Miller, S. I. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. *J Bacteriol* **175**, 4475-84 (1993).

205. Pegues, D. A., Hantman, M. J., Behlau, I. & Miller, S. I. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* genes: evidence for a role in protein secretion. *Mol Microbiol* **17**, 169-181 (1995).

206. Murray, R. A. & Lee, C. A. Invasion genes are not required for *Salmonella enterica* serovar typhimurium to breach the intestinal epithelium: evidence that *Salmonella* pathogenicity island 1 has alternative functions during infection. *Infect Immun* **68**, 5050-5 (2000).

207. Steele-Mortimer, O. *et al.* The invasion-associated type III secretion system of *Salmonella enterica* serovar Typhimurium is necessary for intracellular proliferation and vacuole biogenesis in epithelial cells. *Cell Microbiol* **4**, 43-54 (2002).

208. Chen, L. M., Kaniga, K. & Galan, J. E. *Salmonella* spp. are cytotoxic for cultured macrophages. *Mol Microbiol* **21**, 1101-15 (1996).

209. Lee, C. A. *et al.* A secreted *Salmonella* protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. *Proc Natl Acad Sci U S A* **97**, 12283-8 (2000).

210. Deiwick, J. & Hensel, M. Regulation of virulence genes by environmental signals in *Salmonella typhimurium*. *Electrophoresis* **20**, 813-7 (1999).

211. Wilson, J. A. & Gulig, P. A. Regulation of the *spvR* gene of the *Salmonella typhimurium* virulence plasmid during exponential-phase growth in intracellular salts medium and at stationary phase in L broth. *Microbiology* **144** , 1823-33 (1998).

212. Miller, S. I. PhoP/PhoQ: macrophage-specific modulators of *Salmonella* virulence? *Mol Microbiol* **5**, 2073-2078 (1991).

213. Ahmer BM, van Reeuwijk J, Watson PR, Wallis TS, Heffron F.*Salmonella* SirA is a global regulator of genes mediating enteropathogenesis.*Mol Microbiol.* **31**,971-982 (1999).

214. Miller, S. I. and Mekalanos, J.J. Constitutive expression of the phoP regulon attenuates *Salmonella* virulence and survival within macrophages. *J Bacteriol* **172**, 2485-2490 (1990)

215. Garcia-Del Portillo, F., Pucciarelli, M. G. & Casadesus, J. DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. *Proc Natl Acad Sci U S A* **96**, 11578-83 (1999).

216. Heithoff, D. M., Sinsheimer, R. L., Low, D. A. & Mahan, M. J. An essential role for DNA adenine methylation in bacterial virulence. *Science* **284**, 967-70 (1999).

217. Torreblanca, J. & Casadesus, J. DNA adenine methylase mutants of *Salmonella typhimurium* and a novel *dam*-regulated locus. *Genetics* **144**, 15-26 (1996).

218. Pucciarelli, M. G., Prieto, A. I., Casadesus, J. & Garcia-del Portillo, F. Envelope instability in DNA adenine methylase mutants of *Salmonella enterica*. *Microbiology* **148**, 1171-82 (2002).

219. Fahlen, T. F., Wilson, R. L., Boddicker, J. D. & Jones, B. D. Hha is a negative modulator of transcription of *hilA*, the *Salmonella enterica* serovar Typhimurium invasion gene transcriptional activator. *J Bacteriol* **183**, 6620-9 (2001).

220. Cohn, J., Sessa, G. & Martin, G. B. Innate immunity in plants. *Curr Opin Immunol* **13,** 55-62 (2001).

221. Hardt, W. D. & Galan, J. E. A secreted *Salmonella* protein with homology to an avirulence determinant of plant pathogenic bacteria. *Proc Natl Acad Sci U S A* **94**, 9887-92 (1997).

222. Collier-Hyams, L. S. *et al.* Cutting edge: *Salmonella* AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. *J Immunol* **169**, 2846-50 (2002).

223. Yamamoto, T. *et al.* Disruption of the genes for ClpXP protease in *Salmonella enterica* serovar Typhimurium results in persistent infection in mice, and development of persistence requires endogenous gamma interferon and tumor necrosis factor alpha. *Infect Immun* **69**, 3164-74 (2001).

224. Mouslim, C., Hilbert, F., Huang, H. & Groisman, E. A. Conflicting needs for a *Salmonella* hypervirulence gene in host and non-host environments. *Mol Microbiol* **45**, 1019-27 (2002).

225. Levin, B.R. The evolution and maintenance of virulence in microparasites. *Emerg Infect Dis* **2**, 93-102 (1996).

226. Buchmeier, N. & Libby, S. Dynamics of growth and death within a *Salmonella typhimurium* population during infection of macrophages. *Can J Microbiol* **43**, 29-34 (1997).

227. Cano, D. A. *et al. Salmonella enterica* serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation. *Infect Immun* **69**, 6463-74 (2001).
228. Coburn, G. A., Miao, X., Briant, D. J. & Mackie, G. A. Reconstitution of a minimal RNA degradosome demonstrates functional coordination between a 3' exonuclease and a DEAD-box RNA helicase. *Genes Dev* **13**, 2594-603 (1999).
229. Yamanaka, K. & Inouye, M. Selective mRNA degradation by polynucleotide phosphorylase in cold shock adaptation in *Escherichia coli*. *J Bacteriol* **183**, 2808-16 (2001).
230. Rhen, M., Eriksson, S., Clements, M., Bergström, S. & Normark, S. Basis of persistent infection. *Trends Microbiol*, in press (2002).

231. Mellemgaard, A. & Gaarslev, K. Risk of hepatobiliary cancer in carriers of *Salmonella* typhi. *J Natl Cancer Inst* **80**, 288 (1988).
232. Welton, J. C., Marr, J. S. & Friedman, S. M. Association between hepatobiliary cancer and typhoid carrier state. *Lancet* **1**, 791-4 (1979).