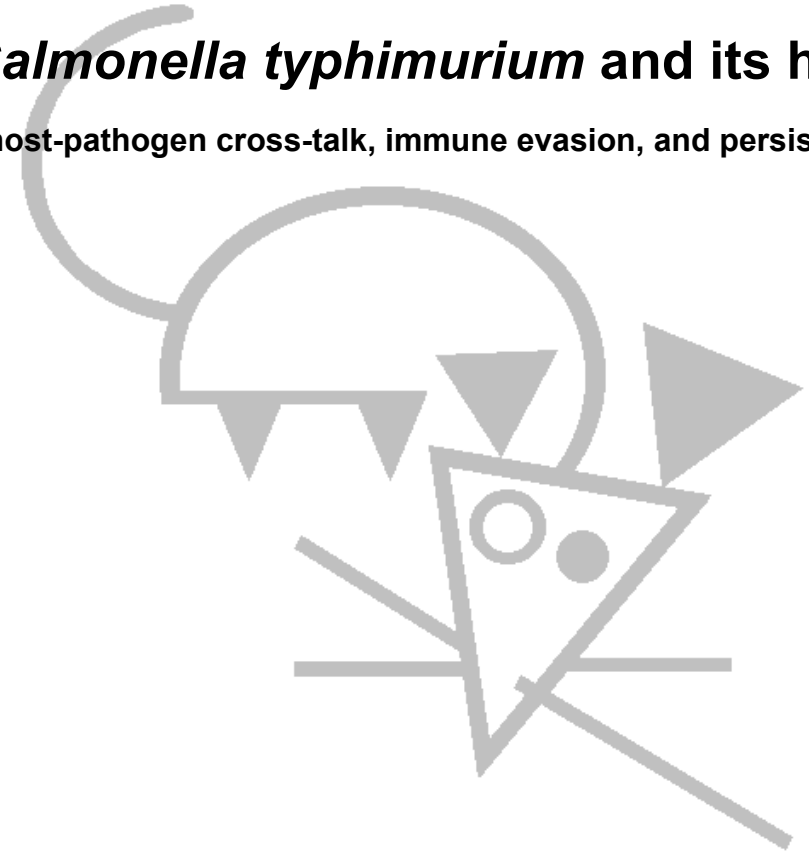
# *Salmonella typhimurium* and its host:

**host-pathogen cross-talk, immune evasion, and persistence**



**Angela van Diepen**

# *Salmonella typhimurium* and its host:

## host-pathogen cross-talk, immune evasion, and persistence

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*aan mijn ouders*
*voor Sjaak*

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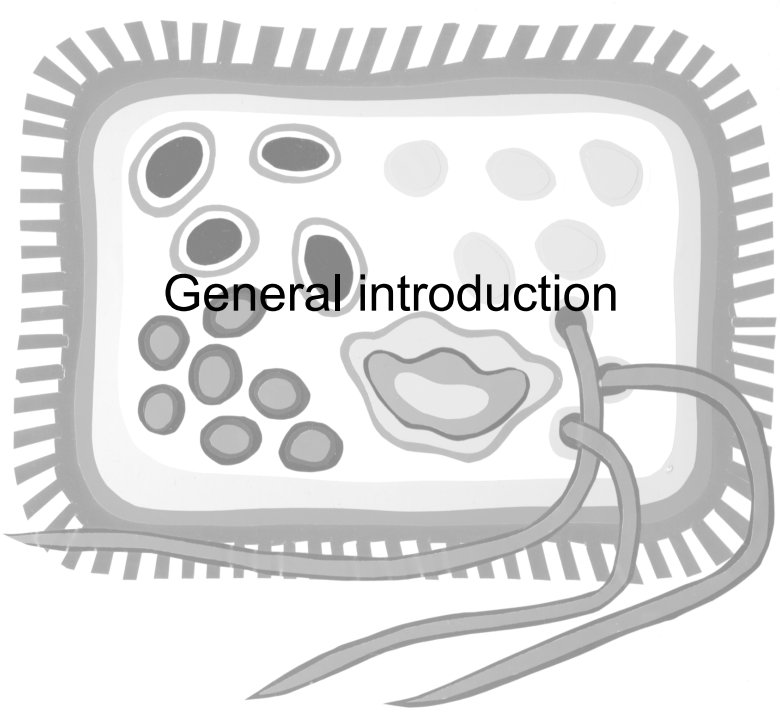


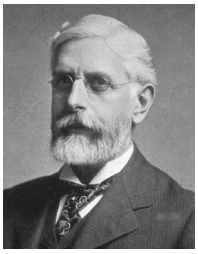
# General introduction

Partly adapted from:

**Riny Janssen, Tahar van der Straaten, Angela van Diepen, and Jaap T. van Dissel.** 2003. Responses to reactive oxygen intermediates and virulence of *Salmonella typhimurium*. Microbes. Infect. **5:**527-534

## History

The genus *Salmonella* is a member of the Enterobacteriaceae, a family of microorganisms that reside within the gastrointestinal tracts of humans and higher animals. Already in the early 1980s Theobald Smith pointed out that not all members of the Enterobacteriaceae behaved the same. He noticed that the organisms that were pathogenic to humans and animals failed to ferment lactose, while the organisms that were thought to be normal inhabitants of the intestinal tracts of humans and higher animals did ferment lactose. This observation led to the early separation of two genera, later called *Salmonella* and *Shigella*, from the rest of the Enterobacteriaceae on the basis of their pathogenicity. The *Salmonellae* are named after Dr. Daniel E. Salmon (1850-1914), a veterinary medical scientist who pioneered research in bacterial diseases and in immunology. His efforts in research on *Salmonella* led to the development of killed typhoid vaccines and to the naming of the bacterial genus in his honor. In 1885 he discovered the first strain of *Salmonella* from the intestine of a pig with hog cholera, later called *S. choleraesuis*. It is argued that this pathogen should in fairness be called Smithella, since it was Theobald Smith who was the true discoverer of the first member of the *Salmonellae* (77). However, it was his supervisor Daniel E. Salmon who wrote the paper “The bacterium of swine plague” (141). Since 1885 a lot more *Salmonella* strains were discovered and nowadays, 2,463 different strains are known (132).



Daniel Elmer Salmon (1850-1914)

## Taxonomy

Originally, the *Salmonellae* were named according to the one serotype-one species concept proposed by Kaufmann (78) and nomenclature was based upon host specificity, the presence of specific surface antigens (i.e. lipopolysaccharide (LPS), O antigens, and flagellar H antigens), and sensitivity to phages. This resulted more than 2,100 different *Salmonella* “species” that were named after their favorite host or the place where they were originally isolated. However, with DNA-DNA hybridization techniques it became clear that all of the *Salmonella* strains, with the exception of the *S. bongori* strain, were related at the species level (28) and therefore, belonged to a single species, which was called *Salmonella* (*S.*) *enterica* (125). *S. enterica* has been divided into six subspecies (spp.) on

the basis of genetic similarity and host range: *enterica* (or *choleraesuis*, Group I), *salamae* (Group II), *arizonae* (Group IIIa), *diarizonae* (Group IIIb), *houtenae* (Group IV), and *indica* (Group VI). *S. bongori* was originally classified as ssp. V, but since it differed too much from the other *Salmonella* it is generally considered a separate species (136). Group I contains most of the serotypes that are pathogenic to humans, including *S. typhi* and *S. typhimurium*. The *Salmonellae* are nowadays classified as *S. enterica* with numerous subspecies and serovars (9). For example, *S. typhimurium* is now officially referred to as *S. enterica* spp. *enterica* serovar Typhimurium. Although this is the official classification, the common species names, used before reclassification, are still widely used.

## The pathogen

The *Salmonellae* are rods that are approximately 2-3 × 0.4-0.6 µm in size with parallel sides and rounded ends. They are gram-negative, non-acid-fast bacteria that do not form spores and show no granules. The *Salmonellae* are motile because of the presence of flagellae, with the exception for the non-motile *S. gallinarum* and *S. pullorum*, the pathogens that cause fowl typhoid and pullorum disease in birds, respectively. Most of the *Salmonella* strains cannot survive in animals and humans and, as a result, do not cause disease. Only a few of the *Salmonella enterica* strains are pathogenic to humans and animals. *Salmonella* infections are one of the most common food-borne infections in the world. Annually, an estimated 1.41 million cases occur in the United States and are responsible for ±600 reported deaths (103). *Salmonella* infections have become a major food borne disease in the developed world, but are even a greater health problem in the developing countries where a lot of people, especially children, get infected and die due to infection with typhoidal as well as non-typhoidal *Salmonella* strains (reviewed in (56)). Severe *Salmonella* infections in the Western world are mainly a problem in the immunocompromized, the elderly, in people with AIDS and very young children. These groups of people can suffer from very severe infections and can die as a consequence. Recurrent infection with a *Salmonella* strain that persisted in the host after a previous episode has also been described for people with AIDS or other immune defects like IL12Rβ1 receptor deficiency (17, 52, 69, 152).

## Bacterial organization

The bacterial cell is composed of a nucleoid containing the chromosomal DNA, cytoplasm, and a cell envelope. All structural components have different functions and help the bacterium to survive and replicate in certain environments and to protect the cell against damage.

**Chromosomal DNA**

As for most bacteria, the chromosomal DNA is a single, covalently linked, ring-shaped molecule. The *S. enterica* serovar Typhimurium strain LT2 chromosome (4,857 kilobases) and 94-kb virulence plasmid have been sequenced and revealed 4,597 suspected genes (100), encoding proteins involved in many processes and many of which were previously unknown. The chromosomal DNA is surrounded by the cytosol that is densely packed with ribosomes that often form polysomes, i.e. special structures that are formed when mRNA is translated by more than one ribosome at the same time.

**Cell envelope**

The cell envelope is composed of an inner cell membrane surrounded by a cell wall and an outer membrane. The cell envelope plays a very important role in the adaptation strategies of *Salmonella* since the structural components are adapted to take up nutrients, to exclude certain toxic compounds, and to adhere to surfaces or cells. The cell membrane is a lipid bilayer composed of phospholipids and is very much alike that of other biological membranes. The inner membrane is surrounded by a cell wall, which is a thin layer of peptidoglycan that confers structural strength and helps determine cellular shape. The region between the cell membrane and the outer membrane is called the periplasm. The periplasm has an osmotic strength that under most conditions is greater than the surroundings thereby maintaining the turgor necessary for growth of the bacterium. The periplasm is iso-osmotic to the cytosol (155) and contains catabolic enzymes, binding proteins involved in the uptake of nutrients, enzymes involved in inactivation of toxic compounds, and enzymes promoting the biogenesis of major envelope protein or polymers (reviewed in (122)).

The outer membrane is built up outside the peptidoglycan layer and these two structures are connected by the outer membrane lipoproteins and porins. The outer membrane is also a bilayer, but the composition is rather different from that of the cell membrane and has the capacity to resist damaging chemicals. The inner leaflet of the outer membrane is similar to the cell membrane and is built up of phospholipids. The outer leaflet, however, does not contain phospholipids, but contains lipopolysaccharide (LPS) instead, which is involved in excluding hydrophobic compounds. Within the outer membrane special channels are formed by porins through which passive diffusion of hydrophilic compounds and certain ions takes place. Other structural components of the outer membrane are the pili and the flagellae. The pili are organelles of attachment to surfaces and minor proteins of pili, termed adhesins, play a role in *Salmonella*-host interaction. The flagellae are organelles of bacterial locomotion composed of basal body, hook, and flagellin forming the helical filament. Flagellae and LPS are antigenic and host immune responses are often directed against these surface H and O antigens, respectively (135).

### *Salmonella* lipopolysaccharide

LPS is the major constituent of the outer membrane of *Salmonella* that is involved in protection of the bacterial cell and is a potent inducer of host immune responses. LPS is composed of three major structural parts, the hydrophilic O-antigen polysaccharide, the hydrophobic lipid A, and the connecting core oligosaccharide (134) (Fig. 1A). The lipid A portion is also called endotoxin, as this the bioactive component that is responsible for some of the pathophysiology (septic shock) associated with severe *Salmonella* infection. Lipid A is the pathogen-associated molecular pattern (PAMP) that is recognized by Toll-like receptor (TLR) 4, leading to MyD88-mediated signal transduction and activation of the phagocytic cell. During infection, lipid A is bound by an acute phase serum protein (LBP, for LPS binding protein) and is delivered to CD14. CD14 is a cell surface protein expressed by macrophages (and other cell types) that delivers LPS to TLR4 that then induces intracellular signaling and activation of the macrophage.

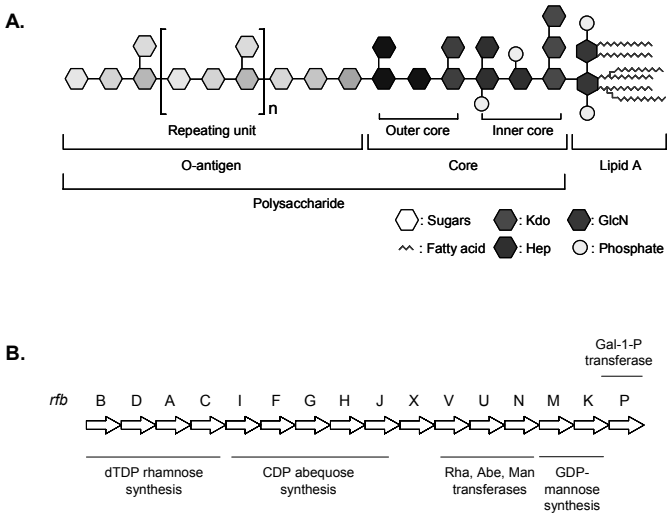



**Figure 1.** LPS structure of *S. enterica* serovar Typhimurium (A) and organization of the *rfb* operon encoding genes involved in the formation of the O-antigen (B).

The LPS core region is a short series of sugars and is composed of two 3-deoxy-D-manno-octulosonic acid (KDO) residues and a heptose. The core is required for the outer membrane to function as a barrier to antibiotics (144, 176) and connects the lipid A to the O-antigen polysaccharide. The O-antigen is an immunogenic repeating oligosaccharide of 1-40 units and each unit is composed of three sugars (mannose, rhamnose, and abequose). The components that are involved in the formation of the O-antigen are all encoded by genes of the *rfb* operon (73) (Fig. 1B). The presence of an intact O-antigen is important for *Salmonella* as it may enhance bacterial virulence and mediate resistance to complement-mediated killing, as the shorter the LPS chain, the more sensitive these mutants get to complement-mediated serum lysis and the less these *S. enterica* serovar Typhimurium mutants are able to colonize the intestines (89, 116, 146).

## Clinical manifestations of *Salmonella* infection

Human infection with *Salmonella* may occur in five different (clinical) forms including enteric fever and its asymptomatic chronic carrier state, gastroenteritis, bacteremia, and extra-intestinal localized complications, i.e. in the bones (osteomyelitis), joints (arthritis), or vasculature (endovasculitis). The strictly human serovars typhi and most of the paratyphi cause enteric fever.

### Asymptomatic chronic carrier state

The most well known asymptomatic carrier of *Salmonella* is Typhoid Mary Mallon, a 40-year-old Irish woman who emigrated to the United States to start working as a cook. She was shown to be a healthy carrier of *S. typhi* and spread the disease to at least 45 people of whom three died (121). This illustrates the problem of chronic carriage of *S. typhi* since chronic carriers may show no signs of illness but shed the bacteria through their stools being the cause of spread of *S. typhi* to other individuals, especially by those working in the food industry. Both the typhoidal as well as the non-typhoidal *Salmonella* strains are able to persist within the host, although this is rare for the non-typhoidal strains. Chronic carriage, which is clinically defined as the situation in which the bacteria are shed in the stool for periods exceeding 1 year, occurs only in about 0.1% of non-typhoidal *Salmonella* cases and might even represent reinfection instead of true chronic carriage. Usually, the bacteria are shed during 6 weeks or 3 months depending on the serotype. In typhoidal infections, however, chronic carriage occurs more often as approximately 2-5% of untreated typhoidal infections results in a chronic carrier state. Better hygiene care can diminish the risk of spread of the bacteria.

### Gastroenteritis

Gastroenteritis can be caused by several *Salmonella* strains, including *S. enterica* serovar Enteritidis and serovar Typhimurium. Infection occurs via ingestion of food or water that is contaminated with animal waste. *Salmonella*-induced enteritis leads to the development of an inflammatory reaction that is characterized by the infiltration of neutrophils. Patients show an acute onset of nausea and vomiting that is followed by diarrhea, abdominal pain, and fever after an incubation time that usually lies between 6 and 72 h depending upon the host and the inoculum. Enteric infection with *Salmonella* is hardly distinguishable from that caused by other enteric pathogens like *E. coli* or *Shigella* as the symptoms are not very specific. The infection is usually cleared within 5 to 7 days without treatment, although *Salmonella* pathogens can be found in the stool four to five weeks after resolution of gastroenteritis (80, 107, 143). *Salmonella* gastroenteritis can be life-threatening in the elderly, children, and other immunocompromised individuals. The worldwide incidence of acute gastroenteritis has been estimated at 1.3 billion by the WHO, resulting in approximately 3 million deaths (124). *S. enteritidis* has surpassed *S. typhimurium* as the major source of gastroenteritis.



### Enteric (typhoid) fever

Enteric fever is a severe disease caused by the human-specific strains *S. typhi* or *S. paratyphi*. Infection occurs through the ingestion of food or water that is contaminated with human waste and disease occurs within 5 to 21 days post-infection. Patients suffer from a systemic infection resulting in high fever, diarrhea, constipation, and sometimes a characteristic rash. Sometimes, very severe complications such as gut perforation, hemorrhage, and septic shock can occur (80, 107, 143). As for gastroenteritis, the severity of the disease depends on the type of strain and the immune status of the host. This type of *Salmonella* disease is a very serious threat since about 10-15% of the immunocompetent people will die due to the infection when no antibiotics are administered and even when proper antibiotic treatment is started mortality rates can be as high as 5-7% in some regions throughout the world. It was estimated that typhoid fever caused approximately 21 million illnesses and 200,000 deaths during 2000 and that paratyphoid fever caused an additional 5 million illnesses (29). Fortunately, most people clear the infection and due to better living conditions and hygiene care the number of cases has declined dramatically in the Western countries. In the developing countries, however, typhoid fever remains a significant problem of morbidity and mortality (38) since typhoid fever is endemic in many developing countries, particularly India, South and Central America, and Africa. These nations share several characteristics that form a risk for spreading typhoid fever; inadequate human waste treatment and limited water supply in combination with a rapid growth of the population, an increased urbanization, and an overloaded healthcare system (105).

### Bacteremia

Approximately 5% of individuals with gastrointestinal illness caused by non-typhoidal *Salmonella* develop bacteremia, a serious and potentially fatal condition in which the bacteria pass the intestinal barrier and enter the bloodstream. Bacteremia is a serious complication of non-typhoidal *Salmonella* infections and can be lethal if not treated with antibiotics. Bacteremia has been most often described for the immunocompromised like HIV infected patients or patients with genetic defects in cellular immunity like Interleukin 12 receptor $\beta$1 (IL12R$\beta$1) deficiency (2, 3, 33, 164) or Interferon $\gamma$ receptor 1 (IFN$\gamma$R1) deficiency (76, 117, 164). In these groups of patients also recurring infections with the same *Salmonella* isolate have been described (17, 52, 69, 152).

## Infection with *Salmonella*

Infection with *Salmonella* occurs by the fecal-oral route, i.e. via ingestion of food or water that is contaminated with animal or human waste. A generally known risk factor is the consumption of raw eggs and non-pasteurized milk, but a risk factor that cannot be ruled out is the food-handler. Especially in places where there is no good hygiene care, the risk of infection is high.

**Natural infection**

Natural infection with *Salmonella* occurs through the ingestion of contaminated food or water. The first natural barrier of the host is the low pH of the stomach. This pH usually is below 1.5 and most of the bacteria are killed. However, if for some reason the pH is slightly above 1.5, *Salmonella* can escape killing since it has evolved mechanisms to survive at low pH. Also when there are large numbers of bacteria present in the food or water, some of the bacteria will pass the stomach intact. Once *Salmonella* has passed, it enters the small intestines where it encounters several defense mechanisms like the thick mucus layer and competing naturally occurring intestinal flora. In the small intestine *Salmonella* will eventually encounter membranous epithelial (M) cells overlying the Peyer's patches. The Peyer's patches are organized mucosa-associated lymphoid tissues in the gut that are overlaid by specialized follicle-associated epithelium (FAE) in which the M cells reside. These M cells function as antigen-sampling cells transporting material across the FAE to the underlying lymphoid tissues where protective immune responses are initiated (reviewed in (115)). Some pathogens use these M cells to pass the intestinal lining and to invade the body (reviewed in (72)). Since reduced amounts of mucus are present at the FAE surface, *Salmonella* preferentially invades these M cells. In addition, M cells have an irregular brush border and a thinner glycocalyx than enterocytes, promoting invasion. *Salmonella* is then transported through the cytoplasm to the underlying lymphoid cells where it it preferentially infects phagocytes within the lamina propria. The phagocytes infected with *Salmonella* then enter the lymphatics and bloodstream, allowing for spread to the liver and the spleen (165). Depending on the type of *Salmonella* strain two major types of diseases occur in humans. When the gut is colonized by the typhoidal strains *S. typhi* or *S. paratyphi*, the bacteria spread to the lymph nodes, become systemic, reaching the liver and spleen and causing a chronic inflammatory response (typhoid fever). Non-typhoidal strains, on the other hand, reside within the Peyer's patches and induce a local inflammatory response mediated by cytokines, chemokines, and neutrophils (salmonellosis).

**Experimental infection**

The most widely used in vivo model for *Salmonella* infection is the mouse model, although studies are also performed in chickens, cows, guinea pigs, and rats. The human-specific *Salmonella* strains *S. enterica* serovar Typhi and Paratyphi cannot be used in these models due to their host-specificity. However, *S. enterica* serovar Typhimurium, the causative agent of gastroenteritis in humans, causes a disease in mice that is comparable to that of enteric fever in humans and therefore serves a good model for human infection with *S. enterica* serovar Typhi and is most widely used.

The natural route of infection is the oral one, but in the experimental setting infections can also be initiated after injecting the bacteria intravenously, intraperitoneally, or subcutaneously. Once *Salmonella* has disseminated into the bloodstream and thus has become systemic, all these systems are equivalent to the natural infections. Within a few

days after local infection the bacteria spread to the spleen and liver where they reside and replicate within macrophages. The disease caused by *S. enterica* serovar Typhimurium is characterized by the influx of inflammatory cells (macrophages and neutrophils), which, together with the bacterial replication, result in hepatosplenomegaly, focal necrosis, and bacteremia. Depending on the dose and the type of mouse used in the infection model, the infection can either cause death within a few days, or an immune response is generated and the mice survive being protected against a second infection (96, 118). Vaccination is therefore an effective tool for the prevention of *Salmonella* infections (95).

**Four stages of infection**

Once *S. enterica* serovar Typhimurium has become systemic, the infection in mice is characterized by four different phases (Fig. 2). In the bloodstream, the bacteria are rapidly killed by resident macrophages and granulocytes (phase 1) (20, 160). In humans complement-mediated killing is also important in innate defense mechanisms. In mice, however, complement is not that potent and cannot kill virulent *S. enterica* serovar Typhimurium, although it might be involved in opsonisation of the bacteria to promote uptake and killing by macrophages and granulocytes (86-88, 142, 154, 172). Bacteria that survive reside within the liver and spleen where they survive and replicate within polymorphonuclear cells or macrophages or extracellularly (36, 37, 137). Bacteria that have adapted to the intracellular macrophage environment divide exponentially within these cells during the first week (phase 2). Survival and replication within macrophages is essential for immuno-pathogenesis as mutants unable tot do so are avirulent (43). Eventually, bacterial growth is halted by the macrophages resulting in a plateau-phase (phase 3). Then the adaptive immune response is initiated and mainly T cells mediate the elimination of *S. enterica* serovar Typhimurium during this late (fourth) phase.

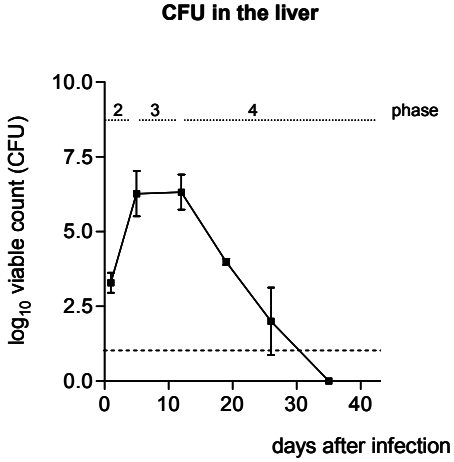



**Figure 2.** Three of the four phases of primary *S. enterica* serovar Typhimurium infection in the livers of C3H/HeN mice. The first phase is characterized by rapid killing of the bacteria by resident macrophages and complement. During the second phase, when the bacteria have spread to the liver and spleen and reside within macrophages, they start dividing exponentially. Bacterial growth is halted during the third phase by the macrophages and the bacteria are eventually cleared during the fourth phase that is mainly mediated by T cells.

## *Salmonella*: an intracellular pathogen

*S. enterica* serovar Typhimurium is a facultative intracellular pathogen that preferentially invades mononuclear cells and is able to survive and replicate within these professional phagocytes. Like some other intracellular pathogens, *S. enterica* serovar Typhimurium has the capacity to adhere to host cells and to induce its own ingestion, even by nonprofessional phagocytes. These processes are induced by proteins that are expressed at the bacterial surface (adhesins and invasins) and can interact with host cell receptors (5, 6, 23, 24, 45, 51, 59, 74, 75, 133, 161). This leads to tha activation of intracellular signaling pathways resulting in cytoskeletal rearrangements and endocytosis (as in professional phagocytes).

The interaction between bacteria and host cells also induces the synthesis of new proteins by the bacteria. This probably reflects an adaptive response to a new environment and illustrates the cross talk between bacteria and host cells. *S. enterica* serovar Typhimurium contains two very important gene clusters in localized regions of the chromosome that are involved in the invasion of and survival within phagocytes. These regions are called *Salmonella* pathogenicity islands (SPI-1 and SPI-2) and they contain several genes that are involved in the delivery of virulence proteins into the host cell. They encode type III secretion systems (TTSSs) that are needle-like structures through which proteins are injected into the host cell (46) (93). The action of the proteins encoded by these genes leads to the uptake of the bacteria and to intracellular survival and replication.

### *Salmonella* Pathogenicity Island 1

SPI-1 contains genes that encode proteins involved in the uptake of *Salmonella* by intestinal epithelial cells and the induction of intestinal secretory and inflammatory responses (reviewed in (179)). Upon contact with the cells via invasins and adhesins, *Salmonella* starts producing the first TTSS, a needle-like structure that spans the inner and outer membrane of the bacterial envelope and secretes the translocon and at least 13 effector proteins into the host cell cytosol to induce several cellular changes promoting the uptake of the *Salmonella* (reviewed in (179)). The effector proteins encoded by genes of the SPI-1 induce cytoskeleton rearrangements that lead to a process called membrane ruffling and leads to the phagocytosis of *Salmonella*, even by non-professional phagocytes. SPI-1 mutants are attenuated when administered orally, however, when given intraperitoneally, these mutants are as virulent as the wild type strain indicating that SPI-1 does not play a role in survival and replication within the liver and spleen (47).

One of the genes encoded by SPI-1 that is involved in *Salmonella* virulence is *sipB*, which is injected into the host cell cytosol upon entry. SipB binds to and activates caspase-1 (64), an IL-1$\beta$ converting enzyme capable of cleaving the pro-forms of the inflammatory cytokines IL-1$\beta$ and IL-18 (65). Caspase-1 activation then leads to the secretion of IL-1$\beta$ and IL-18 and results in macrophage death. This *Salmonella*-induced cell death is characterized by DNA fragmentation and membrane instability leading to lactate dehydrogenase (LDH) leakage and probably does not reflect a host response to infection,



but rather a bacterial strategy to promote disease by enabling cell-to-cell spread (8) since it has been shown that *Salmonella* mutants deficient in *sipB* are not cytotoxic and cannot induce apoptosis (64). In addition, *Salmonella* induces less cell death in cells deficient for caspase-1 and induces no acute inflammation in caspase-$1^{-/-}$ mice and is less virulent in these mice compared to wild-type mice (111).

### *Salmonella*-containing vacuole (SCV)

Once inside the macrophage, *Salmonella* resides within a vacuole that is modulated by the bacterium. The SCV, as it is called, will undergo a few maturation steps during this intracellular lag period of 2-3 h to prevent bacterial killing and to promote bacterial survival and replication. The SCV is entirely *Salmonella*-specific (7, 14, 49, 67, 108) and its formation is an active process that is induced by *Salmonella* (1, 18, 50, 140) and includes the translocation of several bacterial proteins into the host cell cytosol and the cytoskeleton remodelling (10, 81, 104, 106). The SCV is different from the endosomes, although it does acquire the early endosome and recycling compartment markers such as EEA1 and transferrin receptor (reviewed in (54)), but these are recycled from the SCV once it matures. During maturation the SCV acquires the late endosome/lysosomal glycoproteins Lamp 1, Lamp 2 and CD63, but excludes the lysosomal enzymes and mannose 6-phosphate receptors (reviewed in (54)). Once the SCV has completely matured and *Salmonella* has had the time to adapt to this intracellular environment (e.g. after ~3 h), a milieu has been created that enables bacterial growth. In non-phagocytic cells, at the same time, membrane tubules called *Salmonella* induced filaments (Sif) are formed that originate in the SCV and extend into the cell (10, 12, 60, 140). However, for reasons unknown, these Sifs are not formed in macrophages (7), although *sifA* encoding the SPI-2 effector protein SifA is required for intracellular survival and replication and for in vivo virulence (11, 153). It has been suggested that the SCV eventually acidifies and fuses with the lysosomes, while others have stated that fusion with the lysosomes is prevented by *Salmonella* (61, 112). After this ~3 h lag period of SCV maturation, *Salmonella* starts expressing the SPI-2 genes to enable intracellular maintenance and growth.

### *Salmonella* Pathogenicity Island 2

The proteins encoded by genes of SPI-2 are effectors or structural proteins that form a second TTSS involved in intracellular survival and replication. SPI-2 encodes 31 genes that are organized in four operons involved in production of structural components and several effector proteins such as SseB, SseC, and SseD (reviewed in (173)). In addition to these structural and effector proteins SPI-2 encodes at least three chaperone proteins called SscA, SscB and SseA (173). The SPI-2 TTSS is involved in virulence of *S. enterica* serovar Typhimurium and is activated once the bacterium is inside the cell (21) (83, 126)and facilitates intracellular bacterial replication and in vivo virulence (21, 63, 120). SPI-2 has been shown to be involved in many processes including inhibition of fusion of the *Salmonella*-containing vacuole (SCV) and the lysosomes (SpiC) and evasion of NADPH

oxidase dependent superoxide production, all of which are involved in prolonged intracellular survival and replication (48, 163, 168).

## Host (mouse) immune response to *Salmonella*

In the host's defense against *Salmonella* several processes play a role. During the first three phases of *Salmonella* infection the innate immune system plays an important role in containing the extra- and intracellular growth. The fourth phase of infection, the elimination phase, is mediated by the adaptive immune response that leads to the T and B cell-mediated killing and elimination of *Salmonella*.

### Innate immune response

The innate immune response involves aspecific defense mechanisms that are not acquired upon exposure, but are constitutively present. Initial innate immune responses involved in defense against *Salmonella* include gastric acid, antimicrobial peptides (reviewed in (123)), complement (172), opsonins (25, 71), cytokines (reviewed in (174)) and lysozyme. Upon adhesion and invasion of the macrophages or granulocytes, *Salmonella* encounters innate defense mechanisms used by these phagocytic cells to resist infection such as antimicrobial defense mechanisms inside the phagosomes (including low pH, nitrites, oxygen radicals, nitric oxide, and antimicrobial peptides such as defensins, cathelicidins, and thrombocidins), and the secretion of cytokines and chemokines such as IL-1β, IL-6, GM-CSF, MIP-1β, and melanocyte growth stimulating factor (175). Cellular innate immunity is initiated by the recognition of bacterial components called Pathogen-associated molecular patterns (PAMPs) that are recognized via pattern recognition receptors, leading to the induction of an innate response to kill and eliminate *Salmonella* (53). Cells expressing such pattern recognition receptors that are involved in innate defense against *Salmonella* include neutrophils, macrophages/monocytes, NK cells, and dendritic cells (DC's) (53). These cells are involved in the engulfment and killing of *Salmonella*, antigen presentation, and production of cytokines and chemokines in response to the infection. These mechanisms act together to kill and eliminate *Salmonella* and to prevent systemic infection.

### Major cell types involved in innate immune response to *Salmonella*

Neutrophilic granulocytes (neutrophils) are phagocytic cells that are necessary for the initial destruction of *Salmonella* and are also involved in the lysis of hepatocytes that are invaded by *Salmonella* during the first few days of infection (26, 27). Neutrophils are efficient in killing *S. enterica* serovar Typhimurium as these cells are capable of producing large amounts of damaging antimicrobial products like lysozyme and radicals such as superoxide. Especially when bacteria have been opsonized with serum components (complement, antibodies) bacteria are taken up at a high rate and are killed efficiently.



Macrophages/monocytes are phagocytic cells that play a crucial role in innate defense. Monocytes circulate in the blood while macrophages reside in the tissues. Macrophages are mainly activated by T cells, but may also be stimulated upon infection by live bacteria or upon contact with PAMPs (including LPS, porins and outer membrane proteins, fimbrial proteins, flagella, lipoproteins, glycoproteins, and peptidoglycan) (62). Macrophages have evolved mechanisms to respond to such PAMPs by expressing pattern recognition receptors that recognize the PAMPs and initiate the innate immune response to clear the infection (157). Macrophages that are of special interest in *Salmonella* infection are the Kupffer cells in the liver and macrophages in the spleen since these are in the target organs of *S. enterica* serovar Typhimurium. Macrophages play a special role in *Salmonella* infection, since they are crucial in innate defense against *Salmonella* but also act as a Trojan horse to mediate spread to the to the liver and spleen (84). The innate defense mechanisms of macrophages are mainly stimulated by IFN$\gamma$ and TNF$\alpha$ that activate the macrophages in such a way that they should be able to kill and eliminate the intracellular *Salmonella*. However, despite the multitude of antimicrobial defense systems that are present in these phagocytic cells as part of the innate defense system, *Salmonella* has developed mechanisms to resist such killing and for some time is able to survive and even replicate within these cells.

DC's are antigen-presenting cells that have been shown to contain intracellular *S. enterica* serovar Typhimurium, especially those in the Peyer's patches of the small intestine after oral infection (158). After phagocytosis of *Salmonella*, these cells are capable of presenting *Salmonella* antigen to T and B cells leading to the development of an adaptive immune response.

**Innate resistance/susceptibility**

Susceptibility to *S. enterica* serovar Typhimurium in mice is determined by several factors as many processes play a role in the defense against *Salmonella.* One major factor contributing to resistance is the *Ity* (for immunity to *S. typhimurium*) locus. *Ity* controls the growth rate of *Salmonella* in cells of the reticuloendotheial system and is present in two allelic forms, resistant alleles ($Ity^r$) and susceptible alleles ($Ity^s$) and. Mice expressing the $Ity^r$ alleles are relatively resistant to *Salmonella* infection as they can control growth of the bacteria. Those expressing the $Ity^s$ alleles show an increased growth rate of *Salmonella* and cannot control the infection and are therefore susceptible. *Ity* appeared to be identical to *Bcg* and *Lsh*, two loci that were discovered to be involved in resistance to *Mycobacterium* and *Leishmania* respectively (128, 150). This locus plays an important role in regulating innate resistance to these pathogens that is mediated by macrophages. By positional cloning it was revealed that *Ity*/*Bcg*/*Lsh* is homologous to the human *Nramp1* (natural resistance associated macrophage protein 1) (169). *Nramp1* is a gene locus on chromosome 1 that is only expressed in macrophages in the reticuloedothelial organs (spleen and liver) and by the macrophage cell lines J774A and RAW264.7 (55, 170) and encodes a divalent metal ($Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$) pump phosphoglycoprotein (90–100 kDa) that is rapidly recruited to the bacteria-containing phagosome (170, 171) and is involved in

resistance against *Salmonella* (127, 169). Expression of *Nramp1* is greatly increased by activation of macrophages with IFNγ and LPS (55). Mice expressing the resistant allele of *Nramp1* (*Nramp1*$^{G169}$) can control the growth rate of *S. enterica* serovar Typhimurium in vivo, allowing the development of an acquired, predominantly, T cell-mediated immune response, which is essential for the eventual clearance of *S. enterica* serovar Typhimurium (66, 98, 99). However, mice expressing the sensitive allele *Nramp1*$^{D169}$ cannot control the growth rate of *S. enterica* serovar Typhimurium and will die due to the infection.

Another locus involved in resistance against *Salmonella* is *lps*. Two alleles of the *lps* gene have been assigned; *lps*$^{n}$ (responsive) *lps*$^{d}$ (hyporesponsive). Mice expressing the *lps*$^{d}$ allele do not mount an immune response upon injection with LPS and are hypersusceptible to infection with *S. enterica* serovar Typhimurium while those expressing the *lps*$^{n}$ allele do mount an immune response and are resistant to infection. The *lps* gene appeared to be identical to the Toll-like receptor 4 (*tlr4*) gene (129). This *tlr4* gene encodes an important part of the LPS receptor complex and is part of the TLR family of pattern recognition receptors involved in innate immunity. TLR4 is expressed by all cells of the immune system as well as by several non-immune cells and activation of TLR4 by LPS has been shown to lead to the production of cytokines, chemokines, and NO (85, 114) (167). Null mutations in *tlr4*, as seen in C3H/HeJ mice, lead to hyporesponsiveness to LPS and cause these mice to be hypersusceptible to *S. enterica* serovar Typhimurium and other Gram-negative bacteria (68, 118, 119, 129).

**Adaptive immune response**

Eventually, in immunocompetent (ity$^{r}$) mice an adaptive immune response is generated that is mediated by T and B cells to completely eliminate *Salmonella* from the body (99, 102, 109). B cells that recognize a certain antigen presented by DC's or macrophages will mature and respond to the *S. enterica* serovar Typhimurium infection by the production of antibodies (109). Mice with reduced B cell function are susceptible to infection with virulent *S. enterica* serovar Typhimurium infection, but are able to resist infection with an attenuated Δ*aroA* mutant suggesting that other defense mechanisms play a more important role (109). It is of no doubt that antibodies produced by B cells do play an important role in clearing the infection, but despite the presence of these antibodies, reactivation of the infection can occur when IFNγ is neutralized (110), suggesting that the production of antibodies by B cells is not enough to completely eliminate the bacteria from the body. For complete elimination of primary infection and protection against secondary infection with *S. enterica* serovar Typhimurium, T cells, especially CD4$^{+}$ T cells, are absolutely necessary since depletion of T cells early in infection leads to a very severe and life-threatening infection (113) and infection of nude mice or MHCII deficient mice cannot even clear the attenuated Δ*aroA* mutant (66, 149). CD8$^{+}$ T cells also play a role in defense against *Salmonella* infection although little is known about their exact function (91, 98, 99).

## Reactive oxygen intermediates (ROI)

One of the major early defense mechanisms against microorganisms is the production of toxic superoxide by the phagocyte NADPH oxidase and the subsequent generation of superoxide derivatives, both in vitro (97) and in vivo (147, 148, 166). Reactive oxygen intermediates (ROI) play an important role by targeting vulnerable lipid proteins, certain enzymes, and DNA (70, 82), thereby damaging the bacteria. ROI play a crucial role in *Salmonella* infection since mice deficient in a functional NADPH oxidase system are highly susceptible to infection (97).

### Sources of ROI

Every aerobically growing organism is exposed to ROI formed as a by-product of respiration. Therefore part of the mechanisms that *Salmonella* has evolved o cope with superoxide stress is aimed at fighting endogenously formed ROI. Under experimental conditions, superoxide stress can be generated by addition of redox-cycling agents such as menadione and paraquat, which raise the intracellular levels of ROI.

Upon invasion of macrophages, *Salmonella* is exposed to large amounts of superoxide in its direct environment, generated by the anti-microbial defense mechanism of the eukaryotic cell. ROI formed by the NADPH-oxidase upon contact with or uptake of *Salmonella* may cause microbial damage, and will ultimately lead to bacterial death, unless appropriate microbial defenses are activated. The phagocyte NADPH oxidase is composed of two membrane-bound components $gp91^{phox}$ and $p22^{phox}$, and four cytosolic factors, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and RacGTPase (phox for phagocyte oxidase). The active NADPH-oxidase is formed after recruitment and assembly of these components, resulting in the formation of cytochrome $b_{558}$ that accepts electrons from NADPH and donates them to molecular oxygen (reviewed in (4)). Thus, upon stimulation of the phagocyte with opsonized microorganisms or any other activating agent, the oxygen consumption increases dramatically (“respiratory burst”) and a large amount of superoxide is produced. Superoxide is believed not to pass over membranes, but it can diffuse through anion selective pores and will in this manner reach the periplasmic space of Gram-negative bacteria like *Salmonella*. Spontaneous or enzymatic dismutation of superoxide results in the generation of hydrogen peroxide, which is more reactive than superoxide and unlike this compound, can diffuse readily across cell membranes. Together with Fe(II), hydrogen peroxide can form hydroxyl radicals, which are an even more potent oxidant species.

Phagocytes also generate nitric oxide (NO) using the inducible nitric oxide synthase. Together with superoxide, the highly reactive and toxic peroxynitrite is formed. In murine models, the role of NO in anti-microbial defense is well established, but its importance in human defense is to date less clear, although NO can be produced by human macrophages (40). It also appears that production of superoxide and NO are separated in time. Superoxide is produced early, i.e., immediately after uptake of *Salmonella*, whereas NO is produced at a later stage (166).

**Chronic Granulomatous Disease**

The importance of the superoxide-mediated defense system is made evident by a rare inherited syndrome, chronic granulomatous disease (CGD), in which the patient's phagocytes fail to produce any superoxide. This leads to susceptibility to life-threatening microbial infections in these patients, mainly by *Staphylococcus aureus*, *Aspergillus* species, *Candida* species, *Pseudomonas* species, and *Salmonella* species (90). These infections can cause lymphadenitis, pyodermia, pneumonia, skin abscesses, and hepatic abscesses. CGD can be caused by mutations in either one of the genes encoding $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, and $gp91^{phox}$ of the NADPH oxidase complex (31, 139, 162). CGD affects about 1 in 500,000 individuals and 60% of these cases show an X-linked deficiency in $gp91^{phox}$ resulting either in absence, inactivity, or reduced activity of the protein. Approximately 40% of the patients have autosomal recessive deficiencies and lack $p47^{phox}$ (±30%), $p67^{phox}$ (±5%), or $p22^{phox}$ (±5%) (19, 22, 30, 34, 138, 151).

**Oxidative damage**

Superoxide radicals inactivate iron-sulfur clusters present in bacterial enzymes, for instance, enzymes involved in branched amine acid synthesis (82). Therefore, superoxide dismutase mutant strains are auxotrophic for branched amino acids. As a result of iron-sulfur cluster damage, iron is released into the cytosol (Fig. 3). Hydrogen peroxide can cause damage to membranes, enzymes and DNA directly, however, in conjunction with iron, hydroxyl radicals are formed in the Fenton reaction. Hydroxyl radicals are highly reactive and will not diffuse over log distances but cause damage at the site of production. Fe(II) is present in the backbone of DNA and it is likely that most of the cell death that occurs secondary to hydrogen peroxide exposure is caused by DNA damage via hydroxyl radicals (reviewed in (70)). DNA repair mechanisms are therefore crucial for *Salmonella* in order to cope with ROI. Their relative importance is exemplified by the fact that RecA mutants are attenuated in vivo whereas catalase mutants are not (15). The amount of available iron in host cells is limited and bound to a range of host proteins including transferring, lactoferrin, hemoglobin, ferritin and cytochromes. Bacteria have evolved elaborate, high-affinity mechanisms to ensure uptake of sufficient iron even in this environment. Therefore, intracellular iron levels must be tightly controlled. Genes involved in the uptake of iron are regulated by *fur*, the ferric iron uptake repressor. When complexed to Fe(II) Fur generally represses the iron uptake genes. The *fur* regulon will only be expressed when the amount of iron is limiting and Fe(III) needs to be taken up from the environment. Just how toxic a role iron can play in oxidative damage mediated by hydrogen peroxide, is illustrated by the fact that chelation of intracellular Fe(II) protects the bacteria from killing by hydrogen peroxide. In addition, superoxide dismutase mutants are more sensitive to hydrogen peroxide-mediated killing, presumable due to increased complexed intracellular iron levels in these mutants (101). Interestingly, catalase does not have an effect on this process, indicating that not the actual hydrogen peroxide concentration but the intracellular level of Fe(II) is rate limiting in this process (101). Recently, it was also shown that Fur complexed to Fe(II) can enhance the expression of



several proteins including the cytoplasmic iron superoxide dismutase (SodB) (35, 94). This positive regulation by Fur is mediated by a small anti-sense RNA encoded by *ryhB*, which is regulated by *fur*. This small anti-sense RNA is expressed under iron limitation and inhibits expression of iron-storage genes.

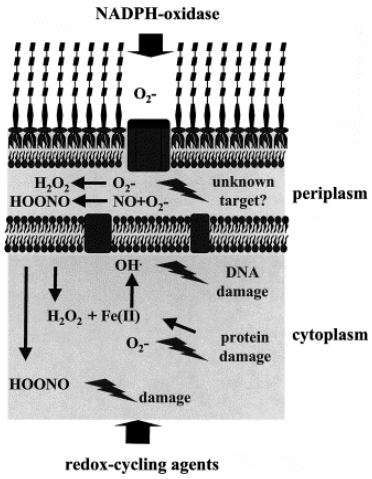



**Figure 3.** Schematic overview of superoxide-mediated damage. Superoxide produced by the NADPH-oxidase passes the outer membrane and may cause damage to periplasmic targets or can be converted into hydrogen peroxide, which will pass the cytoplasmic membrane. In conjunction with Fe(II), hydroxyl radicals are formed that cause DNA and protein damage. Superoxide and NO will form peroxynitrite, which can pass over membranes and cause damage. Superoxide produced as a by-product of respiration or generated by redox-cycling agents may cause damage to [Fe-S] clusters, resulting in the release of Fe(II) which, in conjunction with hydrogen peroxide, will form hydroxyl radicals.

The genes regulated by this anti-sense RNA include two genes encoding enzymes in the tricarboxylic acid cycle, *acnA* and *fumA*, two ferritin genes, *ftnA* and *bfr*, and *sodB* (94). Therefore, under high-iron conditions, *fur* repression not only leads to decreased expression of proteins involved in iron uptake, but also to increased expression of proteins involved in binding iron in the cytoplasm of bacteria. Taken together, these data show that there is a complex interplay between genes involved in defense against oxidative stress and genes involved in controlling intracellular iron levels.

## Defense mechanisms of *Salmonella* against ROI

Exposure of *Salmonella* to ROI results in extensive alterations in protein expression patterns. Although genome-wide transcriptional profiling of the response of *Salmonella* has not been evaluated, exposure of *Escherichia coli* (*E. coli*) to superoxide stress has shown that the expression of a total of 112 genes was modulated by exposure to the redox-cycling agent paraquat (130). Approximately 60% of the genes were upregulated and 40% down-regulated under these conditions (130). Similar experiments performed with hydrogen peroxide have shown that under these conditions, 140 genes are induced by *E. coli* (178). These data indicate that the defense against ROI involves complex mechanisms and, although insight into ROI defense mechanisms has increased extensively over the past decade, the exact function of many of the genes whose expression is modulated under oxidative stress is still unknown.

**SoxR/S system**

Exposure of *E. coli* or *Salmonella* to elevated levels of intracellular superoxide results in activation of the SoxR/S regulon (58, 131). This regulon is composed of at least ten genes with diverse functions (reviewed in (41, 156)). For instance, the cytoplasmic superoxide dismutase, which can neutralize superoxide, is regulated by the SoxR/S system. Other genes regulated by this system include those involved in uptake of superoxide or oxidizing compounds (e.g. *micF* which regulates the expression of pore protein OmpF), those involved in maintenance of the cellular redox state (e.g., the *zwf*-encoded glucose-6-phosphate dehydrogenase) and those involved in protection against superoxide-induced damage. The latter group includes genes involved in the repair of DNA damage, e.g. *nfo*, encoding an endonuclease, and genes involved in repairing damaged iron-sulfur-cluster-containing proteins. In addition, *fur*, the ferric uptake repressor is regulated by the SoxR/S system (177). SoxR is a constitutively expressed transcription factor whose activity is regulated by reduction or oxidation of its iron-sulfur cluster (reviewed in (156)). When this cluster is in a reduced state, the transcription of factor SoxR is inactive. Oxidation of the iron-sulfur cluster in conditions of oxidative stress result is in a conformational change of the protein, leading to its activation. Activated SoxR is a transcription factor whose only known target gene is *soxS* which in turn will activate the whole regulon.

**OxyR system**

The OxyR system is activated upon exposure to hydrogen peroxide, and the activation of this transcription factor also involves oxidation of the tetrameric protein (reviewed in (41, 156)). In this case, oxidation of the cysteine residues in this complex results in the formation of di-sulfide bridges. Only this oxidized form of OxyR is mediated by glutathione. The genes activated by OxyR include that of a cytosolic catalase, KatG, that can inactivate hydrogen peroxide, the glutathione reductase, glutaredoxin, and genes involved in protection of DNA and RNA against oxidative damage (reviewed in (156)). Fur, the ferric uptake repressor is regulated by both the SoxR/S and the OxyR system (177). This is not surprising given the role in intracellular iron in damage caused by ROI.

**Other regulators of superoxide sensitivity**

Several genes involved in resistance against superoxide or hydrogen peroxide are not regulated by the SoxR/S or OxyR system. For instance *katE*, encoding another cytosolic catalase is regulated by *rpoS*, the sigma factor involved in stationary-phase survival. RpoS is not only involved in responses of *Salmonella* to oxidative stress but also in responses to carbon starvation and acid stress (145). The transcriptional regulator, SlyA, positively and negatively regulates the expression of a set of unknown genes. Mutants in *slyA* are highly sensitive to superoxide-generating agents, indicating that some of the genes regulated by SlyA are involved in the oxidative stress response (13). The same holds true for the global regulator ArcA, since *arcA* mutants are also more sensitive to ROI (92).



**Other genes**

Recently, Gralnick et al. (57) identified the YggX protein and proposed that this protein is involved in blocking superoxide damage to [Fe-S] clusters, since an overexpression mutant is more resistant to redox-cycling agents. This protein is not controlled by the SoxR/S system, indicating that genes involved in prevention of damage to proteins can also be found outside this regulon.

In addition to cytoplasmic superoxide dismutases, *Salmonella* and *E. coli* also contain periplasmic superoxide dismutases, *S. enterica* serovar typhimurium and several other *Salmonella* strains express even two of these copper-zinc superoxide dismutases, designated SodCI and SodCII (39). In addition, a putative SodCIII protein has been identified in *Salmonella* (44). *E. coli*, on the other hand, only contains the sodCII gene. These periplasmic superoxide dismutases protect against extracellular superoxide, for instance, that were produced by the macrophages' NADPH-oxidase (32, 42). To date, no clear role of SodCIII in the defense against oxidative stress has been established.

Recently, the *mntH* gene, the *E. coli* and *Salmonella* homologue of NRAMP, was identified as being important for resistance against ROI. In *E. coli* and *Salmonella*, NRAMP homologue is a divalent metal ($Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$) pump phosphoglycoprotein able to transport manganese, and intracellular manganese is thought to be able to neutralize hydrogen peroxide (79). As a result, *mntH* mutants are more sensitive to hydrogen peroxide. However, an important role for this gene in virulence has not been established (79). This indicates that metals other than iron are involved in the ROI defense.

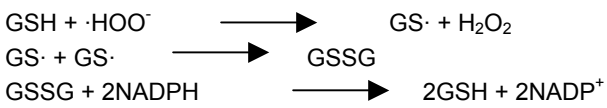
**Protection against oxidative damage**

The genes involved in defense of *S. enterica* serovar Typhimurium against oxidative stress do not all act the same. Some proteins may directly scavenge the oxygen species while others act by producing antioxidants. The cytoplasmic SOD's encoded by *sodA* and *sodB* and the periplasmic SOD's encoded by *sodCI* and *sodCII* are enzymes that catalyze the reaction in which superoxide radicals are converted to oxygen and hydrogen peroxide as follows:

$$SOD_{oxidized} + \cdot O_2^- \longrightarrow SOD_{reduced} + O_2$$
$$SOD_{reduced} + \cdot O_2^- + 2H^+ \longrightarrow SOD_{oxidized} + H_2O_2$$

The hydrogen peroxide produced in this reaction might be damaging to the cells, but *S. enterica* serovar Typhimurium has catalases encoded by *katE* and *katG* to neutralize it. In addition, peroxidases might also play a role in destroying hydrogen peroxide in a NADH- or NADPH-dependent manner.

Glutathione (GSH) is an antioxidant that is synthesized by glutathione synthetase that is very important in the defense against hydrogen peroxide, superoxide radicals, and hydroxyl radicals. GSH reacts with these radicals to form a stable glutathione radical GS· which will dimerize to form oxidized glutathione (GSSG). Glutathione reductase then transfers an electron from NAPDH to the GSSG leading to the re-formation of the reduced GSH $NADP^+$.




Another type of defense against oxidative stress is repair of induced damage. Oxygen radicals can cause cell, protein or DNA damage. Therefore, *S. enterica* serovar Typhimurium has mechanisms to repair the damage (reviewed in (41)). Examples include RecA, a protein encoded by *recA* that is involved in the recombinational DNA repair pathway important for cell survival upon exposure to hydrogen peroxide (15, 16), as well as endonuclease IV encoded by *nfo* and exonuclease III encoded by *xth* involved in excision repair (159). In conclusion, defense mechanisms of *S. enterica* serovar Typhimurium against oxidative stress are diverse and complex since many genes are involved and compensatory systems as well as systems overlapping with other types of stress defense systems have been described. Further research on *S. enterica* serovar Typhimurium genes that are involved in superoxide stress is necessary for better knowledge on the response to one of the most powerful defense mechanisms of the host: oxidative stress.

## Scope of this thesis

One goal of this thesis was to gain more insight into the mechanisms by which *S. enterica* serovar Typhimurium is able to persist and reactivate at a later timepoint. This was done by investigating the possibility of reactivation in a mouse model of latent *S. enterica* serovar Typhimurium infection and determining which mechanisms are involved in prevention of growth during the phase of persistence. The other goal was to get more insight into the strategies that are used by *S. enterica* serovar Typhimurium to survive within macrophages and mice and to resist superoxide produced by the macrophages in response to infection with the pathogen. Chapter 1 gives an overview of what is currently known about *Salmonella*, the interaction with the host, and systems that play a role in the defense against superoxide and in survival within macrophages.

Chapter 2 describes a novel in vivo mouse model for reactivating *S. enterica* serovar Typhimurium infection to elucidate which mechanisms are involved in persistency and reactivation at a later time point. Since depletion of $CD4^+$ T cells and neutralization of IFN$\gamma$, as shown by others, resulted in reactivation of the *S. enterica* serovar typhimurium infection we investigated in Chapter 3 whether neutralization of TNF$\alpha$, another very potent activator of macrophages and a cytokine shown to be involved in suppression of growth early in infection, was able to cause reactivation of the *S. enterica* serovar typhimurium infection as well.

In the research on persistency and survival of *S. enterica* serovar Typhimurium within host cells, we have created and selected for mutants that were less able to induce cell damage and were able to survive for a longer period of time within RAW264.7 macrophages. Chapter 4 deals with these LPS mutants and shows that mutants with the

rough phenotype are attenuated, although able to survive within macrophages in vitro and to cause a local infection in the lymph nodes.

To analyze the systems used by *S. enterica* serovar Typhimurium to resist superoxide stress we have created many mutants by random MudJ transposon insertion and selected for increased susceptibility to superoxide as described in Appendix 1. One of the mutants obtained in this way was studied in further detail and has been described in Chapter 5. This mutant AVD101 is a mutant in which the MudJ transposon had inserted into the promotor region of pnp, the gene encoding PNPase which is involved in the degradation of mRNA and in the growth adaptation at low temperatures and is considered a regulator of virulence and persistence of *S. enterica* serovar Typhimurium. We have described an additional role for PNPase in the resistance to superoxide and for intracellular survival within macrophages.

In Chapter 6 we describe the isolation and characterization of DLG294, an *S. enterica* serovar typhimurium mutant that has a MudJ transposon insertion in a gene designated *sspJ* that has rendered this mutant hypersusceptible to superoxide and to be attenuated in vitro. The phenotype of this *sspJ* mutant was further studied in Chapter 7. We have determined the in vivo phenotype of this mutant in C3H/HeN and C57BL/6 mice and in $p47^{phox-/-}$ mice that were unable produce any superoxide. To find out whether the in vitro attenuation of DLG294 was due to differences in the state of activation of the macrophages we compared the gene expression profiles of RAW264.7 macrophages that had been infected with the wild-type strain or DLG294 using Affymetrix gene chips and is described in Chapter 8. Since we did not observe many differences in gene expression profiles of the wild-type and DLG294-infected cells, we concluded that the difference in outgrowth in RAW264.7 cells must have been due to the lack of expression of *sspJ*. Because it was still unclear why DLG294 was attenuated in RAW264.7 macrophages we have studied the phenotype DLG294 in Chapter 9 using a phenotypical array and have studied the intracellular behavior of DLG294 by looking at the intracellular gene expression profile of the intracellular bacteria and have compared that to that of the wild-type strain using a *Salmonella* gene array. Finally, the results are summarized and discussed in Chapter 10.

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chapter two

# Gamma irradiation or $CD4^+$ T cell depletion causes reactivation of latent *Salmonella enterica* serovar Typhimurium infection in C3H/HeN mice

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## Abstract

*Salmonella* is a gram-negative pathogen that can cause a range of diseases in both animals and man varying from mild diarrhea to severe disseminated infections like typhoid fever. Upon infection, a host develops an immune response to limit bacterial growth and kill and eliminate the pathogen. *Salmonella* has evolved mechanisms to evade the host immune system and remain dormant within the body, only to reappear (reactivate) at a later time point when the immune system is abated. The exact mechanism by which *Salmonella* is able to persist is unknown, but evading the immune response likely plays an important role. We have developed an in vivo model for studying reactivation of *Salmonella enterica* serovar Typhim*urium* infection in mice. Upon subcutaneous infection, C3H/HeN ($Ity^r$) mice showed an increase in bacterial numbers in livers and spleens reaching a peak on day 19. After full recovery of the infection, these mice were irradiated or depleted for $CD4^+$ T cells. The mice showed a decrease in numbers of leukocytes and $CD4^+$ T cells, respectively, and displayed a secondary infection peak in livers and spleens with a course similar to the primary infection. From this we concluded that $CD4^+$ T cells are involved in active suppression of *S. enterica* serovar Typhimurium during persistency.

The role of $CD4^+$ T cells during primary infection with *S. enterica* serovar Typhimurium is well established. This is the first study to describe a role of $CD4^+$ T cells during the phase of latent *S. enterica* serovar Typhimurium infection.

## Introduction

*Salmonellae* are Gram-negative, facultative intracellular pathogens that can cause a range of diseases in both animals and man varying from mild diarrhea to severe disseminated infections such as typhoid fever. Like other intracellular bacteria, *Salmonella* has the capacity to adhere to host cells and induce its own uptake, even by nonprofessional phagocytes. Natural infection with *Salmonella* occurs through the intestinal tract. Once *Salmonella* has survived the antimicrobial mechanisms of the gastrointestinal tract, e.g. low pH, normal intestinal flora, intestinal mucus layer, and has reached the lining of the intestine, *Salmonella* preferentially penetrates the M cells that transport the bacteria from the lumen to the underlying Peyer's patches, or alternatively, *Salmonella* passes the epithelial cells (14, 30). After passing the epithelial barrier *Salmonella* is taken up by phagocytic cells, especially macrophages. These cells are important components of the host innate defense and are equipped with a multitude of antimicrobial mechanisms. Following ingestion by macrophages, bacteria reside within phagosomes that fuse with lysosomes to form phagolysosomes in which the bacteria can be rapidly killed due to acidification and bacteriolysis (reviewed in (10)). However, despite this multitude of antimicrobial mechanisms present as part of the innate defense of phagocytic cells, *Salmonella* is able to enter, survive and even replicate within these cells and can cause chronic or persistent infections by evasion or disturbance of the host defense (15). This ability of *Salmonella* to enter and replicate within phagocytic cells is essential for its survival, as mutants unable to do so are avirulent (5). Although the exact mechanisms for intracellular survival of *Salmonella* after phagocytosis are still unknown, it has become clear that *Salmonella* responds to the specific host environment by expressing factors crucial for intracellular survival and for circumvention of the host defense systems (3, 6, 7, 15, 23).

Upon infection, the host will try to develop an immune response to limit bacterial growth and to eventually kill and eliminate the pathogen. B cells, T cells and macrophages are important for host resistance and their protective effects are mediated by several cytokines such as IFN$\gamma$, IL-12, and TNF$\alpha$ (4, 16, 19-21, 26). This integrated response results in activation of macrophages that in turn kill *Salmonella*. Although the macrophages are the main host cells, necessary for survival and replication of *Salmonella* within the host, and mediate the *Salmonella*-induced pathology, these same macrophages also play a crucial role in host defense against *Salmonella* (31). They are necessary for the early local control of infection, and, subsequently, for the induction of acquired immunity (11, 18) as well as for restriction of bacterial growth in immune mice (31). During a *Salmonella* infection, macrophages are mainly activated via TLR4 signaling and via several cytokines produced by NK cells and T lymphocytes.

Following full activation of the macrophages and the acquired immune system, the host should be able to eliminate and clear *Salmonella* from the body. However, *Salmonella* has evolved mechanisms to evade the host immune system, to persist reside within the body, and to reappear (reactivate) at a later time point. Several studies and case reports



have shown that patients who underwent total body irradiation or received an organ transplant and were treated with glucocorticoids or other immunosuppressive therapy, as well as patients suffering from HIV infection (1, 9, 12) or Interleukin 12 receptor β1 deficiency (27) can suffer from recurrent (systemic) infections with a *Salmonella* strain that persisted within the host.

By investigating the possibility of *S. enterica* serovar Typhimurium to persist and reactivate after immune intervention in a mouse model of latent *S. enterica* serovar Typhimurium infection, we aim to gain insight into the mechanisms by which *S. enterica* serovar Typhimurium is able to persist and to reactivate at a later time point. This study shows that total body irradiation or selective $CD4^+$ T cell depletion in C3H/HeN ($Ity^r$) mice that were infected with *S. enterica* serovar Typhimurium and had apparently cleared the bacteria form the organs, leads to reactivation of the *S. enterica* serovar Typhimurium infection. In addition, this is the first study that describes the role of $CD4^+$ T cells during persistent *S. enterica* serovar Typhimurium infection in mice.

## Materials and Methods

**Mice.** Six- to eight-week old female *Salmonella*-resistant ($Ity^r$) C3H/HeN mice were obtained from Harlan (Horst, The Netherlands). Mice were maintained according to the institutional guidelines. Water and food were given ad libitum. All studies involving animals were carried out in accordance with, and after approval of, the animal research ethics committee of the Leiden University Medical Center.

**Bacteria.** For in vivo infection experiments, single colonies of *S. enterica* serovar Typhimurium strain 14028s were grown overnight in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm). The overnight cultures were diluted in fresh LB medium and grown to the end of log phase and were then washed and diluted in sterile PBS. The CFU in the inoculum were determined by plating serial dilutions.

**Antibodies.** Monoclonal antibodies (mAbs) directed to mouse T cell surface antigen CD4 were obtained from the supernatant of cultured hybridoma GK1.5 (rat anti-mouse CD4, American Type Culture Collection (ATCC)). The hybridoma was cultured in protein-free hybridoma medium (Gibco). The supernatant was isolated and concentrated using a capillary dialyzer. The concentrated supernatant was filter-sterilized and, after determination of the protein concentration, stored at –20°C until use. FITC-conjugated rat anti-mouse CD4 (L3T4) and PE-conjugated rat anti-mouse CD8 (Ly-2) monoclonal antibodies were obtained from BD Biosciences.

***S. enterica* serovar Typhimurium infection.** Mice were inoculated subcutaneously in the flanks with 0.1 ml bacterial suspension containing $3 \times 10^4$ CFU *S. enterica* serovar Typhimurium 14028s. For each group on each time point 3-6 mice were used. Mice were sacrificed by carbon dioxide inhalation and blood was taken immediately by cardiac puncture. Part of the blood was prevented from coagulation by adding 40 U heparin/ml and another part was used to obtain serum. To determine the bacterial load within spleens, livers, and inguinal lymph nodes, these organs were aseptically removed and single cell suspensions were prepared by using sterile 70-µm-mesh-size cell strainers (Falcon). Cells were pelleted by centrifugation for 10 min and were lysed in distilled water. The bacterial number per organ was determined bacteriologically by plating serial dilutions. The lowest number of bacteria that can be detected in this way is 30 CFU for the spleens and lymph nodes and 50 CFU for the livers.

**Leukocyte count and blood cell differentiation.** Blood was obtained by cardiac puncture and 40 U heparin/ml was added to prevent coagulation. The number of leukocytes was determined by counting the number of nucleated cells in the blood and we used 5 µl blood to make blood smears for the differentiation of the blood cells. Blood smears were fixed in methanol and stained with Giemsa and were used to determine the relative percentages of the different types of cells in the blood. This, together with the total leukocyte numbers was used to calculate the number of lymphocytes, monocytes, and PMN present in the blood on the different time points. The number of lymphocytes was used together with the FACS analysis data, to calculate the number of $CD4^+$ and $CD8^+$ T cells in the blood on the different time points after infection.

**Gamma irradiation and in vivo T cell depletion.** Immune intervention was carried out either by sublethal total body gamma irradiation or in vivo depletion of $CD4^+$ T cells on a time point when bacteria could no longer be detected bacteriologically within livers, spleens, and inguinal lymph nodes. For total body gamma irradiation, mice were put in a small perspex box and were irradiated until a dose of 6 Gy was reached. $CD4^+$ T cells were depleted using 200 µg rat anti-CD4 GK1.5 antibody injected intraperitoneally. Mice received a second and a third injection with 100 µg of this antibody on day two and four after the first injection.

**Flow Cytometry.** To determine $CD4^+$ and $CD8^+$ T cell counts in blood, 100 µl heparinized blood was used. Before staining, erythrocytes were lysed on ice for 10 minutes using lysisbuffer (155 mM $NH_4Cl$, 100 mM $KHCO_3$, 10 mM EDTA). After washing with PBS, the cells were stained during 30 minutes with FITC-conjugated rat anti-mouse CD4 mAb and PE-conjugated rat anti-mouse CD8 mAb, respectively. Flow cytometry was performed on a FACSCaliber System (Becton Dickinson).



**Detection of *S. enterica* serovar Typhimurium-specific antibodies.** Induction of *S. enterica* serovar Typhimurium -specific antibodies was determined in a whole cell ELISA in which Maxisorp plates (Nunc) were coated with whole *S. enterica* serovar Typhimurium 14028s. Briefly, *S. enterica* serovar Typhimurium 14028s was grown ON in LB medium and after washing with PBS was resuspended in 0,5 mM $Na_2HPO_4$, pH 9.6. Maxisorp plates were filled with 100 μl of this suspension and were dried ON at 37°C. Non-specific binding was blocked by incubating the plates with 3% FCS in PBS for 2 h at 37°C. Serial dilutions of the sera were added to the wells. Sera from naïve mice were included as a control. The plates were incubated at 37°C for 2 h. After washing the plates four times with PBS-Tween (0.05%) per well 100 μl 1:10,000 diluted peroxidase-labeled goat-anti-mouse IgG antibodies (SouthernBiotech, Birmingham, AL35260, USA) were added and the plates were incubated at 37°C for another 2 h. Plates were washed again with PBS-Tween (0.05%) and substrate solution (0.11 M NaAc, 1 mg/ml TMB, 0.01% $H_2O_2$) was added to the wells. The coloring reaction was stopped by addition of $H_2SO_4$ and the wavelength absorbance was measured at 490 nm using an ELISA plate reader (VICTOR$^2$ 1420 multilabel counter, PerkinElmer Life and Analytical Sciences). Titers are defined as the dilution for which:

$$OD_{450} \text{ (sample)} > OD_{450} \text{ (naïve serum)} + 2 \times \text{standard deviation.}$$

**Calculation of bacterial growth.** The growth rate was determined according to the equation: k = [ln N(t peak) – ln N(day 1)] / t, in which N(t peak) is the number of viable bacteria per organ on the peak of infection after time t (min) has elapsed, and N (day 1) represents the bacterial number on day 1 after infection.

**Statistical analysis.** For comparison between treatments we used Student's *t* tests and for correlation analysis a Pearson correlation analysis was performed. For both analyses, a P value <0.05 was considered significant.

## Results

**Replication of *S. enterica* serovar Typhimurium 14028s during a primary infection in C3H/HeN mice.** Mice were inoculated subcutaneously in the flanks with $3 \times 10^4$ CFU *S. enterica* serovar Typhimurium 14028s. During the first 5 days after the infection, the body weight of the infected mice was comparable to that of the age-matched weight controls (Fig. 1A). The infected mice showed a decrease in body weight between days 12 and 19. During this period, some of the mice also showed signs of illness like ruffled fur and malaise. During the next week, the mice fully recovered and showed an increase in body weight. The infection with *S. enterica* serovar Typhimurium 14028s induced severe hepatosplenomegaly reaching a peak on day 19 (Fig. 1C and 1E). The isolated livers and spleens were weighed and were used to determine the pathology

induced by the *S. enterica* serovar Typhimurium infection. Pathology was calculated as the percentage organ weight of the total body weight as described before (28). The induction of pathology during the first 19 days after infection was similar for the spleens and livers as is shown by a statistically significant correlation between the induced liver and spleen pathology (R=0.763; P<0.001). The pathology induced by *S. enterica* serovar Typhimurium became less severe during the next week and eventually stabilized at 7.5% for the livers and 1-1.25% for the spleens.

Fig. 1B shows that *S. enterica* serovar Typhimurium 14028s was detectable in the lymph nodes already on day 1 after infection and was able to replicate reaching a peak on day 5 after infection. From there, the infection spread to the spleens and livers and within these organs bacterial loads up to $3 \times 10^7$ CFU per organ were reached (Fig. 1D and F). The bacterial loads were highest on day 19, which correlated with the induced pathology in the livers (R=0.590; p=0.001) and spleens (R=0.577; P=0.002). The bacterial loads eventually declined, which coincided with the increase in body weight and a reduction in spleen and liver pathology (Fig. 1A, 1C and 1E).

**Reactivation of the *S. enterica* serovar Typhimurium infection by gamma irradiation.** On day 41, when the bacterial loads in the organs were reduced below the detection limit, the mice received a sublethal total body irradiation (6 Gy). The infection control group was not treated. The irradiated mice showed signs of illness, like ruffled fur and malaise between day 54 and 61. Due to the irradiation, the spleenweight on average decreased by ~40% compared to the infection controls (0.18 g vs 0.33 g) and the liverweight showed ~8% reduction (1.86 g vs 2.03 g). The effects of the irradiation on the bacterial numbers in the organs are shown in Fig. 2. In the infection control group, 60% of the mice showed detectable amounts of bacteria in the livers (Fig. 2B), but the averages were around or below the detection limit and no increase in bacterial numbers could be observed. The bacterial numbers in the lymph nodes and spleens stayed below or around the detection limits (Fig. 2A and 2C).

In the irradiated mice population, on the other hand, we observed an increase in bacterial numbers of *S. enterica* serovar Typhimurium in the livers and spleens (Fig. 2B and 2C) upon immune intervention and in all the mice the infection peaked on day 54. This secondary infection (i.e. reactivation) was milder than the primary infection as is shown by lower maximal bacterial numbers in the organs reached, but otherwise followed a course that was similar to that of the primary infection peak (Table 2, Fig. 2). In the lymph nodes we also observed a slight increase in bacterial numbers on day 54 (Fig. 2A), although the growth rate differed from that of the primary infection.

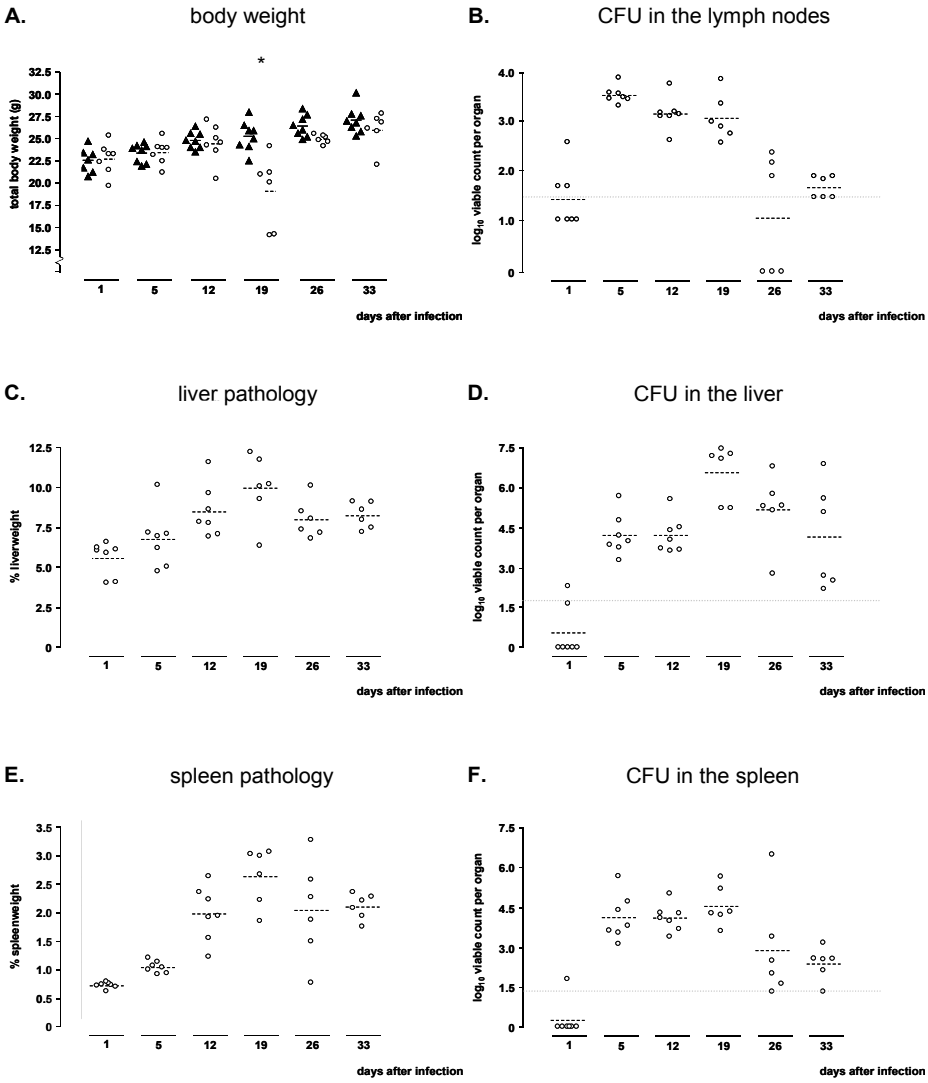


**Figure 1.** Total body weight (A), liver and spleen pathology (C and E) and bacterial numbers within the lymph nodes (B), livers (D), and spleens (F) of C3H/HeN ($Ity^r$) mice during a primary infection with *S. enterica* serovar Typhimurium 14028s (open circles). The crosses in A represent the total body weights of mice that were not infected with *S. enterica* serovar Typhimurium and that served as weight controls. Averages from 2 independently performed experiments are shown. On day 0, C3H/HeN ($Ity^r$) mice were infected subcutaneously in the flanks with ~$3 \times 10^4$ CFU *S. enterica* serovar Typhimurium 14028s. The actual dose was confirmed by plating serial dilutions of the inoculum. At the indicated time point, the livers, spleens, and lymph nodes were aseptically removed and weighed. The pathology in the livers and spleens was calculated as the percentage organ weight of the total body weight. The viable count within the organs was determined by making lysates and plating serial dilutions of the lysates (n=6-8 per group). The results are expressed as $\log_{10}$ viable counts (means $\pm$ standard errors of the means). Asterisks indicate statistically significant differences ($P<0.05$) compared to the infection controls (Students *t* test) and the gray dashed lines represent the detection limit of the microbiological method.

**Leukocyte counts in the blood.** On each time point we determined the leukocyte counts in the blood for each mouse and differentiated the types of blood cells present at the different time points after infection (Table 1). During the growth phase in the primary infection, there was a statistically significant correlation between time and the total number of leukocytes (R=0.544; p<0.001), meaning that the number of leukocytes increased during the primary infection peak. The number of leukocytes did not change further, but stabilized up to the day of treatment on day 41 (Table 1). Giemsa stained blood smears were used to determine the percentages of the different types of cells in the blood on the different time points after infection. The observed increase in the number of leukocytes was mainly due to the increase in the number of granulocytes (Table 1) whereas the number of lymphocytes and monocytes did not change during the primary infection as well as in the infection control group on the later time points. FACS analysis on the lymphocyte population revealed no change in the number and ratio of $CD4^+$ and $CD8^+$ T cells during the primary infection peak (data not shown).

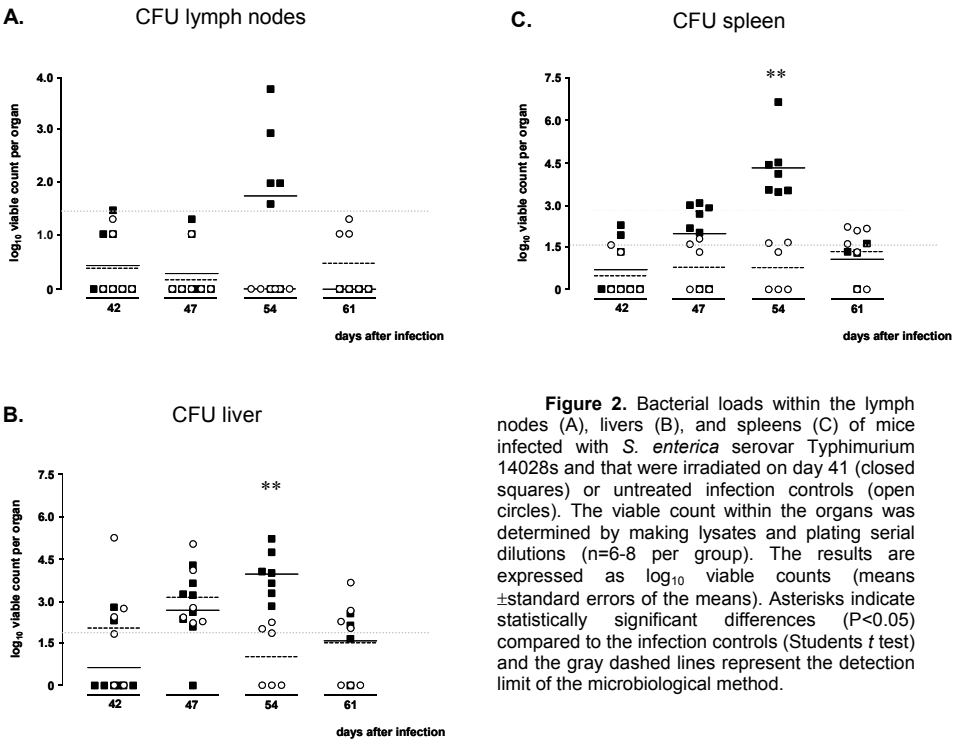


**Figure 2.** Bacterial loads within the lymph nodes (A), livers (B), and spleens (C) of mice infected with *S. enterica* serovar Typhimurium 14028s and that were irradiated on day 41 (closed squares) or untreated infection controls (open circles). The viable count within the organs was determined by making lysates and plating serial dilutions (n=6-8 per group). The results are expressed as $\log_{10}$ viable counts (means ±standard errors of the means). Asterisks indicate statistically significant differences (P<0.05) compared to the infection controls (Students *t* test) and the gray dashed lines represent the detection limit of the microbiological method.

**Table 1. Number of leukocytes, lymphocytes, monocytes, and granulocytes in the blood[a]**

| day | treatment | leukocytes ($n \times 10^5$) | lymphocytes ($n \times 10^5$) | monocytes ($n \times 10^5$) | granulocytes ($n \times 10^5$) |
|---|---|---|---|---|---|
| 1 | --- | 54.7 ± 8.3 | 38.7 ± 12.9 | 1.1 ± 0.5 | 14.3 ± 8.9 |
| 5 | --- | 85.1 ± 13.5 | 48.3 ± 9.4 | 1.8 ± 1.4 | 29.9 ± 7.3 |
| 12 | --- | 77.3 ± 24.4 | 30.4 ± 12.7 | 0.8 ± 0.7 | 42.3 ± 13.6 |
| 19 | --- | 100.7 ± 28.5 | 33.8 ± 13.8 | 2.8 ± 2.2 | 52.2 ± 13.1 |
| 26 | --- | 78.0 ± 13.4 | 39.3 ± 5.6 | 1.2 ± 1.0 | 33.8 ± 11.7 |
| 33 | --- | 110.8 ± 37.6 | 52.2 ± 10.3 | 1.8 ± 1.9 | 53.3 ± 29.2 |
| 42 | --- | 86.2 ± 29.7 | 53.7 ± 18.0 | 0.7 ± 0.7 | 27.9 ± 14.2 |
| 42 | irr[b] | 23.5 ± 9.4 | 7.4 ± 3.8 | 0.0 ± 0.0 | 15.5 ± 6.3 |
| 43 | depl[c] | 50.2 ± 11.3 | 26.3 ± 6.9 | 1.1 ± 0.9 | 21.6 ± 13.0 |
| 47 | --- | 87.8 ± 23.9 | 58.5 ± 20.6 | 1.0 ± 0.5 | 24.7 ± 7.6 |
| 47 | irr | 9.1 ± 4.4 | 5.1 ± 2.6 | 0.2 ± 0.2 | 3.1 ± 2.3 |
| 47 | depl | 61.1 ± 21.2 | 31.4 ± 11.8 | 1.5 ± 1.6 | 26.2 ± 17.1 |
| 54 | --- | 50.8 ± 29.0 | 29.0 ± 22.4 | 0.6 ± 0.7 | 19.3 ± 10.2 |
| 54 | irr | 10.6 ± 5.1 | 5.8 ± 3.0 | 0.1 ± 0.1 | 2.8 ± 3.4 |
| 54 | depl | 89.6 ± 21.1 | 39.0 ± 16.2 | 2.1 ± 1.4 | 39.0 ± 16.2 |
| 61 | --- | 94.6 ± 24.1 | 45.6 ± 14.3 | 1.0 ± 1.1 | 41.9 ± 27.4 |
| 61 | irr | 29.5 ± 7.6 | 8.7 ± 2.2 | 0.2 ± 0.2 | 15.8 ± 4.5 |
| 61 | depl | 55.0 ± 14.2 | 29.2 ± 8.5 | 0.7 ± 0.4 | 23.2 ± 11.9 |

[a] values are mean numbers/ml ± stdev
[b] mice that received a total body irradiation (6 Gy)
[c] mice that were depleted for CD4+ T cells by injection of anti-CD4 antibodies

The irradiated mice, however, showed reduced leukocyte counts in the blood already on day 1 after the irradiation (day 42) when compared to the untreated infection controls and the numbers remained lower up to day 61 after infection (Table 1). Table 1 also shows that the numbers of granulocytes declined in the irradiated mice and were lowest on day 47 and 54. On day 61 the number of granulocytes had not recovered fully yet, but showed an increase compared to day 54. The number of monocytes seemed to be decreased by the irradiation, but the numbers are too low to reach statistical significance. The number of lymphocytes, on the other hand, was reduced dramatically already on day 1 after the irradiation compared to the infection controls and remained significantly lower up until day 61. We used these lymphocyte numbers together with the FACS analysis data to calculate the number of $CD4^+$ and $CD8^+$ T cells and, as expected, observed that both the $CD4^+$ and

the $CD8^+$ T cells were reduced to the same extent in the irradiated mice compared to the infection controls (Fig. 3A and 3B). These data suggest that the observed reactivation of the *S. enterica* serovar Typhimurium infection upon irradiation could have been due to the reduction in the numbers of either granulocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, or a combination of cells.

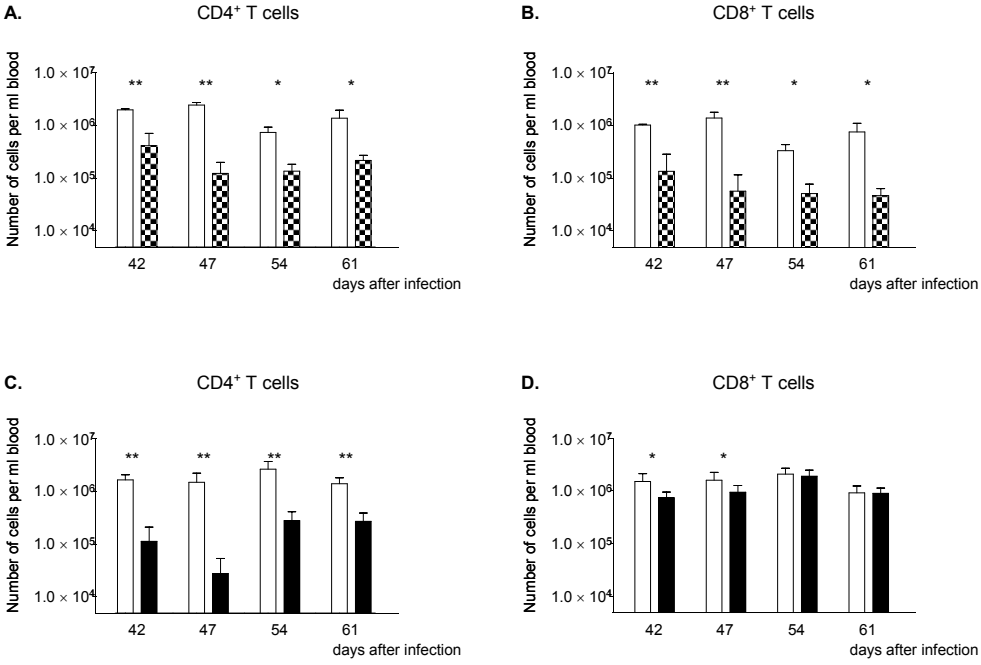


**Figure 3.** Number of $CD4^+$ T cells (A and C), and $CD8^+$ T cells (B and D) per ml blood of C3H/HeN mice infected with *S. enterica* serovar Typhimurium that received no further treatment (white bars), mice that received a total body irradiation (checkered bars), or mice that were depleted for T cells (black bars). The number of $CD4^+$ and $CD8^+$ T cells was calculated by combining data from leukocyte counts, cell differentiation, and FACS analysis.

**Reactivation following T cell depletion.** Reactivation of a latent *S. enterica* serovar Typhimurium infection in people has been described for patients suffering from AIDS. These patients showed reduced $CD4^+$ T cells counts and suffered from recurring infections with the same *S. enterica* serovar Typhimurium strain. This strongly suggests a role for the $CD4^+$ T cells in the suppression of *S. enterica* serovar Typhimurium during the persistence phase. Since the irradiated mice also showed a reduction in the numbers of $CD4^+$ T cells as well as the numbers of granulocytes and $CD8^+$ T cells, we wondered whether only reducing the number of $CD4^+$ T cells by in vivo depletion could also result in the reactivation of a latent *S. enterica* serovar Typhimurium infection in C3H/HeN mice. The primary infection and change in the number of leukocytes followed a course similar to that shown in Fig. 1 and Table 1, respectively (data not shown). In the infection control group

we observed no reactivation of the infection as the bacterial numbers stayed around or below the detection limits in all the organs up to day 61. In the lymph nodes of the mice that were depleted for $CD4^+$ T cells, we observed no detectable outgrowth of *S. enterica* serovar Typhimurium (Fig. 4A). In the livers and spleens, on the other hand, we observed an increase in bacterial numbers that was significantly different from those in the infection controls and this reactivation reached a peak on day 47 (Fig. 4B and 4C). As for the irradiated mice, this reactivation peak was lower than the peak observed for the primary infection, but followed a course that was similar to that of the primary infection peak, although the growth rate was slightly lower (Table 2). Of note, neither spleenweight nor liverweight significantly changed upon $CD4^+$ T cell depletion as the average spleen- and liverweights were 0.39 g and 2.03 g, respectively for the $CD4^+$ T cell depleted mice compared to 0.35 g and 2.10 g for the infection controls. Table 1 shows that the total number of leukocytes and lymphocytes slightly, though significantly, changed upon injection with the rat anti-CD4 antibody compared to the infection controls with an exception for day 54. FACS analysis on the lymphocyte population revealed a strong decrease in the number of $CD4^+$ T cells in the depleted mice indicating that the injection of the rat-anti CD4 antibody resulted in a successful depletion (~95% reduction on days 42 and 47) of the $CD4^+$ T cell population (Fig. 3C) and, as expected, had no effect on the number of $CD8^+$ T cells (Fig. 3D). From this we concluded that $CD4^+$ T cells are involved in the suppression of *S. enterica* serovar Typhimurium during persistency.

**Table 2. Growth rates of *S. enterica* serovar Typhimurium in the spleen, liver, and inguinal lymph nodes[a]**

| | liver ($hr^{-1}$) | spleen ($hr^{-1}$) | lymph nodes ($hr^{-1}$) |
|---|---|---|---|
| primary | 0.030 ± 0.003 | 0.038 ± 0.006 | 0.048 ± 0.004 |
| irradiation | 0.026 ± 0.005 | 0.030 ± 0.005 | 0.012 ± 0.004 |
| T cell depletion | 0.017 ± 0.018 | 0.013 ± 0.007 | ND |

[a] values are growth rates ± stdev
ND, not done
Growth rate = k
$k=[\ln N(t) - \ln N(t=0)] / t$

**Anti-*Salmonella* IgG antibodies in the serum of *S. enterica* serovar Typhimurium infected mice.** To determine whether the mice had antibodies to the pathogen, serum was collected from the mice that were sacrificed on each time point and anti-*Salmonella* IgG antibodies were detected using a whole cell ELISA. The infected mice showed no antibodies to *S. enterica* serovar Typhimurium on days 1 and 5, but started producing antibodies between day 5 and 12, when the infection reached its peak in the lymph nodes and spleens.

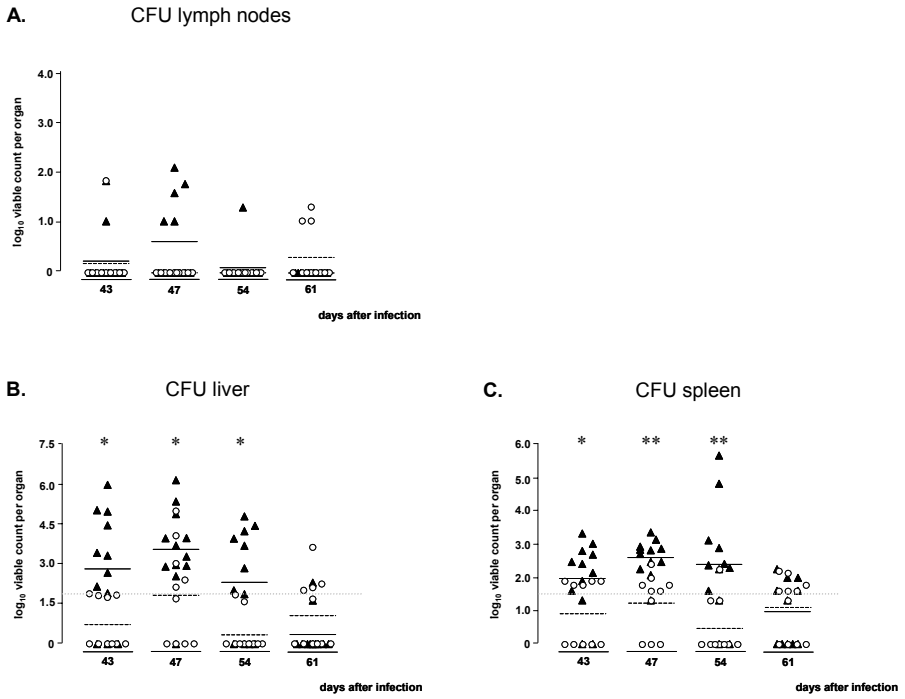


**Figure 4.** Bacterial loads within the lymph nodes (A), livers (B) , and spleens (C) of mice infected with *S. enterica* serovar Typhimurium 14028s and that were depleted for $CD4^+$ T cells (closed triangles) or untreated infection controls (open circles). On day 39 after infection, the mice were treated with 200 μg rat anti-CD4 GK1.5 and/or anti-CD8 2.43 antibodies injected intraperitoneally. Mice received a second and a third injection with 100 μg of these antibodies on day two and four after the first injection. The infection controls were injected intraperitoneally with an equal volume of PBS. At the indicated time points, livers, spleens, and lymph nodes were aseptically removed and weighed. The viable counts within the organs were determined by making lysates and plating serial dilutions of the lysates (n=6-8 per group) and are expressed as $\log_{10}$ viable counts (means ± standard errors of the means). Asterisks indicate statistically significant differences compared to the infection controls (Students *t* test).

The titers increased further until day 43 after infection and then remained around a $\log_3$ dilution factor of 8.5 (Fig. 5). The mice that were irradiated or depleted for $CD4^+$ T cells and that showed a reactivation of the *S. enterica* serovar Typhimurium infection had serum antibody levels that were similar to those of the infection controls. So despite the fact that these mice have serum antibodies to *S. enterica* serovar Typhimurium they still showed reactivation of the *S. enterica* serovar Typhimurium infection. To exclude the possibility that mice displaying the strongest reactivation of the *S. enterica* serovar Typhimurium infection showed lower serum levels of anti-*Salmonella* IgG, the serum antibody titers were plotted against the number of bacteria in the organs and showed no statistically significant correlation (data not shown). From this, we concluded that the observed reactivation of the *S. enterica* serovar Typhimurium infection was not due to a reduction in the serum antibody levels, but was due to the effects of the irradiation and depletion on the blood cells. The

observed reactivation of the *S. enterica* serovar Typhimurium infection in the irradiated mice might have been due to the reduction in the numbers of either granulocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, or a combination of cells, while in the depleted mice, the reactivation was due to a reduction in the number of $CD4^+$ T cells in the blood.

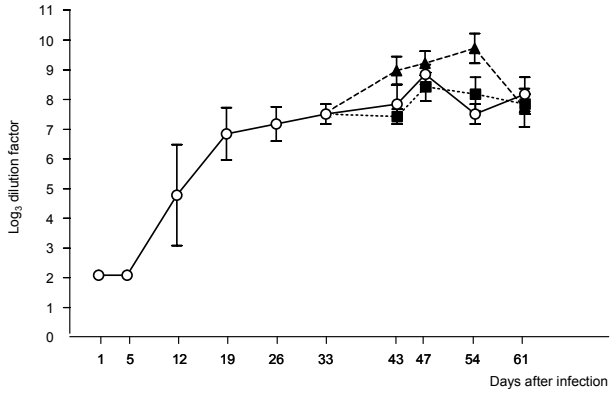


**Figure 5.** Anti-*Salmonella* IgG antibodies in the serum of *S. enterica* serovar Typhimurium infected mice that received no further treatment (open circles), received a total body irradiation (closed squares), or were depleted for $CD4^+$ T cells (closed triangles). Titers are defined as the $OD_{450}$(serum sample) > $OD_{450}$(naïve serum) + 2 × stdev.

## Discussion

The main findings of the present study on reactivation of *Salmonella* after clearance of a primary systemic infection in C3H/HeN ($Ity^r$) mice are that after total body irradiation or selective $CD4^+$ T cell depletion, the number of bacteria in the livers and spleens increased at a rate identical to that in the primary infection. The main difference between the two outgrowth curves was that upon reactivation, the *S. enterica* serovar Typhimurium infection appeared to be controlled more rapidly than in the primary infection, i.e. by about two instead of three weeks. The presence of high levels of *Salmonella*-specific antibodies did not prevent the reactivation, but could play a role in the differences in outgrowth rate between the irradiated and $CD4^+$ T cell depleted mice, since antibodies might act as opsonins for granulocytes which were not affected by $CD4^+$ T cell depletion.

We have used subcutaneous *S. enterica* serovar Typhimurium infection of C3H/HeN ($Ity^r$) mice in the inguinal region to set up a model for reactivation of *S. enterica* serovar Typhimurium infection. By infecting subcutaneously, a reservoir is established near the draining lymph nodes from where *Salmonella* readily spreads via the lymph stream and becomes systemic, finally reaching the liver and spleen (2). This model gives rise to a more subtle infection than the intraperitoneal, intravenous, or oral models that result in peracute and overwhelming infections. Another advantage over oral infection is that the subcutaneously injected bacteria can be dosed precisely, while in an oral infection, the actual dose depends on the amount of bacteria that pass the stomach. Using this subcutaneous infection model, we have been able to set up a new in vivo model for reactivation of latent *S. enterica* serovar Typhimurium infection in which total body

irradiation or in vivo depletion of $CD4^+$ T cells in C3H/HeN ($Ity^r$) mice that fully recovered from a primary infection with *S. enterica* serovar Typhimurium resulted in the outgrowth of bacteria that resided within the body. In our reactivation model we have accepted that some of the control mice showed bacterial numbers in the organs that were slightly above the detection limits. Otherwise, we would have needed many more animals to find only a few in which *S. enterica* serovar Typhimurium persisted and reactivated upon irradiation or T cell depletion.

Upon total body irradiation, the mice showed a reduction in both granulocyte and $CD4^+$ and $CD8^+$ T cell numbers and, as a result, they showed a strong reactivation of the *S. enterica* serovar Typhimurium infection. Reactivation of a latent *S. enterica* serovar Typhimurium infection in humans has been mainly described for patients suffering from AIDS (1, 8, 9, 13). These patients all showed strongly reduced $CD4^+$ T cells counts and suffered from recurring infections with the same *S. enterica* serovar Typhimurium strain. This strongly suggests a role for the $CD4^+$ T cells in the suppression of *S. enterica* serovar Typhimurium during the persistence phase. The studies describing reactivation of *Salmonella* infections in HIV infected individuals are all studies that were done before the introduction of the highly active antiretroviral therapy (HAART) (1, 8, 13) or were done in developing countries where patients receive no such effective therapies (9). HAART therapy has been shown to be very effective in restoring the $CD4^+$ T cell population in patients with AIDS, thereby reconstituting immunity. Hung et al have shown that the risk of recurrent non-typhoidal *Salmonella* bacteremia has decreased significantly by the introduction of HAART therapy in Taiwan (12). In our in vivo reactivation model by irradiation, the mice showed reduced numbers of both granulocytes and $CD4^+$ and $CD8^+$ T lymphocytes. We investigated whether reducing the number of $CD4^+$ T cells, as occurs in HIV infected individuals, could also result in a reactivation of (a latent) *S. enterica* serovar Typhimurium infection C3H/HeN mice. Therefore, we performed another set of reactivation experiments in which we depleted the mice for $CD4^+$ T cells. Like for the irradiated mice, these $CD4^+$ T cell depleted mice showed a reactivation of the *S. enterica* serovar Typhimurium infection in both the livers and spleens. Strikingly, this reactivation took place despite the presence of high anti- *S. enterica* serovar Typhimurium antibody titers (Fig. 5). This is consistent with the observation that protection against *Salmonella* requires both immune serum and T cells (22). The reactivation peak followed a course similar to that of the primary infection, although slightly less severe. This could be due to the presence of *S. enterica* serovar Typhimurium -specific antibodies that might suppress extracellular bacterial growth, but cannot prevent the bacteria from reactivating. A more likely explanation could be that these antibodies act as opsonins for granulocytes thus promoting granulocyte-mediated uptake and killing of *S. enterica* serovar Typhimurium (29). This is also consistent with the observation that the growth rate upon irradiation was similar to that of the primary infection peak while after T cell depletion it was lower, especially in the spleen (Table 2). If the antibodies, which are highly abundant (Fig. 5), favor granulocyte-mediated uptake and killing of *S. enterica* serovar Typhimurium, this would indeed reduce the growth rate in $CD4^+$ T cell depleted mice as compared to the irradiated mice since the



granulocytes disappeared upon irradiation, but were still present in the CD4 depleted mice (Table 1). Furthermore, the pool of phagocytic cells in the spleens would be lower in the spleens of the irradiated mice as was evident from a reduction in spleenweight (~40% reduction compared to the infection controls), while in the $CD4^+$ T cell depleted mice no significant reduction in spleen weight was observed. Since $CD4^+$ T cell depletion still resulted in reactivation, apparently, granulocytes cannot completely compensate completely for the absence of $CD4^+$ T cells.

Very recently, Monack et al. have described a model for chronic carriage of *S. enterica* serovar Typhimurium in $Ity^r$ mice (24). In contrast to our latent infection model in which bacteria could no longer be detected in the lymph nodes after 43 days, these mice show high numbers of bacteria in the mesenteric lymph nodes up until 268 days after oral infection and periodical fecal shedding as observed for chronic carriers of *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi in man and serves a good model for chronic carriage. They have shown that in their chronic carriers *S. enterica* serovar Typhimurium persisted within macrophages in the mesenteric lymph nodes. Apparently, these macrophages were not able to eliminate the pathogen. When IFNγ the most potent activator of macrophages produced by T cells, was neutralized in this in vivo chronic carriage model, these mice showed reactivation of the *S. enterica* serovar Typhimurium infection (24). This indicates that IFNγ plays an essential role in the control of chronically persistent *S. enterica* serovar Typhimurium infection, but is not able to activate the macrophages in such a way that they can completely eliminate the pathogen as the bacteria persisted in the macrophages of the mesenteric lymph nodes from which they could spread to the environment. IFNγ is produced by NK cells and by activated $CD4^+$ and $CD8^+$ T cells in response to cytokines like IL-12 and IL-18 that are produced by infected macrophages (19, 20). IFNγ then acts together with bacterial components like LPS to activate the macrophages to display maximal capacity to kill the pathogen. In the reactivation model described by Monack et al. they have neutralized IFNγ and thereby have prevented the activation of the infected macrophages by all these types of IFNγ producing cells, resulting in the reactivation of the *S. enterica* serovar Typhimurium infection. In our reactivation model of latent infection, however, we have depleted the mice for the $CD4^+$ T cells and, as a result, have prevented the production of IFNγ by this type of cell. In our reactivation model upon $CD4^+$ T cell depletion, the IFNγ producing NK and $CD8^+$ T cells were still present (Fig. 3D). Apparently, the amounts of IFNγ produced by the $CD8^+$ T cells and by the NK cells are not sufficient to appropriately activate macrophages and prevent reactivation. Our data, together with those described by Monack et al., indicate that IFNγ produced by $CD4^+$ T cells is necessary to suppress bacterial growth during the persistence phase and suggests that IFNγ produced by NK cells and $CD8^+$ T cells do not play an important role in this process.

It has been known for several years that $CD4^+$ T cells play a very important role in the clearance of the bacteria during a primary infection with *S. enterica* serovar Typhimurium. Previous studies have shown that mice depleted for $CD4^+$ T cells on the day of infection are highly susceptible to *S. enterica* serovar Typhimurium and die due to the lack of $CD4^+$

T cell-mediated defense against *Salmonella* (25). This is the first study, however, that describes the role of $CD4^+$ T cells in preventing reactivation of the *S. enterica* serovar Typhimurium infection in $Ity^r$ mice during the persistence phase. We have set up an in vivo reactivation mouse model that is very suitable for further studies on reactivating *S. enterica* serovar Typhimurium infections and might give further insights into the strategies that are used by *S. enterica* serovar Typhimurium to persist without being noticed by the host's immune system. Such knowledge might eventually lead to the development of new treatments to prevent recurrent infections with *S. enterica* serovar Typhimurium.

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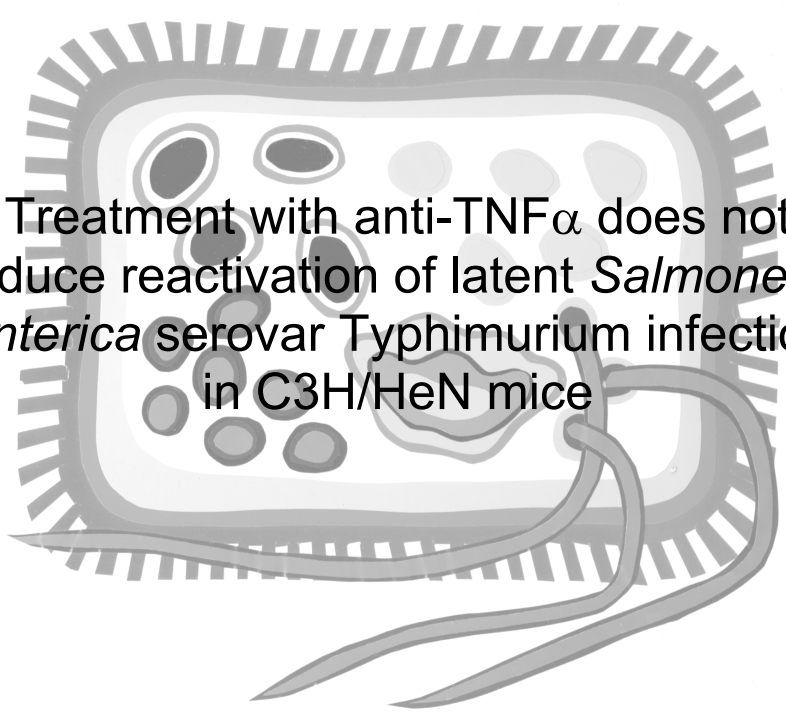
chapter three



# Treatment with anti-TNF$\alpha$ does not induce reactivation of latent *Salmonella enterica* serovar Typhimurium infection in C3H/HeN mice

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## Abstract

TNFα is a tightly regulated non-specific effector molecule that has pro-inflammatory and immuno-regulatory effects. The action of TNFα appears to be unopposed and results in damage to tissue, e.g. cartilage or bowel mucosa. In some inflammatory disorders, TNFα is induced, but fails to decrease leading to high amounts of this potent and damaging pro-inflammatory cytokine. Nowadays, patients suffering from these disorders can be successfully treated with the TNFα blocking agents Infliximab or Ethanercept. However, this treatment carries an increased risk of manifest infection with *Mycobacteria* and might lead to reactivation of latent infections, as has been described for intracellular pathogens such as *Mycobacteria*.

Reactivation of latent *Salmonella* infection has been described for patients with an impaired immune system due to medication or underlying disease, and a role for IFNγ and $CD4^+$ T cells during the phase of persistency of *Salmonella* has been described. Since TNFα plays an important role in defense to primary *Salmonella* infections, we tested whether treatment with TNFα neutralizing agents may lead to reactivation of a latent *Salmonella* infection. In the C3H/HeN mouse model of latent *S. enterica* serovar Typhimurium infection, in contrast to previous findings on reactivation after CD4+ T cell depletion or anti-IFNγ treatment, neutralization of TNFα did not lead to reactivation and suggests only a minor role for TNFα during the phase of latency.

## Introduction

TNFα is a non-specific effector molecule that is mainly produced by phagocytes (neutrophils and macrophages) and that has pro-inflammatory and immuno-regulatory effects. TNFα can act both as a membrane-associated protein and as a soluble cytokine after cleavage from the cell surface by the TNFα converting enzyme (2, 16, 20). Both soluble and membrane-bound TNFα are able to bind to the TNF receptor (TNFR) 1 as well as to TNFR2. Soluble TNFα preferentially binds to and activates the TNFR1, while TNFR2 is mainly engaged and activated by the membrane-bound form of TNFα. Activation of TNFR1 leads to a whole range of cellular and tissue responses such as induction of cytokine and chemokines production, MHC class I and II expression, cell adhesion molecule expression, inhibition of cell growth, apoptosis, tissue repair and damage, neurotoxicity and neuroprotection. TNFR2 activation leads to thymocyte proliferation, skin necrosis, and T-cell proliferation and apoptosis. TNF receptors are expressed on the surface of most cell types (30), so TNFα exerts its effects on almost every cell and organ within the body. Therefore, the production of TNFα is strictly regulated during infection. TNFα production is induced rapidly, but also degraded at a high rate.

In some inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, TNFα is induced, but fails to decrease. In these patients, the regulation of TNFα appears to be disturbed leading to the production of high amounts of this potent but damaging pro-inflammatory cytokine. Recently, new therapeutics have been developed to lessen the damage induced by TNFα , i.e. Infliximab (Remicade®, Centocor Inc) and Ethanercept (Enbrel®, Wyeth/Immunex), which are TNFα-blocking agents that are presently applied in the treatment of patients with rheumatoid arthritis and Crohn's disease. This treatment is highly beneficial for these patients. However, an important disadvantage of treatment with neutralizing antibodies to TNFα is the increased susceptibility to infection with intracellular bacteria. By its ability to activate macrophages to enhanced microbicidal activity and role in the formation of granulomas, TNFα has been shown to play a central role in immunity to bacterial infections with *Mycobacteria* (reviewed in (1, 9)), *Listeria moncytogenes* (4), *Salmonella* (22) and some other bacterial pathogens (6-8, 11, 12, 15, 21, 23, 25).

Besides the increased susceptibility to primary infection with intracellular bacteria, another complication of treatment with anti-TNFα is reactivation of latent infection with such bacteria, as has been described for *Mycobacteria.* Keane et al. reported 70 cases of tuberculosis (TB) among ~147,000 patients treated with TNFα blocking agents world-wide (14). The early onset after start of anti-TNFα treatment and low background incidences of TB is suggestive of reactivation rather than primary infection (14) and shows a role for TNFα in controlling latent infection with *Mycobacteri*a. In line with these observations, it has been speculated that treatment with neutralizing antibodies to TNFα could lead to reactivation of latent *Salmonella* infection as well.

Reactivation of infection of non-typhoidal *Salmonella* strains that had persisted within the host have been described for patients with an impaired functioning immune system



such as HIV-infected individuals (5, 10, 13), with Interleukin 12 receptor β1 deficiency (26), or patients with hematologic malignancies who underwent total body irradiation or who received a solid organ transplant and were treated with glucocorticoids or other immunosuppressive therapy.

Recently, we have shown that $CD4^+$ T cells play an important role during the phase of latency in C3H/HeN ($Ity^r$) mice and that depletion of the $CD4^+$ T cells resulted in reactivation of latent infection (29). Monack et al. have shown that neutralization of IFNγ resulted in reactivation of latent *S. enterica* serovar Typhimurium as well (19). $CD4^+$ T cells and IFNγ are important for activation of macrophages by inducing the release of TNFα, which via an autocrine loop acts on the macrophages' TNF receptor and leads to the formation of an angry macrophage expressing enhanced bacterial killing. Thus, given the pivotal role that TNFα plays in the cellular immune response, some problems with reactivation of persistent infections were to be expected in those receiving treatment with anti- TNFα.

The role of TNFα in mice during a primary infection with *Salmonella* enterica serovar Typhimurium has been known for years (18), however, its role in controlling latent infection is unclear. Therein, we used the mouse model for latent *S. enterica* serovar Typhimurium infection as described in detail recently (29) to address the question whether reactivation of the persistent *S. enterica* serovar Typhimurium infection is induced upon neutralization of TNFα by treating the mice with Ethanercept after full recovery from a primary infection with *S. enterica* serovar Typhimurium.

## Materials and Methods

**Mice.** Six- to 8-week-old female *Salmonella*-resistant ($Ity^r$) C3H/HeN mice were purchased from Harlan (Horst, The Netherlands). Mice were maintained under standard conditions according to institutional guidelines. Water and food were given ad libitum. Experiments were approved by the local Animal Ethical Committee.

**Bacterial strains and growth conditions.** For the infection experiments wild-type *S. enterica* serovar Typhimurium 14028s was used. Single colonies of the different strains were grown in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm). For the in vivo experiments, the overnight cultures were diluted in fresh LB medium and grown to the end of log phase and were then washed and diluted in sterile PBS. The CFU in the inoculum were determined by plating serial dilutions.

**In vitro infection experiment.** RAW264.7 cells were seeded in a 24-wells plate at a density of $1 \times 10^5$ cells per well and allowed to adhere at 37°C in RPMI medium supplemented with 10% fetal calf serum. The cells were left unaffected or were incubated with 100 U recombinant IFNγ (Pharmingen International) and/or 0.5 mg anti-TNFα

antibodies (Enbrel) for 18 h prior to infection. Bacteria were washed in PBS and were added to the cells at a 10:1 multiplicity of infection. The bacteria were spun onto the cell by centrifugation for 30 min at 270 × g. Cells were incubated for 30 min at 37°C and 5% $CO_2$ to allow bacterial endocytosis. After washing the cells with PBS, medium containing 100 μg/ml gentamicin was added and incubated at 37°C for another 30 min to kill the extracellular bacteria. The cells were then washed again. This point was designated time point zero. Next, medium containing 10 μg/ml gentamicin was added to the cells to kill any remaining extracellular bacteria and prevent reinfection. At 0, 24, and 48 hours the cells were lysed in 1 ml $H_2O$ and serial dilutions were made to determine the number of bacteria.

**In vivo *S. enterica* serovar Typhimurium infection.** Mice were inoculated subcutaneously in the flanks with $3 \times 10^4$ CFU *S. typhimurium* 14028s. For each group on each time point 3-4 mice were used. Fecal samples were taken and mice were sacrificed by carbon dioxide inhalation. Blood was obtained immediately by cardiac puncture. Part of the blood was prevented to coagulate by adding 40 U heparin/ml and another part was used to obtain serum. To determine the bacterial load within spleens, livers, mesenteric and inguinal lymph nodes, these organs were aseptically removed and single cell suspensions were prepared by using sterile 70-µm-mesh-size cell strainers (Falcon). Cells were pelleted by centrifugation for 10 min and were lysed in distilled water. The bacterial number per organ was determined bacteriologically by plating serial dilutions. The lowest number of bacteria that can be detected in this way is 30 CFU for the spleens and lymph nodes and 100 CFU for the livers. The feces was weighed and suspended in PBS and was plated on selective (SS) agar to determine the number of *S. enterica* serovar Typhimurium.

**Leukocyte count and blood cell differentiation.** The number of peripheral blood leukocytes was determined by counting the number of nucleated cells in the heparinized blood. In addition, we used 5 μl blood to make blood smears for the differentiation of the blood cells. Blood smears were fixed in methanol for 15 min and were stained with Giemsa for 30 min. In the blood smears the relative percentages of the different types of cells were determined. Next, the number of lymphocytes, monocytes, and PMN present in the blood on the different time points was calculated.

**Treatment with neutralizing TNFα antibodies and dexamethasone.** Immune intervention was carried out either by intraperitoneal injection of the mice with with 300 μg Ethanercept (a concentration known to have an effect in mice, personal communication with R. Flierman, Dpt. of Rheumatology, LUMC, The Netherlands) or 6 mg/kg dexamethasone on a time point when bacteria could no longer be detected bacteriologically within livers, spleens, and inguinal lymph nodes. Mice received a second and a third injection with 300 μg of Ethanercept or 6 mg/kg dexamethasone on day two and four after the first injection.



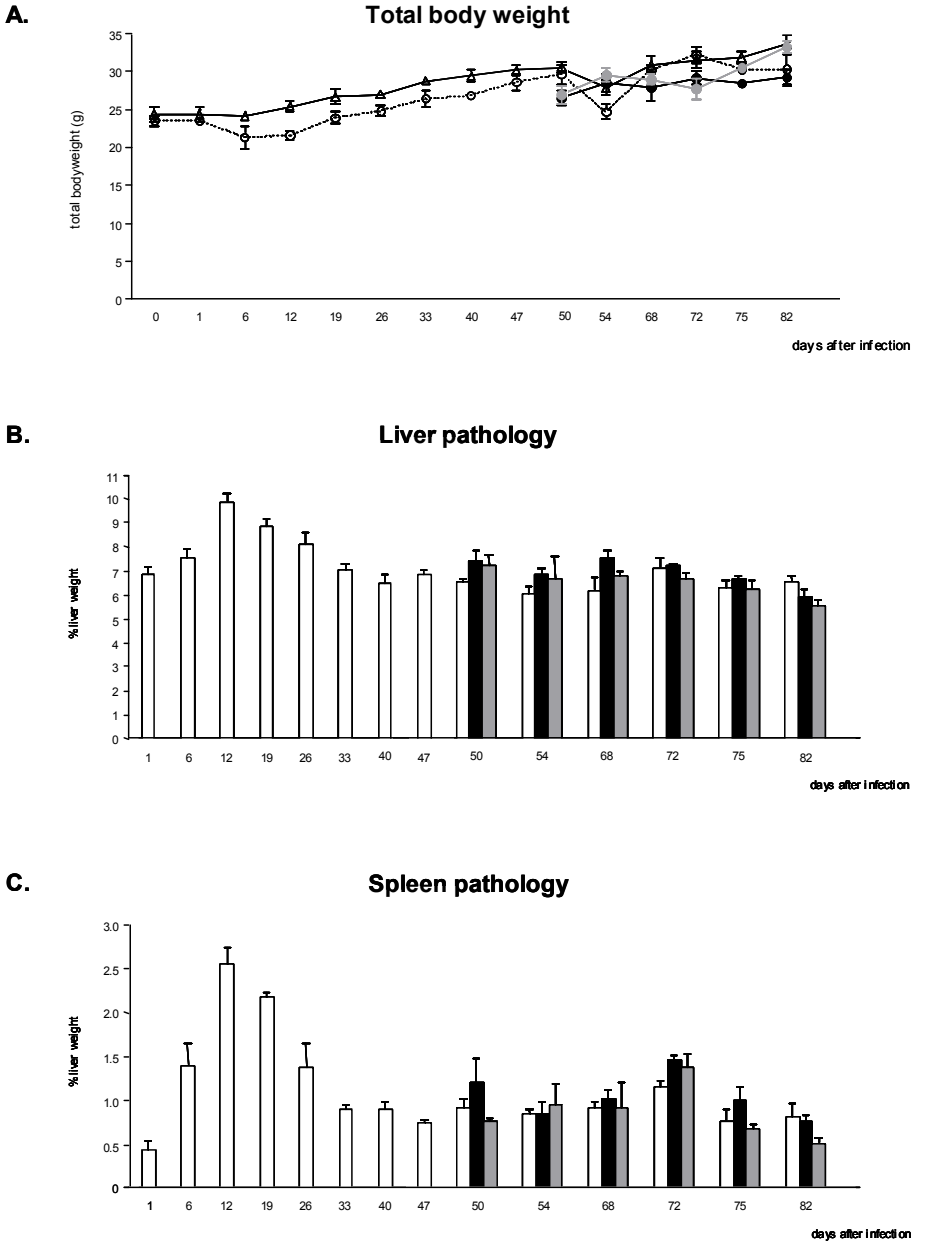
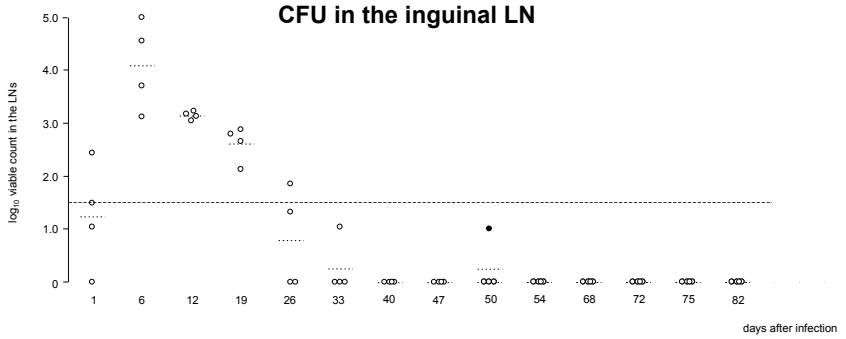



**Figure 1.** Total body weight (A), liver and spleen pathology (B and C) and bacterial numbers within the lymph nodes (D), livers (E), and spleens (F) of C3H/HeN ($Ity^r$) mice. On day 0, C3H/HeN ($Ity^r$) mice were injected subcutaneously in the flanks with ~$3 \times 10^4$ CFU *S. enterica* serovar Typhimurium 14028s. The dose was confirmed by plating serial dilutions of the inoculum. Four age-matched mice were not infected and served as weight controls (white triangles). After full recovery from the primary infection, the mice were injected i.p. with PBS (white dots and bars), anti-TNF$\alpha$ antibodies (black dots and bars), or dexamethasone (grey dots and bars).

**D.**

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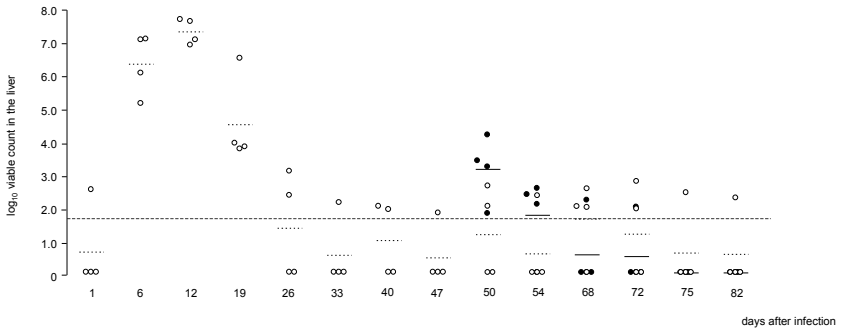


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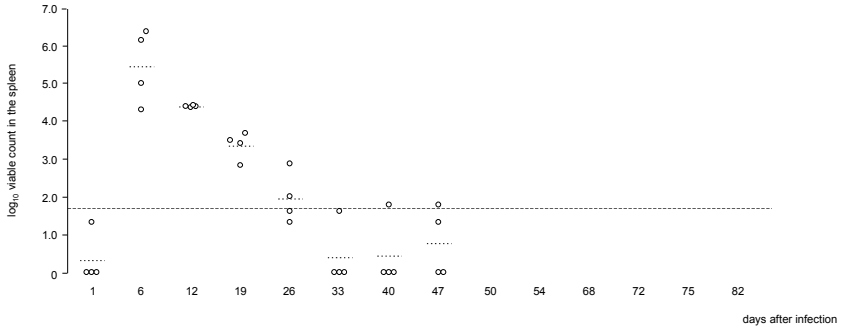


**Figure 1 continued.** At the indicated time points, the livers, spleens, and lymph nodes were aseptically removed and weighed. The pathology in the livers and spleens was calculated as the percentage organ weight of the total body weight. The viable count within the organs was determined by making lysates and plating serial dilutions of the lysates (n=3-4 per group). The results are expressed as $\log_{10}$ viable counts (means $\pm$ standard deviations). The gray dashed lines represent the detection limit of the microbiological method.



**Detection of antibodies raised against *S. enterica* serovar Typhimurium.** Induction of *S. enterica* serovar Typhimurium-specific antibodies was determined in a whole cell ELISA as described in (28). Maxisorp plates (Nunc) were coated with whole *S. enterica* serovar Typhimurium 14028s and after blocking, serial dilutions of the sera were added to the wells. Sera from naïve mice were included as a control. The wavelength absorbance was measured at 490 nm using an ELISA plate reader (VICTOR$^2$ 1420 multilabel counter, PerkinElmer Life and Analytical Sciences). Titers are defined as the dilution for which:

$$OD_{450} \text{ (sample)} > OD_{450} \text{ (naïve serum)} + 2 \times \text{standard deviation.}$$

**Statistical analysis.** For comparison between treatments we used Student's *t* tests and a P value <0.05 was considered significant.

## Results and Discussion

**No reactivation following neutralization of TNFα.** Mice were inoculated subcutaneously in the flanks with $3 \times 10^4$ CFU *S. enterica* serovar Typhimurium 14028s. After full recovery from the primary infection, the mice were injected with neutralizing antibodies to TNFα. The mice lost weight during the primary infection reaching a nadir between day 6 and 12 and gained weight during the next few weeks following a course comparable to that of the weight controls (Fig. 1A). The mice showed a primary infection reaching bacterial loads up to $1 \times 10^6$ and $2.5 \times 10^4$ CFU for the spleen and lymph nodes respectively on day 6 and $1.5 \times 10^7$ for the liver on day 12.

On day 48, 50, and 54, when the bacterial numbers in the organs were reduced to below the detection limit, the mice were treated with anti-TNFα antibodies. Infection controls were injected with equal volumes of PBS. None of the mice showed signs of illness and they all gained weight (Fig. 1A) and showed no consistent change in liver and spleen weight (Fig. 1B and 1C). Anti-TNFα treatment had no effect on the bacterial numbers in the organs (Fig. 1D-F), although treatment with anti-TNFα antibodies did induce a transient increase in bacterial numbers in the livers (Fig. 1E). In both groups, the mice showed detectable amounts of bacteria in the livers and spleens (Fig. 1B and 1C), but the averages were around or below the detection limit and no increase in bacterial numbers could be observed. In the feces on the other hand, we were able to detect bacteria on the later timepoints in these groups of mice. In our reactivation model we accepted that some of the control mice still showed some low number of bacteria in the organs, just above the limit of detection. Otherwise, we would have needed many more animals to find only a few in which *S. typhimurium* persisted and reactivated upon treatment.

**Leukocyte counts in the blood.** On each time point after infection and immune intervention, the number of leukocytes in the blood was determined and the types of blood

cells were differentiated using Giemsa stained blood smears (Table 1). During the growth phase of the primary infection, the number of leukocytes increased which was mainly attributable to the increase in the number of granulocytes (Table 1). During recovery from the infection, the leukocyte counts declined and eventually stabilized. Treatment of the mice with neutralizing antibodies to TNFα did not result in a change in the number of leukocytes, nor in the numbers of lymphocytes, granulocytes, and monocytes (Table 1).

**Anti-*Salmonella* IgG antibodies in the serum of *S. enterica* serovar Typhimurium infected mice.** From each mouse on each timepoint, serum was collected to determine the anti-*Salmonella* IgG antibody titer to the pathogen using a whole cell ELISA. Between days 6 and 12, when the primary infection peaked in the organs, the mice started producing antibodies to *S. enterica* serovar Typhimurium 14028s (Fig. 2). The antibody titers increased further to a $\log_3$ dilution factor of around 8 (Fig. 2). The mice that were treated with anti-TNFα antibodies had serum antibody levels that were similar, or slightly lower on days 50 and 5 to those of the infection controls so lack of reactivation could not be explained by altered antibody production in these mice.

**In vitro infection of IFNγ stimulated RAW264.7 macrophages.** To examine the role of TNFα in vitro in RAW264.7 macrophages, we performed in vitro infection experiments in which we stimulated the cells overnight with 100 U IFNγ and infected the cells as described in Materials & Methods. Just prior to infection, cells were treated with 10 μg/ml Anti-TNFα antibodies or were left untreated. At timepoint zero and after 24 h the number of intracellular bacteria was determined. In the untreated cells, the bacteria were able to grow out within 24 h to $1.5 \times 10^6$ CFU. Pre-treatment of the cells with 100 U IFNγ resulted in no outgrowth after 24 h, while treatment with anti-TNFα antibodies had no effect. When cells were activated with IFNγ and anti-TNFα treatment had no effect on the number of bacteria (Fig. 3), indicating that anti-TNFγ treatment cannot neutralize the effect of IFNγ.

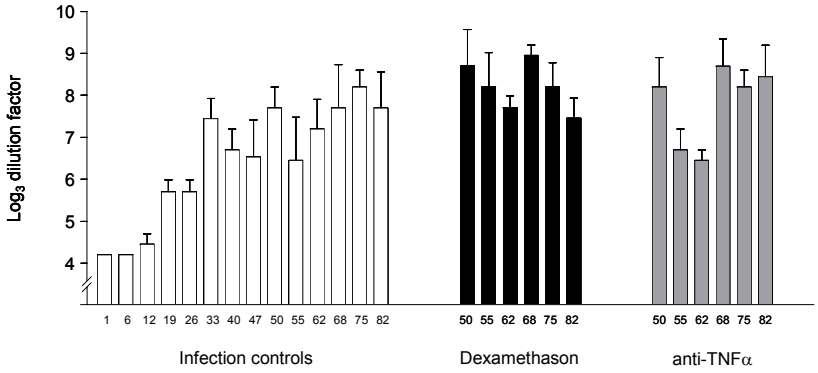


**Figure 2.** Anti-*Salmonella* IgG antibodies in the serum of *S. enterica* serovar Typhimurium infected mice that received no further treatment (white bars), were treated with anti-TNFα antibodies (black bars), or were treated with dexamethasone (grey bars). Titers are defined as the $OD_{450}$(serum sample) > $OD_{450}$(naïve serum).

**Table 1. Number of leukocytes, lymphocytes, monocytes, and granulocytes in the blood[a]**

| day | treatment | leukocytes ($n \times 10^5$) | lymphocytes ($n \times 10^5$) | monocytes ($n \times 10^5$) | granulocytes ($n \times 10^5$) |
|---|---|---|---|---|---|
| 1 | --- | 64.3 ± 9.1 | 42.3 ± 5.6 | 4.8 ± 0.8 | 17.2 ± 8.9 |
| 5 | --- | 73.8 ± 54.0 | 28.0 ± 17.9 | 9.8 ± 6.8 | 35.9 ± 7.3 |
| 12 | --- | 77.2 ± 15.2 | 27.2 ± 10.9 | 9.9 ± 5.4 | 40.1 ± 13.6 |
| 19 | --- | 95.3 ± 23.8 | 38.2 ± 8.7 | 10.6 ± 6.3 | 46.5 ± 13.1 |
| 26 | --- | 80.8 ± 34.7 | 44.9 ± 16.1 | 5.1 ± 3.1 | 30.8 ± 11.7 |
| 33 | --- | 46.5 ± 4.2 | 32.0 ± 3.2 | 2.5 ± 1.8 | 12.0 ± 29.2 |
| 40 | --- | 120.0 ± 14.1 | 90.8 ± 4.0 | 6.2 ± 3.3 | 23.1 ± 6.9 |
| 47 | --- | 104.3 ± 23.8 | 76.4 ± 20.0 | 5.3 ± 1.6 | 22.6 ± 5.4 |
| 50 | --- | 117.7 ± 38.7 | 80.1 ± 22.4 | 5.4 ± 2.6 | 32.1 ± 17.1 |
| 50 | αTNF[b] | 155.0 ± 28.9 | 89.5 ± 36.8 | 12.0 ± 6.8 | 53.5 ± 23.1 |
| 50 | dex[c] | 172.5 ± 38.6 | 100.5 ± 17.6 | 10.3 ± 3.5 | 61.7 ± 19.3 |
| 54 | --- | 72.3 ± 16.3 | 48.5 ± 13.5 | 5.1 ± 2.4 | 18.7 ± 8.4 |
| 54 | αTNF | 62.0 ± 16.7 | 37.4 ± 6.1 | 6.3 ± 2.8 | 18.4 ± 8.6 |
| 54 | dex | 50.3 ± 7.3 | 15.8 ± 5.5 | 7.1 ± 2.6 | 27.4 ± 6.4 |
| 68 | --- | 57.0 ± 13.0 | 30.8 ± 6.4 | 4.4 ± 2.0 | 21.8 ± 8.0 |
| 68 | αTNF | 66.5 ± 26.6 | 38.6 ± 15.8 | 4.0 ± 2.0 | 23.9 ± 9.7 |
| 68 | dex | 38.3 ± 12.8 | 18.4 ± 4.1 | 3.3 ± 1.4 | 16.5 ± 8.4 |
| 72 | --- | 58.3 ± 15.5 | 37.1 ± 9.3 | 4.0 ± 1.3 | 17.1 ± 6.1 |
| 72 | αTNF | 66.5 ± 7.3 | 38.7 ± 6.7 | 4.0 ± 1.1 | 23.8 ± 6.0 |
| 72 | dex | 48.5 ± 17.7 | 22.7 ± 6.0 | 2.2 ± 0.3 | 23.6 ± 12.6 |
| 75 | --- | 135.0 ± 30.0 | 94.2 ± 9.3 | 5.6 ± 3.0 | 35.2 ± 19.7 |
| 75 | αTNF | 187.5 ± 42.7 | 106.8 ± 6.7 | 10.9 ± 8.1 | 69.8 ± 30.8 |
| 75 | dex | 142.5 ± 41.1 | 92.1 ± 6.0 | 5.9 ± 0.9 | 44.5 ± 11.8 |
| 82 | --- | 79.0 ± 8.5 | 52.4 ± 11.3 | 1.8 ± 0.5 | 24.8 ± 12.1 |
| 82 | αTNF | 87.0 ± 15.0 | 62.3 ± 19.9 | 1.6 ± 0.3 | 23.1 ± 5.1 |
| 82 | dex | 57.5 ± 14.0 | 37.6 ± 29.1 | 1.6 ± 0.5 | 18.2 ± 6.8 |

[a] values are mean numbers/ml ± stdev
[b] mice that were treated with anti-TNFα
[c] mice that were treated with dexamethasone

**Dexamethasone treatment of mice during latency.** In the experiment shown in Figure 1 we have also treated mice with the glucocorticoid dexamethasone. Glucocorticoids have immunosuppressive effects through the inhibition of several immune functions, including chemotaxis, phagocytosis, and cytotoxicity, and by the down-regulation of cytokine gene expression, including IL-I, IL-2, IL-6, IFN-γ, and TNF-α (3). Glucocorticoids are known to inhibit neutrophil infiltration at inflammatory sites, thereby inhibiting neutrophil-mediated killing via mechanisms that are no completely understood yet. Glucocorticoids exert an anti-inflammatory effect and downregulate the expression of ICAM-1, which is constitutively expressed on neutrophils and vascular endothelial cells and is upregulated by inflammatory cytokines (17, 24, 27) such as TNFα. Treatment with dexamethasone (and other glucocorticoids) might promote reactivation of latent *S. typhimurium* infection by inhibiting the neutrophil-mediated killing. Treatment with dexamethasone resulted in a slight increase in the number of granulocytes and moderate decrease in lymphocyte number (Table 1). Dexamethasone treatment did not have an effect on body weight and hepatosplenomegaly (Fig. 1A, B, and C), nor did it induce reactivation of the latent *S. enterica* serovar Typhimurium infection in the organs (Fig. 1D, E, and F). Also in these mice, antibody production was induced to a similar extent as in the infection controls (Fig. 2).

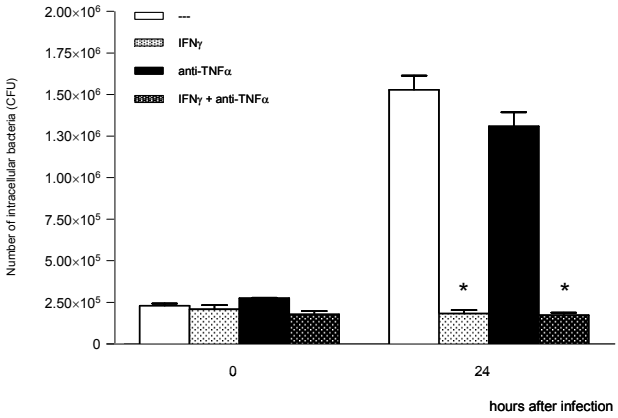


**Figure 3.** Intracellular *S. enterica* serovar Typhimurium 14028s in RAW264.7 macrophages. The cells were left untreated (white bars), stimulated with IFNγ (white bars with black dots), not stimulated but treated with anti-TNFα antibodies (black bars) or stimulated with IFNγ and treated with anti-TNFα antibodies (black bars with white dots). All cells were challenged with *S. enterica* serovar Typhimurium 14028s as described in Materials and Methods and the numbers of intracellular bacteria were determined at 24 h after challenge. Asterisks indicate that the number of intracellular bacteria is significantly different from that of wild-type *S. enterica* serovar Typhimurium 14028s. Data from a representative experiment are shown.

**Concluding remarks.** The main finding of this study is that neutralization of TNFα did not result in reactivation of latent *S. enterica* serovar typhimurium infection in C3H/HeN mice. This is in sharp contrast to the many reports of reactivating Mycobacterial infections after neutralization of TNFα. Both species are intracellular pathogens that preferentially invade mononuclear cells and have developed mechanisms to reside within a host without being noticed by the host immune system and are much alike. For both *Salmonella* and *Mycobacteria* an important role has been ascribed to TNFα in preventing or dealing with a primary infection. Regarding recurrent infections, there are sharp differences. For

*Mycobacteria*, recurrence of latent infections due to anti-TNFα have been described several times now, while this is not the case for *Salmonella*. Recurrent infections with the same *Salmonella* strain have been described for patients with immune disorders such as HIV and recently a role for $CD4^+$ T cells and IFNγ in preventing recurrence of a latent infection has been described. This strongly suggests that TNFα plays only a modest role in preventing reactivation while IFNγ and $CD4^+$ T cells play a much more important role.

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chapter four

# An *rmlC Salmonella enterica* serovar Typhimurium mutant is attenuated in vivo but is able to persist in RAW264.7 macrophages

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## Abstract

*Salmonella* is a facultative intracellular pathogen that can invade and replicate within several cells, including epithelial cells and macrophages. To be able to spread from the intestines into the body and to the liver and spleen, *S. enterica* serovar Typhimurium has to go from cell to cell and it can do so by inducing cell death, although it is currently unknown what the in vivo relevance of *Salmonella*-induced cell death is. To gain more insight into the strategy that is used by *Salmonella* to survive within macrophages and to induce apoptosis, we have created and selected *S. enterica* serovar Typhimurium mutants with increased ability to survive within macrophages without causing enhanced cell-death and analyzed the in vitro cytotoxicity and its in vivo virulence.

In this way, we have selected for an *rmlC* mutant that has truncated LPS chains. This mutant as well as a rough (Ra chemotype) variant displayed increased bacterial intracellular numbers in RAW264.7 macrophages and persisted even after 48 h, while inducing cell death to a similar extent as the wild-type strain. However, despite the increased ability to survive within cells, these LPS mutants were strongly attenuated in mice.

## Introduction

*Salmonella* is a facultative intracellular pathogen that can invade and replicate within several cells, including epithelial cells and macrophages. *Salmonella* is able to infect both humans and animals and depending on the type of *Salmonella* strain and host it can cause a range of diseases. *S. enterica* serovar Typhimurium causes gastroenteritis in humans, but causes typhoid fever-like disease in mice. *S. enterica* serovar Typhimurium is most widely studied in vitro and in vivo and serves a good model for human typhoid fever caused by *S. enterica* serovar Typhi.

Natural infection with *S. enterica* serovar Typhimurium occurs through the ingestion of contaminated food or water. Those bacteria that have survived the acidic environment of the stomach and have reached the intestine will eventually encounter membranous epithelial (M) cells overlying the Peyer's patches. *S. enterica* serovar Typhimurium uses these M cells to pass the intestinal lining and to invade the body by inducing its own uptake through a mechanisms known as *Salmonella*-induced membrane ruffling mediated by type three secretion system proteins that are encoded by genes of *Salmonella*-pathogenicity island 1 (SPI-1) (reviewed in (16)). *S. enterica* serovar typhimurium is transported through the cytoplasm to the underlying lymphoid cells where it predominantly infects the macrophages. *S. enterica* serovar Typhimurium then becomes systemic and spreads to the liver and spleen and causes the chronic inflammatory response that is typical for typhoid fever.

To be able to spread from the intestines into the body and to the organs, *S. enterica* serovar Typhimurium has to go from cell to cell. *S. enterica* serovar Typhimurium can do so by inducing cell death. Two types of *Salmonella*-induced cell death have been described. The first one involves a rapid, caspase-1-dependent, induction and the second one a slower, caspase-1-independent, induction of cell death. The rapid *Salmonella*-induced cell death leads to a strong pro-inflammatory response that is mediated by IL-1$\beta$ and IL-18 and therefore differs from the classical apoptotic mechanisms. It has therefore been stated that *Salmonella* induces programmed necrosis in a caspase 1-dependent manner (2). This type of *Salmonella*-induced necrosis is dependent upon the production and secretion of the SPI1 encoded protein SipB and requires the presence and activation of caspase-1 (5, 10). The second type of *Salmonella*-induced cell death is slower than the rapid caspase-1 induced cell death (at 12-13 h post infection) and is not dependent upon SipB and caspase-1 as *Salmonella* can still induce cell death in the absence of these proteins (8, 15). This type of induced cell death is reminiscent of apoptosis and occurs even in the absence of bacterial replication, is SPI-1 independent and requires a functional SPI-2 and *ompR* (15). It is currently unknown which other mechanisms, besides SPI-2-encoded genes and *ompR* might play a role in this second type of *Salmonella*-induced cell death.

Van der Velden et al. have proposed a model in which the rapid and delayed *Salmonella*-induced apoptosis in infected macrophages is induced under different physiological conditions at distinct time and location during a natural infection. The rapid



SPI-1-dependent induction of cell death resulting in inflammation may be required for the recruitment of phagocytes and for systemic dissemination, while the delayed, caspase-1 independent, apoptosis is required during the systemic phase of infection and is used to spread intercellularly within apoptotic bodies (15). In this model, *Salmonella*-induced cell death is generally thought to reflect a bacterial strategy to promote disease (1) and seen as a virulence mechanism.

Alternatively, cell death upon *S. enterica* serovar Typhimurium infection might also been seen as a host response to infection that is beneficial to the host as cell death exposes *S. enterica* serovar Typhimurium to immune defense mechanisms of the host such as antibodies, complement, and neutrophils. By inducing cell death of infected macrophages, the bacteria are released into the host tissues and blood and can be rapidly killed by complement or can be opsonized and then killed by granulocytes.

Further research on the relevance of *Salmonella*-induced cell death is necessary since it is currently unknown what the in vivo relevance of *Salmonella*-induced cell death is. Although it has been shown that several *Salmonella* mutants are less cytotoxic and cannot induce apoptosis in vitro (11, 13), it is not known whether such mutants are attenuated or not. Therefore, we have selected for *S. enterica* serovar Typhimurium mutants that reside in macrophages and that are still viable after prolonged times of infection when most of the cells have undergone cell death. Analysis of in vitro cytotoxicity and in vivo virulence of such mutants might give more insights into the role of *Salmonella* induced cell-death in in vivo virulence.

## Materials and Methods

**Mice.** Six- to 8-week-old female *Salmonella*-resistant ($Ity^r$) C3H/HeN mice were purchased from Harlan (Horst, The Netherlands). Mice were maintained under standard conditions according to the institutional guidelines. Water and food were given ad libitum. All experiments were approved by the local Animal Ethical Committee.

**Bacterial strains and growth conditions.** The bacterial strains used in this study are enlisted in Table 1. Single colonies of the different strains were grown in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm). For the in vivo experiments, the overnight cultures were diluted in fresh LB medium and grown to the end of log phase and were then washed and diluted in sterile PBS. The CFU in the inoculum were determined by plating serial dilutions.

**Generation of *S. enterica* serovar Typhimurium mutants.** Wild type *S. enterica* serovar Typhimurium 14028s was used as the parental strain to isolate mutants that displayed reduced ability to induce cell death in RAW264.7 cells. Mutants were made by random MudJ transposon insertion. Phage lysate containing the MudJ was made using TT10289 (7). Briefly, P22 phages were added to a 1:10 diluted ON culture of TT10289 and

incubated at 37°C ON while being shaken. After adding 10% chloroform and another incubation at 37°C for 30 minutes, the lysate was centrifuged for 2 minutes to remove the cell debris. This cell lysate containing phages with the MudJ transposon (10 μl) was added to100 μl ON culture of the recipient strain 14028s. After incubation at 37°C for 5 hours, the bacteria were plated on LB agar containing 50 μg/ml kanamycin (Sigma) and 0.1% sodium citrate (Merck) to select for bacteria in which the MudJ transposon had been inserted.

**Table 1. *Salmonella* strains and plasmids used in this study**

| *Salmonella* Strain | Characteristics | Origin or reference |
|---|---|---|
| *S. enterica* serovar Typhimurium | | |
| 14028s | Wild type | ATCC |
| 14028r | Ra chemotype (rough) | This study |
| SF1398Re | Re chemotype (deep rough) | This study |
| TT10289 | LT2 *hisD*9953::MudJ *hisA*9949::Mud1 | {370} |
| AVD16703 | 14028s *rmlC*::MudJ | This study |

**Selection of *S. enterica* serovar Typhimurium mutants.** To select for *S. enterica* serovar Typhimurium mutants we pooled the random MudJ transposon insertion mutants by scraping the plates. The pooled bacteria were washed with PBS and were used for in vitro infection of RAW264.7 cells as described. Only now the cells were seeded in 150 $cm^2$ flasks at a density of $1 \times 10^7$ cells per flask and were allowed to adhere ON at 37°C and 5% $CO_2$ and the pooled bacteria were used to infect the cells at a 10:1 multiplicity of infection. Endocytosis was allowed to proceed for 30 minutes and gentamicin treatment was performed as described for the in vitro infection experiment. At 72 hours after infection, the cells were washed and lysed in milliQ and the lysate was plated on agar plates. The bacteria were again scraped off the plates, washed with PBS and again used for infection of RAW264.7 cells. After this second passage, single colonies were made phage-free by repetetive plating on EBU agar and were tested in a regular in vitro infection experiment.

**Inverse PCR.** To identify the gene in which the MudJ transposon had inserted, the DNA flanking the left end of the transposon was amplified and sequenced using inverse PCR. Genomic DNA of strain AVD16703 was isolated and digested with *HaeIII* (Gibco-BRL) for 4 hours. After inactivating the *HaeIII* enzyme, the sample was treated with T4 DNA ligase (Invitrogen). The digested and ligated DNA was amplified using the following primers: 5'-CCGGGAGGACATTGGATTAT-3' (sense) and 5'-CGTACTTCAAGTGAATCAATAC-3'. The PCR product was purified using the QIAquick PCR purification kit (Qiagen) and was sequenced.



**In vitro infection experiment.** RAW264.7 cells were seeded in a 24-wells plate at a density of $1 \times 10^5$ cells per well and allowed to adhere ON at 37°C in RPMI medium supplemented with 10% fetal calf serum. Bacteria were washed in PBS and were added to the cells at a 10:1 multiplicity of infection. The bacteria were spun onto the cell by centrifugation for 10 min at 270 × g. Cells were incubated for 10 min at 37°C and 5% $CO_2$ to allow bacterial endocytosis. After washing the cells with PBS, medium containing 100 μg/ml gentamicin was added and the cells were incubated at 37°C for another 10 min to kill the extracellular bacteria. The cells were then washed again. This was designated time point zero. Medium containing 10 μg/ml gentamicin was added to the cells to kill the extracellular bacteria and to prevent reinfection. At 0, 24, and 48 hours the cells were lysed in 1 ml milliQ and serial dilutions were made to determine the number of bacteria.

**Cytotoxicity test.** *Salmonella*-induced cytotoxicity was determined using the Cytotox 96® Non-Radioactive Cytotoxicity Assay (Promega) that is based upon the release of lactate dehydrogenase (LDH). From each well, 50 μl supernatant was taken and transferred to a 96 wells plate. As a control for spontaneous release of LDH 50 μl supernatant was taken from non-infected cells on each timepoint. The maximum LDH release was determined by adding 0.9% Triton X-100 to the cells and thereby lysing them and transferring 50 μl to the 96-wells plate. Then 50 μl substrate mix was added to each well and the plate was incubated at room temperature in the dark for 30 min. The coloring reaction was stopped by adding 50 μl stop solution provided with the kit and the OD490 was determined using an ELISA plate reader (VICTOR$^2$ 1420 multilabel counter, PerkinElmer Life and Analytical Sciences). The induced cytotoxicity (%) was calculated as follows:

$$\frac{OD_{490}(\textit{Salmonella}\text{-induced release}) - OD_{490}(\text{spontaneous release})}{OD_{490}(\text{maximum release}) - OD_{490}(\text{spontaneous release})} \times 100\%$$

**In vivo infection experiment.** Mice were inoculated subcutaneously in the flanks with 0.1 ml bacterial suspension containing $3 \times 10^4$ CFU *S. enterica* serovar Typhimurium 14028s, 14028r, or AVD16703. For each group on each time point 4 mice were used. Mice were sacrificed by carbon dioxide inhalation and blood was taken immediately by cardiac puncture. Part of the blood was used to obtain serum and the other part was prevented from coagulation by adding 40 U heparin/ml to count the number of leukocytes and to test the blood for the presence of *Salmonella* by culture in LB medium. To determine the bacterial load within spleens, livers, and inguinal lymph nodes, these organs were aseptically removed and single cell suspensions were prepared by using sterile 70-μm-mesh-size cell strainers (Falcon). Cells were pelleted by centrifugation for 10 min and were lysed in distilled water. The bacterial number per organ was determined bacteriologically by plating serial dilutions.

**Preparation of cell envelopes followed by Proteinase K digestion.** Single colonies were grown ON in 10 ml LB medium at 37°C while being shaken. The ON culture was pelleted for 10 min at 3,000 rpm and cell envelopes were isolated as described in (3). Briefly, the pellets were washed once in ice-cold sonicationbuffer (50 mM Tris-HCl, 2 mM EDTA, pH 8.5) and were then resuspended in 4 ml of this buffer. The suspension was then sonicated with 8 pulses of 20 sec with an amplitude of 20-24 μm and with cooling. The sonicates were pelleted and the pellets containing cell debris and the cell envelopes were resuspended in 100 μl of 2 mM Tris-HCl pH7.8. Protein concentrations in the samples were measured using a BCA (Pierce) protein assay according to the manufacturers recommendations to standardize the samples at 1 mg/ml. The samples were then treated with Proteinase K (250 μg/ml) for 2 hours at 60°C to degrade the proteins present in the cell envelopes and equal volumes of sample buffer were added.

**Electrophoretic separation.** For the separation of the LPS fragments was done by SDS-PAGE by loading 35 μl of the samples on a 16% acryl amide separation gel and a 5% acryl amide stackinggel.

**Silver-staining.** To visualize the LPS fragments, the acryl amide gel was stained using a silver staining method according to Heukeshoven and Dernick (6) with a few modifications. Briefly, the acryl amide gel was incubated in fixing solution (40% ethanol, 10% acetic acid) for 30 min. The gel was then placed ON in incubation solution (30% ethanol, 0.5 M sodium acetate, 0.13% glutardialdehyde, 0.2% sodium thiosulphate). After washing with distilled water, the gel was stained in silver solution (0.1% silver nitrate, containing 0.007% formaldehyde) for 40 min and developed in 0.24 M sodium carbonate containing 0.0035% formaldehyde for 15 min until the bands become intensively dark. The coloring reaction was stopped by placing the gel in 0.04 M EDTA-$NA_2$·2 $H_2O$ and was washed with distilled water. The gel was then preserved in 10% glycerol.

**Statistics.** Statistical analysis was performed using Student's *t* tests and a *P* value <0.05 was considered significant.

## Results

**Isolation of *S. enterica* serovar Typhimurium mutants with reduced ability to induce cell damage.** In a typical in vitro infection experiment of RAW264.7 macrophages with the wild-type strain 14028s, the bacteria start to replicate until they reach a maximum after 24 h. At later timepoints after infection, the bacterial numbers decline due to death of the macrophages and subsequent exposure of the bacteria to gentamicin that is present in the medium or due to eventual killing of the bacteria by the macrophages. To select for mutants with reduced capacity to induce death of the host cell, we have generated *S. enterica* serovar Typhimurium mutants by random MudJ transposon insertion in the wild-



type strain 14028s and pooled them for use in in vitro infection of RAW264.7 cells. As a control we infected cells with wild-type *S. enterica* serovar Typhimurium. At 72 h after infection, the cells were lysed and the lysate was plated onto agar in order to enrich for mutants with a reduced capacity to cause death of the macrophages. The bacteria were again scraped off the plates and were again used for infection of RAW264.7 cells. During this second passage we observed that many cells were no longer attached to the bottom of the tissue culture flask when wild-type bacteria were used for infection, while in the flasks used for infection with the pooled mutants cells remained attached. This suggested that this pool contained mutants with a reduced ability to induce cell damage. After the second passage, several single colonies were isolated and used in an in vitro infection experiment. Mutants H and J displayed increased survival compared to the wild-type strain 14028s at 48 h after infection in in vitro infection (Figure 1) and these were selected for further study.

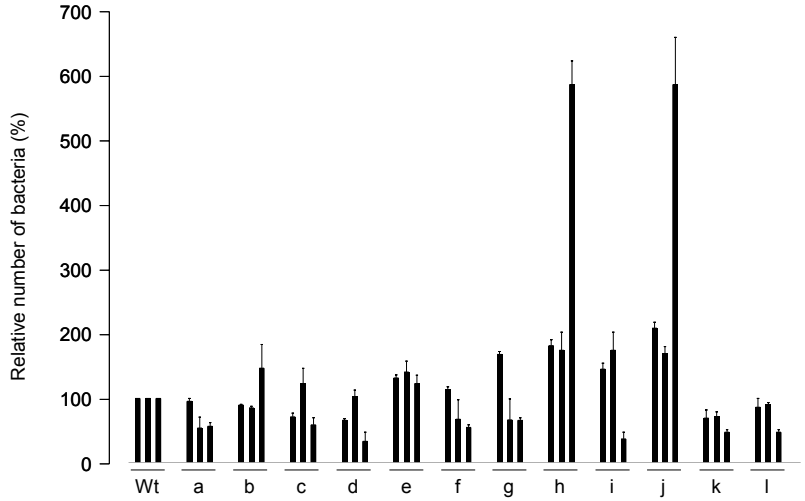


**Figure 1.** Relative number of random MudJ transposon insertion *S. enterica* serovar Typhimurium 14028s mutants in RAW264.7 macrophage-like cells. On t=0, 3, and 24 h the number of intracellular bacteria was determined bacteriologically and the relative number compared to the wild-type strain on each timepoint was calculated. Each first bar represents t=0 h, the second bar t=3 h, and the third bar t=24 h.

**Identification of the gene inactivated by MudJ insertion.** The gene that was inactivated by the MudJ insertion and rendered mutants H and J to be able to survive within RAW264.7 cells even after 48 was identified by inverse PCR. Part of the gene in which the MudJ had inserted (left flanking region) was sequenced and NCBI nucleotide BLAST search revealed homology of the MudJ flanking region to *rmlC*, a gene encoding dTDP-4-deoxyrhamnose 3,5-epimerase (complement 2175118-2175669, accession number NC_003197.1). Sequence analysis also revealed that mutants H and J were identical. The MudJ had been inserted at exactly the same position in the *rmlC* gene. Therefore, we continued our experiments with mutant J only and have named this strain

AVD16703. *RmlC* is part of the *rfb* gene cluster that encodes the enzymes for O-antigen biosynthesis. *RmlC* acts together with *rmlB*, *rmlD*, and *rmlA* to encode L-rhamnose, which is part of the repeating unit of the O-antigen polysaccharide of LPS.

**LPS fragment analysis.** The MudJ insertion in *rmlC* should in theory result in the lack of production of L-rhamnose and result in a lack of the O-antigen. To confirm that the isolated mutant AVD16703 was indeed an LPS mutant we isolated cell envelopes and analyzed the LPS fragments by gel electrophoresis and silver staining. As a control we used the LPS rough mutant 14028r (Ra chemotype) and the deep rough LPS mutant SF1398Re (Re chemotype). LPS of 14208r consists of the lipid A portion and the core region and lacks the O-antigen. The LPS chain of SF1398Re is even shorter as it consists only of lipid A glycosylated with two 3-deoxy-D-manno-octulosonic acid (Kdo) residues and thus lacks both the O-antigen and the core region. Figure 2 shows the LPS fragments of the wild-type strain 14028s show the typical ladder pattern. LPS from mutant SF1398Re is very small and gives only a smear at the bottom of the gel. LPS from our mutant AVD16703 appeared to be very much alike that of the 14028r strain. This 14082r strain is an LPS mutant that is known to lack the O-antigen and consists only of lipid A and the core region.

One striking feature was that mutant AVD16703, although very much alike 14028r in the LPS fragment analysis, showed different colony morphology on LB agar plates. Colony morphology of the Ra chemotype mutant 14028r was rough, while that of mutant AVD16703 was not. On blood agar plates, on the other hand, the colony morphology of AVD16703 was slightly rough, but less clearly compared to the 14028r strain (data not shown).

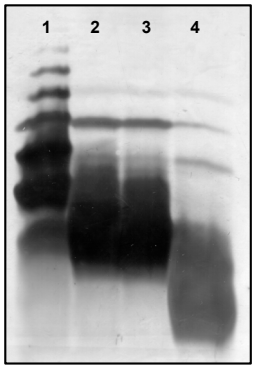


**Figure 2.** SDS-PAGE fractionation of LPS fragments from wild-type *S. enterica* serovar Typhimurium 14028s (lane 1), the *rmlC* mutant AVD16703 (lane 2), the Ra chemotype rough mutant 14028r (lane 3), and the Re chemotype mutant SF1398Re (lane 4). The gel was silver stained.

**In vitro intracellular growth of *S. enterica* serovar Typhimurium mutant strains.** Since LPS fragment analysis revealed that LPS from 14028r and mutant AVD16703 was very much alike, we tested both mutants in an in vitro infection experiment. Since mutant AVD16703 was originally isolated as a strain that was able to survive for a longer period of time and is thought to induce less cell death, we also assessed the intracellular

survival/outgrowth after 48 h. The wild-type strain grows out after 24 h, but after 48 h the number of viable bacteria is reduced (Fig. 3A). In contrast, mutant AVD16703 is able to survive after 48 h. The bacterial numbers are somewhat reduced compared to 24 h, but are still significantly higher than those of the wild-type strain. Remarkebly, the 14028r mutant showed even higher numbers of intracellular bacteria both at 24 and 48 h after infection.

***Salmonella*-induced cytotoxicity.** The reduction in the number of wild-type bacteria at 48 h after infection and the higher numbers of the LPS mutants could be explained in two ways. The intracellular replication of the mutants could have been faster than that of the wild-type, or the infection with the wild-type strain could have caused more cell death than the LPS mutants, resulting in a reduction in the number of viable bacteria. Therefore, we analyzed the induced cytotoxicity by measuring the LDH release in the infected wells. The induced cytotoxicity at 24 h was around 20% for all three strains and increased to

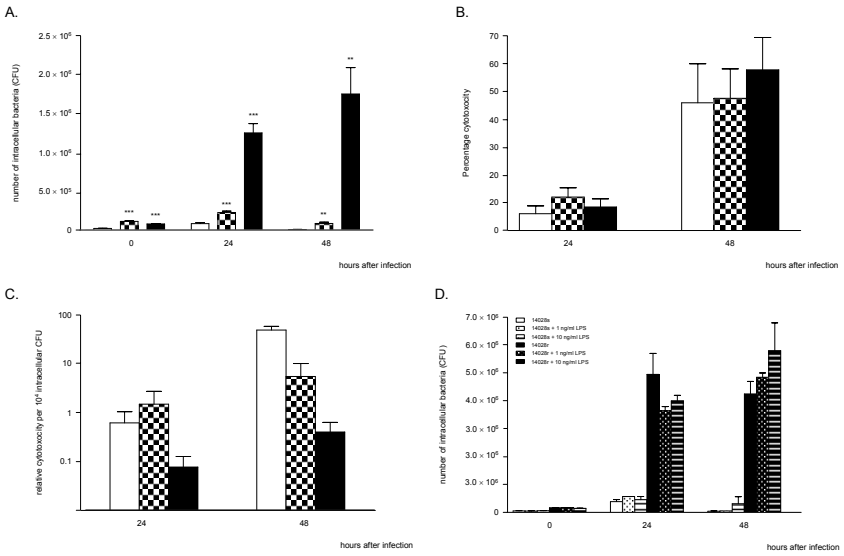


**Figure 3.** Intracellular *S. enterica* serovar Typhimurium in RAW264.7 mouse macrophage-like cells (A), percentage cytotoxicity induced by the infection (B), relative induced cytotoxicity per $10^4$ intracellular CFU (C), and number of intracellular bacteria in the absence or presence of different concentrations of extracellular *E. coli* LPS (D). The cells were challenged with *S. enterica* serovar Typhimurium 14028s (white bars), AVD16703 (checkered bars), 14028s (black bars) as described in Materials and Methods. The numbers of intracellular bacteria were determined at 0, 24, and 48 h after infection and the percentage cytotoxicity 24 and 48 h after infection. Asterisks indicate that the number of intracellular bacteria is significantly different from that of wild-type *S. enterica* serovar Typhimurium 14028s. Mean data of two independently performed experiments ± standard errors of the means are shown.

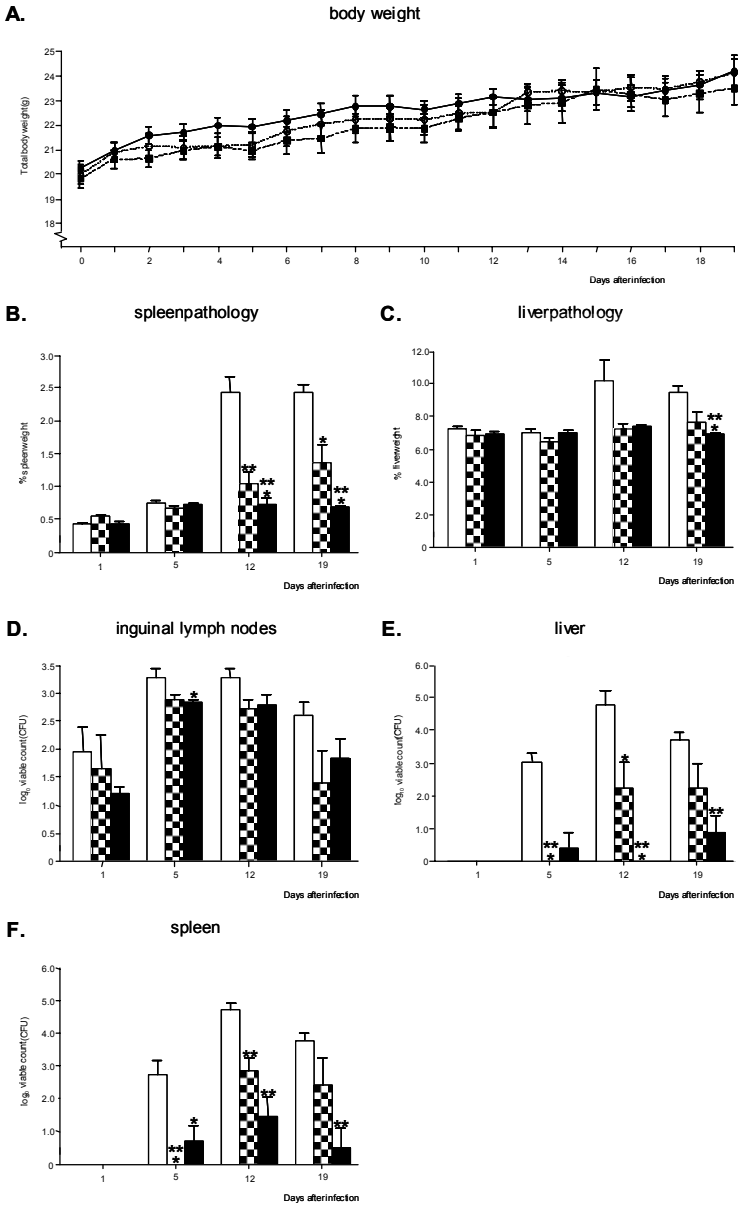


**Figure 4**. Total body weight (A), induced pathology in the spleen (B) and liver (C), and number of bacteria in the inguinal lymph nodes (D), liver (E), and spleen (F). Mice were infected subcutaneously in the flanks with $3 \times 10^4$ CFU of 14028s (white dots and white bars), AVD16703 (black squares and checkered bars), and 14208r (black dots and black bars). At the indicated time points, livers, spleens, and lymph nodes were aseptically removed and weighed. The viable counts within the organs were determined by making lysates and plating serial dilutions of the lysates and are expressed as $\log_{10}$ viable counts (means ± standard errors of the means). Averages from 4 mice per time point and per group are shown. Asterisks indicate statistically significant differences compared to the wild-type-infected mice (Student's *t* test) and the gray dashed lines represent the detection limit of the microbiological method (50 CFU for the livers and 30 CFU for the spleens and lymph nodes

~50% after 48 h (Fig. 3B). The induced cytotoxicity seemed slightly higher for the 14028r strain at 48 h, but this was not statistically significant. When the relative cytotoxicity was calculated as LDH release per $10^4$ intracellular bacteria, it appeared that this was reduced for both mutant AVD16703 and the 14028r strain (Fig. 3C). This indicates that with similar cytotoxicity more mutant *S. enterica* serovar Typhimurium was present in the macrophages as reflected by the relative cytotoxicity (Fig. 3C). Since the mutants have truncated LPS that might have influenced the activation status of the macrophage, we added different concentrations of LPS during the in vitro infection. However, no effect of extracellular LPS was observed (Fig. 3D).

**In vivo infection with AVD16703 and 14028r.** To determine whether the mutant strains are capable of surviving for a longer period of time in mice, we performed an in vivo infection experiment in which C3H/HeN mice were infected subcutaneously in the flanks with ~3 $\times$ $10^4$ CFU of wild-type 14028s, 14028r, or AVD16703. All the mice showed an increase in total body weight during the 19 days after infection. Only in the wild-type infected mice showed growth was halted between days 2 and 5 after infection (Fig. 4A) but the mice showed no reduction in body weight. The mice that were infected with the wild-type strain showed an increase in spleen and liver pathology (Fig. 4B and 4C) while the 14028r-infected mice showed no signs of hepatosplenomegaly. The AVD16703 infected mice showed no increase in liverweight, but did show a slight increase in spleenweight reaching intermediate spleenweights (Fig. 4B and 4C). All the strains tested showed detectable numbers of bacteria in the inguinal lymph nodes already on day 1 after infection and in all groups the numbers increased reaching a peak between days 5 and 12 (Fig. 4D). For the livers and spleen the strains behaved differently. The wild-type infected mice showed high bacterial numbers in the liver and spleen peaking on day 12 after infection, while in the 14028r and AVD16703-infected mice hardly any bacteria could be detected (Fig. 4E and 4F).

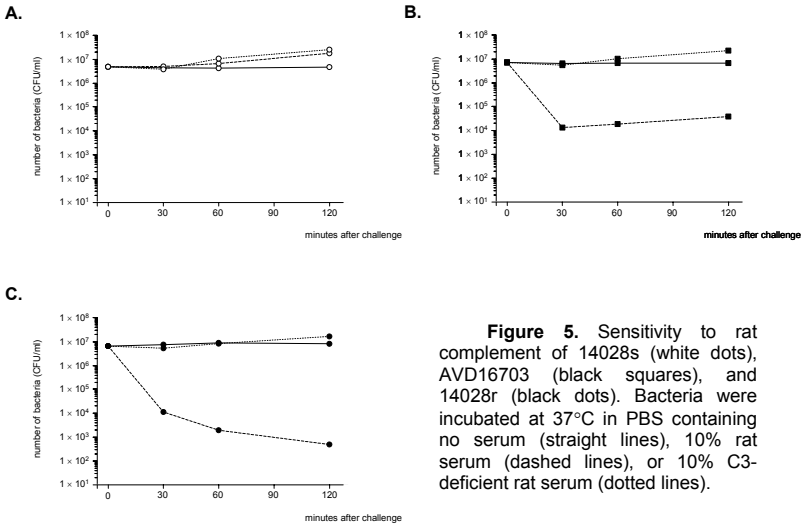


**Figure 5.** Sensitivity to rat complement of 14028s (white dots), AVD16703 (black squares), and 14028r (black dots). Bacteria were incubated at 37°C in PBS containing no serum (straight lines), 10% rat serum (dashed lines), or 10% C3-deficient rat serum (dotted lines).

**In vitro sensitivity to complement.** The in vivo attenuation could have been explained by increased sensitivity to complement-mediated killing as has been described for other LPS mutants (14). Therefore, we determined the rate of killing by complement of the wild-type strain and the two LPS mutant strains AVD16703 and 14028r. First, we determined the in vitro killing of rat serum that had not been heat inactivated. The wild-type strain is not killed when incubated in the presence of 10% rat serum and bacterial numbers are comparable to those bacteria that were incubated in the absence of serum (Fig. 5A). The LPS mutant strains, on the other hand, appeared to be more sensitive to rat complement since bacterial numbers declined already after 30 minutes (Fig. 5B and 5C). AVD16703 then stabilized, while the numbers of 14028r declined even further during the next 90 min (Fig. 5C). Comparable results were obtained when human serum was used (data not shown). When rat serum was used that was deficient for the C3 component of the complement system, the bacteria were not killed (Fig. 5A, B, and C), indicating that the C3-mediated complement is involved in the increased sensitivity to complement-mediated killing of the LPS mutants. However, when mouse serum was used in this experiment, no killing was observed even when the bacteria were incubated in 100% serum. Even when much lower numbers of bacteria were used, these mutants were not killed (data not shown). However, the outgrowth within 4 h of the LPS mutants was less than that of the wild-type strain, indicating that complement-mediated killing could play a role in vivo, when bacterial numbers are very low.

## Discussion

To gain insight into the role of *Salmonella*-induced cell death in virulence, we have selected for mutants that survived for prolonged periods after infection in macrophages, when most of the cells infected with wild-type *S. enterica* serovar Typhimurium 14028s have undergone cell death. We reasoned that such mutants would be less cytotoxic. The relative cytotoxicity of the selected mutants was lower compared to the wild-type strain. These mutants reached higher intracellular numbers in the in vitro macrophage infection assay while inducing a similar cytotoxicity as the wild-type strain did at a much lower number of intracellular bacteria. Also, the LPS mutants were highly attenuated in vivo indicating that *Salmonella*-induced cell death should be regarded as a virulence determinant.

We have selected two mutants from the in vitro infection assay and sequence analysis revealed that these mutants were identical and the MudJ transposon had inserted in the *rmlC* gene encoding dTDP-4-deoxyrhamnose 3,5-epimerase (RmlC). RmlC is involved in the pathway of biosynthesis of dTDP-$_{L}$-rhamnose from glucose 1-phosphate and thymidine triphosphate. The *rmlC* gene is part of the *rfb* gene cluster that is involved in the LPS O-antigen biosynthesis of *S. enterica* serovar Typhimurium (9). RmlC catalyzes the third step in the biosynthesis of dTDP-$_{L}$-rhamnose, which requires three additional enzymes RmlA, B, and D. These enzymes act together to synthesize dTDP-$_{L}$-rhamnose from $_{D}$-glucose-1-



phosphate and dTTP. The sugar dTDP-$_L$-rhamnose is the precursor of $_L$-rhamnose, a major residue in the O-antigen of LPS. An intact LPS chain is essential for colonization and resistance to complement-mediated serum killing as the shorter the LPS chain, the more sensitive these mutants get to complement-mediated serum lysis and the lesser these *S. enterica* serovar Typhimurium mutants are able to colonize the intestines (12, 14). The biosynthesis of dTDP-$_L$-rhamnose by the four enzymes RmlABCD is a very important process for *Salmonella* since humans do not synthesize dTDP-$_L$-rhamnose and therefore cannot be taken up and has to be produced by *Salmonella* itself. It has therefore been stated by others that these four enzymes including RmlC might be very good targets against which new drugs might be designed (4). When looking at the data presented here, this might indeed be a good target for defense against *Salmonella* colonization since our mutant lacked the O-antigen of LPS and as a result showed an attenuated in vivo phenotype in mice and increased sensitivity to human complement, so targeting of the genes involved in $_L$-rhamnose biosynthesis might indeed be a good way of designing a potent drug against human *Salmonella* infection.

Since infection with the RmlC mutant and the 14028r strain resulted in decreased relative cytotoxicity in RAW264.7 macrophages and since SPI-1-inducing conditions had no effect on the early induction of cell death (data not shown), this would suggest that these LPS mutants induce cell death in a SPI-1-independent manner and that SipB and caspase-1 are not required. Therefore, it is most likely that the second, delayed type of *Salmonella*-induced cell death is induced at a higher rate for these mutants. In the model proposed by Van der Velden et al. (15) *Salmonella*-induced cell death is generally thought to reflect a bacterial strategy to promote disease (1) and seen as a virulence mechanism allowing the bacteria to spread from cell to cell within the host. The delayed, caspase-1 independent, type of *Salmonella*-induced cell death is proposed to be required during the systemic phase of infection and is used to spread intercellularly within apoptotic bodies (15). The LPS mutants displayed similar cytotoxicity to infected macrophages as the wild-type strain while showing increased intracellular numbers. This could be due to increased intracellular replication that could continue for a longer period because of the relatively lower induction of cytotoxicity. However, despite the increased numbers of intracellular bacteria, these mutants were highly attenuated in vivo. This suggests that these LPS mutants were killed at a higher rate or were not able spread from the inguinal lymph nodes. Increased killing could have been due to increased complement-mediated killing as the LPS mutants are highly susceptible to rat complement (Fig. 5). However, the LPS mutants were not killed when mouse serum was used and therefore we could not confirm the in vivo attenuation to be due to increased complement-mediated killing. Still, complement-mediated killing is a likely explanation for the in vivo attenuation since mouse complement should be able to kill, eliminate, or at least reduce bacterial loads when bacterial numbers are low and local concentrations of complement are high. Another explanation could be the increased opsonisation of the LPS mutants with mouse complement that does not result in killing by complement itself, but to increased uptake and killing of the bacteria by granulocytes, thereby preventing the bacteria form reaching the liver and spleen.

We have shown that LPS mutants display increased bacterial intracellular numbers in RAW264.7 macrophages that persist even after 48 h while inducing more cell death. This would suggest that the infected cells that were still intact contained more intracellular bacteria without being lysed. This could mean that the LPS mutants are able to grow very fast intracellularly and are able to reach high bacterial numbers inside macrophages. However, despite the increased ability to survive within cells, these LPS mutants are strongly attenuated in vivo due to mechanisms that need to be studied in more detail. Based on the data presented here, one would suggest that the relatively reduced induction of cell death of the LPS mutants is not beneficial to the in vivo virulence of *S. enterica* serovar Typhimurium, despite the increased ability to survive within macrophages. Apparently, *Salmonella*-induced cell death is a process that needs to be regulated and further research on the relevance of *Salmonella*-induced cell death is necessary before it is clear what the in vivo relevance of *Salmonella*-induced cell death is.

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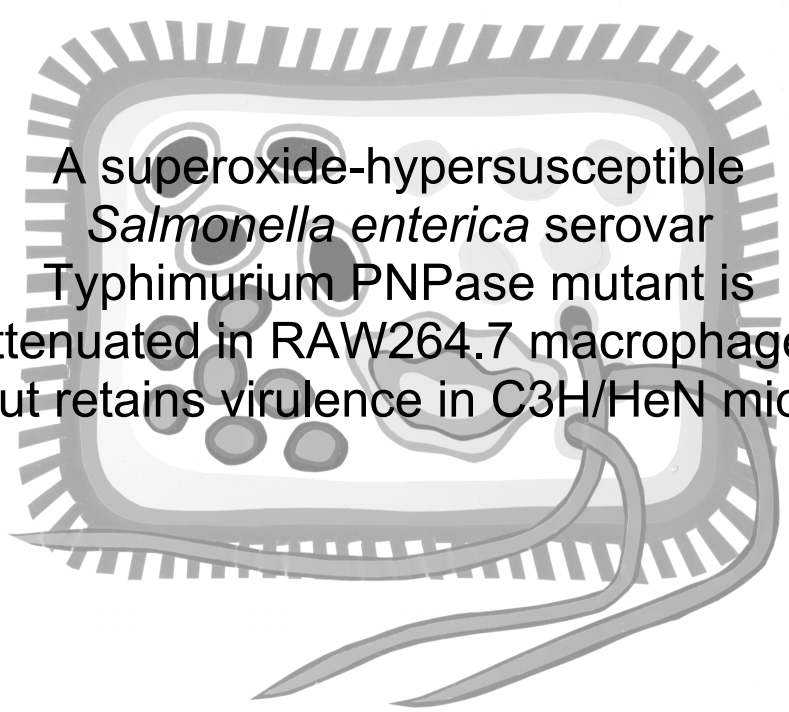
chapter five



# A superoxide-hypersusceptible *Salmonella enterica* serovar Typhimurium PNPase mutant is attenuated in RAW264.7 macrophages but retains virulence in C3H/HeN mice

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*Submitted for publication*

## Abstract

*Salmonella enterica* serovar Typhimurium is a Gram-negative, facultative intracellular pathogen that predominantly invades mononuclear phagocytes. Upon contact with host cells, the intracellular pathogen *Salmonella* promotes its uptake, targeting and survival in its intracellular niche, thereby evading the macrophages' microbicidal effector mechanisms such as oxygen intermediates. Many genes involved in bacterial resistance to superoxide stress have been characterized, but the exact contribution of many of these genes to bacterial survival within macrophages and in vivo virulence in mice is still poorly understood.

By generating *S. enterica* serovar Typhimurium mutants with increased susceptibility to superoxide and investigating the intracellular fate of the strains within macrophages we hope to gain insight into the mechanisms that are essential to *Salmonella* to resist superoxide stress and to survive and replicate within host cells and in mice. Here we describe the isolation of an *S. enterica* serovar Typhimurium mutant lacking the expression of polynucleotide phosphorylase (PNPase). This mutant is hypersusceptible to superoxide and is attenuated in macrophages, yet was more virulent in C3H/HeN mice than the wild-type strain. This diverse fate of a superoxide-hypersusceptible strain underscores the complexity of virulence determinants and demonstrates that care must be taken when extrapolating in vitro findings on intracellular fate to prediction of in vivo virulence.

## Introduction

The Gram-negative, facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium has evolved many mechanisms that allow the bacterium to survive, persist, and even replicate within a host. It predominantly invades mononuclear phagocytes and is able to establish persistent infections by evasion or disturbance of the host defense (13). *S. enterica* serovar Typhimurium can respond to a specific host environment by selectively expressing appropriate factors to prevent the induction or to neutralize the action of antimicrobial effector mechanisms within the macrophage which favor intracellular survival and replication and are necessary for resistance against the defense systems of the host (5, 7, 8, 13, 17). This ability of *S. enterica* serovar Typhimurium to enter and replicate within phagocytic cells is essential for its survival, as mutants unable to do so are avirulent (6). One of the major early defense mechanisms of macrophages against microorganisms is the production of toxic superoxide by the phagocyte NADPH oxidase and the subsequent generation of superoxide derivatives, both in vitro (16) and in vivo (18, 19, 21). Since superoxide is a by-product of normal aerobic metabolism, both eukaryotic and prokaryotic cells have evolved ways to respond to superoxide stress by the activation of genes involved in a protective response (10). In bacteria, these mechanisms include neutralization of phagocyte derived oxidants, repair of damage resulting from such oxidants or prevention of their production all together (17) and are (10) of huge importance for the ability of *S. enterica* serovar Typhimurium to survive within the phagosome. The essential role of the periplasmic Cu,Zn-SOD and the type III secretion system encoded by *Salmonella* pathogenicity island 2 (SPI2) in this defense has been demonstrated, as mutants deficient in one of these systems show reduced survival within macrophages (4, 10, 22).

Research on mutant strains of *E. coli* as well as *Salmonella* has led to the identification of a range of genes that are necessary for survival during oxidative stress generated in vitro. A range of mutants have been isolated and described that are either sensitive to endogenously or to exogenously produced superoxide, or to exogenously as well as endogenously produced superoxide, or to hydrogen peroxide (reviewed in (12)). However, the exact contribution of many of these genes to bacterial survival within macrophages and in vivo virulence in mice is still poorly understood since for a lot of mutants the in vitro sensitivity to superoxide does not translate into attenuated intracellular or in vivo behavior (reviewed in (12)).

By generating *S. enterica* serovar Typhimurium mutants with increased susceptibility to superoxide and investigating the intracellular fate we hope to gain more insight into the mechanisms that are used by *Salmonella* to resist superoxide and to survive and replicate within host cells and in mice. Here we describe the isolation of an *S. enterica* serovar Typhimurium mutant lacking the expression of polynucleotide phosphorylase (PNPase) that is hypersusceptible to superoxide, is severely attenuated in macrophages, but was more virulent in C3H/HeN mice than wild-type *S. enterica* serovar Typhimurium.



## Materials and Methods

**Mice.** Six- to 8-week-old female *Salmonella*-resistant ($Ity^r$) C3H/HeN mice were purchased from Harlan (Horst, The Netherlands). Mice were maintained under standard conditions according to the institutional guidelines in filter top cages. Water and food were given ad libitum. All experiments were approved by the local Animal Ethical Committee.

**Cells and culture conditions.** RAW264.7 were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (50 µg/ml) at 37ºC and 5% $CO_2$.

**Bacterial strains.** The bacterial strains used in this study are enlisted in Table 1. Strain MC1 and MC71 were a kind gift from Dr. Mikael Rhen (Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden). Single colonies of the different strains were grown in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm). For the in vivo experiments, the overnight cultures were diluted in fresh LB medium and grown to the end of log phase and were then washed and diluted in sterile PBS. The CFU in the inoculum were determined by plating serial dilutions.

**Table 1. *Salmonella* strains and plasmids used in this study**

| *Salmonella* Strain | Characteristics | Origin or reference |
|---|---|---|
| *S. enterica* serovar Typhimurium | | |
| 14028s | Wild type | This study |
| AVD101 | 14028s *pnp*::MudJ | This study |
| AVD102 | MudJ transposon insertion in 14028s | This study |
| AVD103 | MudJ transposon insertion in 14028s | This study |
| AVD104 | MudJ transposon insertion in 14028s | This study |
| TT10289 | LT2 *hisD*9953::MudJ *hisA*9949::Mud1 | (11) |

**Generation of *S. enterica* serovar Typhimurium mutants.** Wild type *S. enterica* serovar Typhimurium was used as the parental strain to isolate mutants that displayed increased susceptibility to the superoxide generating agent menadione. Mutants were generated by random P22 MudJ transposon insertion and were selected for superoxide sensitivity by streaking single colonies onto series of M9 agar plates containing 0.5 mg/ml, 0.25 mg/ml, or no menadione (Sigma). A single tip was used to streak on all three plates starting with the plate containing the highest concentration of menadione and ending on the plate containing no menadione. Colonies that did not grow on the plates containing menadione, but that did grow well on the control plate, were selected for further research.

**Disk diffusion assay.** To determine superoxide susceptibility, disk diffusion assays were performed as described by Bauer et al. (1). Briefly, overnight cultures of salmonellae were spread onto M9 plates. A cotton disk containing 30 mmol menadione was placed in the center. After overnight incubation at 37$^{o}$C the diameter of the bacterium-free zone was determined (mm) as a measure of susceptibility.

**Inverse PCR.** To identify the gene in which the MudJ transposon had inserted, the DNA flanking the left end of the transposon was amplified and sequenced using inverse PCR. Genomic DNA of strain AVD16703 was isolated and digested with *HaeIII* (Gibco-BRL) for 4 hours. After inactivating the *HaeIII* enzyme, the sample was treated with T4 DNA ligase (Invitrogen). The digested and ligated DNA was amplified using the following primers: 5'-CCGGGAGGACATTGGATTAT-3' (sense) and 5'-CGTACTTCAAGTGAATCAATAC-3'. The PCR product was purified using the QIAquick PCR purification kit (Qiagen) and was sequenced.

**Replication within macrophages.** RAW264.7 cells ($2 \times 10^5$) were challenged with *S. enterica* serovar Typhimurium at a 10:1 multiplicity of infection (MOI). The bacteria were spun onto the cell by centrifugation for 10 min at 270 $\times$ g to promote bacterial uptake. Cells were allowed to internalize the bacteria for 30 min at 37°C and 5% $CO_2$. After washing the cells with PBS, medium containing 100 μg/ml gentamicin was added to kill the extracellular bacteria and cells were incubated at 37°C for 1 h. The cells were then washed again. This was designated time point zero. Medium containing 10 μg/ml gentamicin was added to the cells to kill any remaining extracellular bacteria and to prevent reinfection. At 0, 24, and 48 hours the cells were lysed in 1 ml $H_2O$ and serial dilutions were made to determine the number of intracellular CFU.

**In vivo infection experiment.** Mice were inoculated subcutaneously in the flanks with 0.1 ml bacterial suspension containing $3 \times 10^4$ CFU *S. enterica* serovar Typhimurium 14028s or 14028s *pnp*::MudJ. For each group on each time point 4-6 mice were used. Mice were sacrificed by carbon dioxide inhalation and blood was taken immediately by cardiac puncture. Spleens, livers, and inguinal lymph nodes were aseptically removed and single cell suspensions were prepared by using sterile 70-µm-mesh-size cell strainers (Falcon). Cells were pelleted by centrifugation for 10 min and were lysed in distilled water. The bacterial number per organ was determined bacteriologically by plating serial dilutions.

**Western Blot analysis.** To analyze the expression of PNPase in wild-type *S. enterica* serovar Typhimurium strains and PNPase mutants total bacterial extracts were prepared by lysing the bacteria in Laemmli's sample buffer (62.5 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 5% b-mercaptoethanol). The proteins were separated by SDS PAGE on an 11% Lugtenberg polyacrylamide gel (14) and were blotted onto nitrocellulose. Western blots were assayed using 1:5,000 polyclonal rabbit serum to PNPase (a kind gift from Dr.



Mikael Rhen, Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden).

**Statistics.** Statistical analysis was performed using Mann-Whitney rank order tests or Student's *t* test and a *P* value <0.05 was considered significant.

## Results

**Isolation and selection of *S. enterica* serovar Typhimurium mutants that are hypersusceptible to superoxide.** A total of 165 independently isolated mutants were selected from the series of M9 plates supplemented with 0, 0.25, or 0.5 mg/ml menadione. The superoxide sensitivity of the mutants was confirmed by disc diffusion assay. Diameters of bacterial-free zones were determined and four mutants were shown to be extremely sensitive to superoxide (Table 2) (bacteria free zone ≥35 mm). These four strains were selected for further research. First, the number of bacteria in RAW264.7 macrophages after 24 h was determined as described in Materials and Methods. Only one of the four mutants was able to reach bacterial numbers comparable to those of the wild-type (Figure 1), whereas the other three were attenuated. One strain, AVD101, did not show any net replication over 24 h and was selected for further analysis. By sequence analysis of the inverse PCR product, it was shown that the MudJ transposon had inserted in the promotor region of *pnp*, the gene encoding polynucleotide phosphorylase (PNPase) (Fig. 2).

**Table 2. Analysis of susceptibility to menadione by disk diffusion assay**

| | Mean zone of growth inhibition (mm) | | |
|---|---|---|---|
| *Salmonella* Strain | Menadione (30 mmol) | $H_2O_2$ (30 μg) | Gentamicin (100 μg) |
| 14028s | 27 | 26 | 26 |
| AVD101 | 35 | 32 | 36 |
| AVD102 | 40 | 34 | 38 |
| AVD103 | 35 | 28 | 30 |
| AVD104 | 37 | nd[a] | nd |

[a] nd, not determined

**In vitro growth of 14028s *pnp*::MudJ in RAW264.7 macrophages.** AVD101 was taken up by the macrophages to the same extent as the wild-type strain (i.e. $\pm 2.5 \times 10^4$ CFU) and bacterial numbers remained as high as those of the wild-type strain at 3 h after infection. However, after 24 h, the wild-type strain had grown out reaching bacterial numbers up to $3.6 \times 10^5$ CFU, whereas AVD101 only reached $1.5 \times 10^5$ CFU (Fig. 3A). We

compared our wild-type and *pnp*::MudJ mutant with the MC1 (wild-type strain) and MC71 (a known PNPase$^{-/-}$ derivative) strains described by Clements et al. (3). The wild-type strain MC1 was able to grow out within 24 h reaching higher numbers that 14028s. The number of intracellular bacteria was lower for the PNPase mutant MC71 compared to the corresponding wild-type strain MC1, but was higher than that of AVD101 (Fig. 3A).

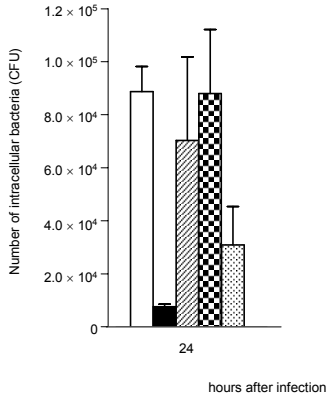


**Figure 1.** Number of intracellular bacteria in RAW264.7 murine macrophage-like cells infected with wild-type *S. enterica* serovar Typhimurium 14028s (white bar) and the superoxide hypersusceptible mutants AVD101 (black bar), AVD102 (dashed bar), AVD103 (checkered bar), and AVD104 (dotted bar). At 24 h after infection of the cells the numbers of intracellular bacteria were determined bacteriologically.

**Western Blot analysis to determine PNP expression.** Expression of PNPase in our mutant AVD101 was analyzed by Western Blot analysis on total cell lysates of overnight LB broth cultures of wild-type and AVD101 and polyclonal rabbit serum raised to PNPase. As control we included strain MC1 expressing wild-type PNPase and MC71 a strain that expresses a truncated and non-functional PNPase. MC1 and 14028s express wild-type PNPase and MC71 the truncated PNPase as described (3) (Fig. 3B). AVD101, however, expresses no PNP and can be considered a PNPase knockout strain (Fig. 3B).

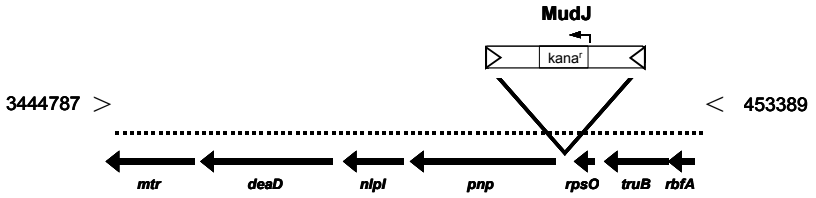


**Figure 2.** Position of the MudJ transposon in the genome of *S. enterica* serovar Typhimurium. The MudJ transposon had inversely inserted in the promotor region of *pnp*, the gene encoding PNPase.

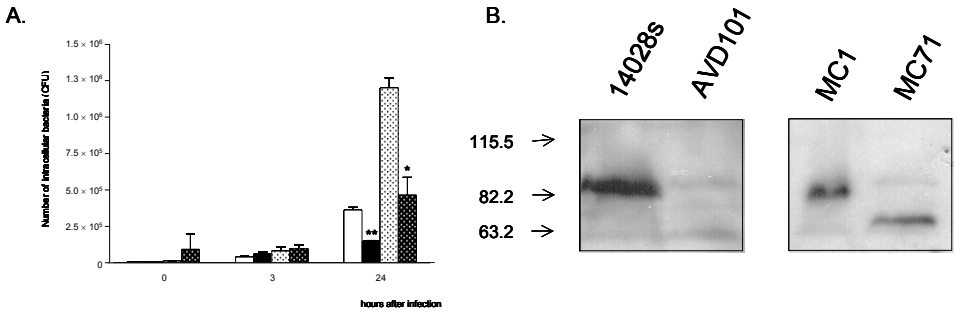


**Figure 3.** Intracellular *S. enterica* serovar Typhimurium in RAW264.7 mouse macrophage-like cells at 0, 3, and 24 h after infection (A) and expression of PNPase in a total extract (B) of *S. enterica* serovar Typhimurium 14028s (white bars, wild-type), AVD101 (black bars, 14028s *pnp*::MudJ), MC1 (white bars with black dots, wild-type), MC71 (black bars with white dots, truncated PNPase). Asterisks indicate that the number of intracellular bacteria is significantly different from that of the parental wild-type *S. enterica* serovar Typhimurium strains. Two asterisks indicate P<0.005 and one asterisk P<0.05.

**In vivo virulence of 14028s and AVD101 in *Salmonella*-resistant C3H/HeN mice.** Because the superoxide hypersusceptible mutant AVD101 appeared to be avirulent in RAW64.7 macrophages, we next assayed the in vivo virulence in *Salmonella*-resistant C3H/HeN mice (Ity$^r$). Mice were infected subcutaneously in the flanks as described (20). AVD101-infected mice showed a strong reduction in body weight after day 6 (Fig. 4A) starting to be statistically significant different from those mice infected with the wild-type strain on day 9 (Fig. 4A). Also, AVD101-infected mice showed signs of illness like ruffled fur and malaise on day 7 while the wild-type-infected mice still appeared healthy. The increase in liver and spleen weight was similar for 14028s and AVD101 infected mice (Fig. 4B and 4C). All the mice showed a moderate increase in the number of bacteria in the inguinal lymph nodes (Fig. 4D) and no statistically significant differences could be observed between the two groups of mice. In the livers and spleens on the other hand, there was a large increase in bacterial numbers in both groups (Fig. 4E and 4F). The number of AVD101 was significantly higher than those of the wild-type strain on day 5 and remained increased as compared to the wild-type up to day 12, although at that timepoint the difference was no longer statistically significant. Thus, mutant AVD101 appeared to be at even more capable of replication than wild-type *S. enterica* serovar Typhimurium 14028s in C3H/HeN mice.

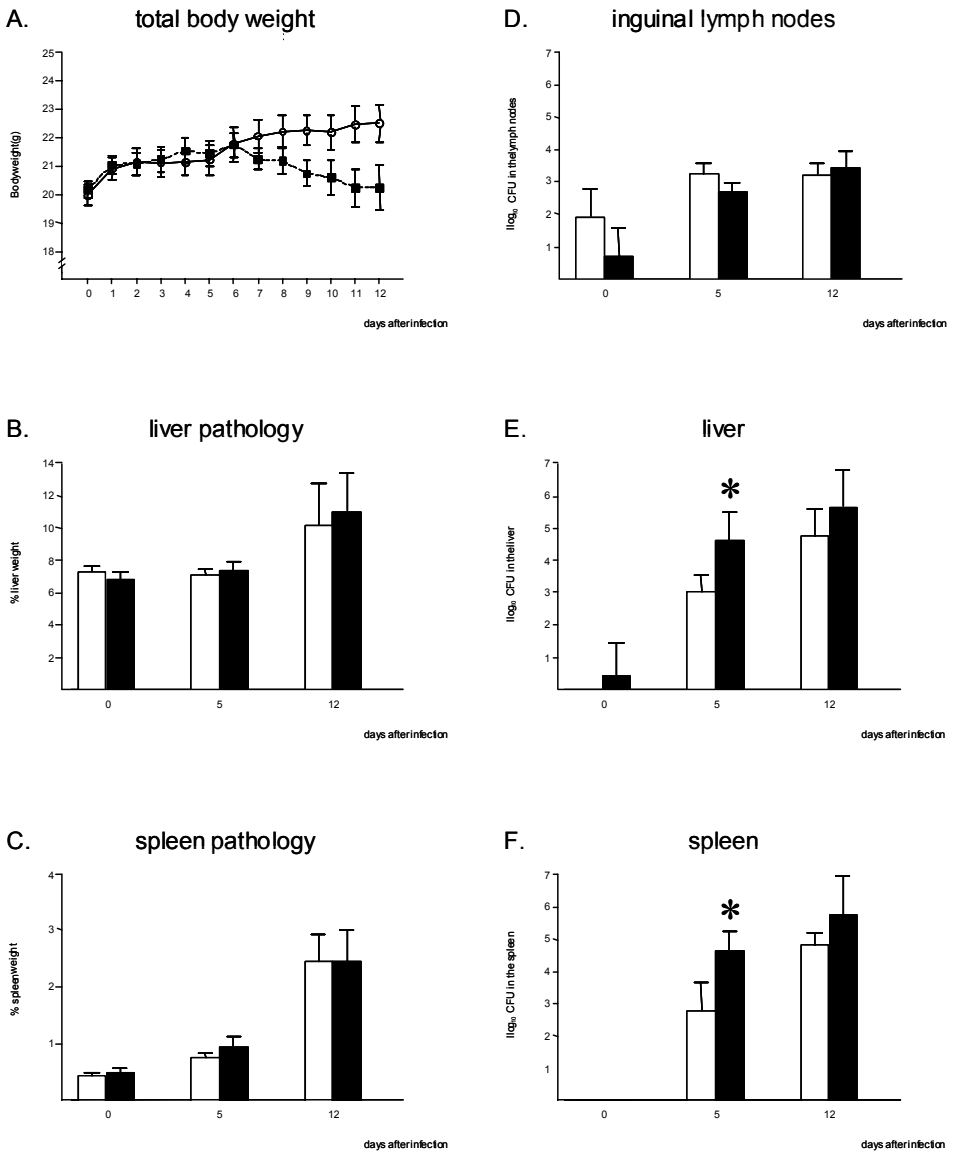


**Figure 4**. Total body weight (A), induced pathology in the spleen (B) and liver (C), number of bacteria in the inguinal lymph nodes (D), liver (E), and spleen (F), and intracellular number of bacteria in the spleen (G). Mice were injected subcutaneously in the flanks with $3 \times 10^4$ CFU of 14028s (white dots and white bars), AVD101 (black squares and black bars). At the indicated time points, livers, spleens, and lymph nodes were aseptically removed and weighed. The viable counts within the organs were determined by making lysates and plating serial dilutions of the lysates, and are expressed as $\log_{10}$ viable counts (means ± standard errors of the means). Averages from 4-6 mice per time point and per group are shown. Asterisks indicate statistically significant differences of AVD101 compared to the wild-type 14028s-infected mice (Student's *t* test) and the gray dashed lines represent the detection limit of the microbiological method (50 CFU for the livers and 30 CFU for the spleens and lymph nodes).

## Discussion

The main findings of this study are that AVD101, a mutant lacking the expression of cold-shock-associated exoribonuclease polynucleotide phosphorylase (PNPase) due to MudJ transposon insertion into the promotor region of *pnp*, displays hypersusceptibility to superoxide in vitro, is attenuated after ingestion by macrophages in vitro, yet appears at least as or even more virulent than the wild-type strain in C3H/HeN ($Ity^r$) mice. Western blot analysis revealed that this mutant AVD101 showed no expression of the protein PNPase and therefore can be considered a PNPase knock-out strain.

Because the MudJ transposon had inserted into the promotor region of *pnp*, the MudJ transposon could have influenced the expression of *pnp* in two ways. The insertion might lead to a complete lack of *pnp* expression resulting in the absence of the protein PNPase. In addition, it might have a polar effect on the genes that are directly down-stream of *pnp* and that could have been affected by the MudJ transposon as well. By Western blot analysis it was confirmed that the mutant strain AVD101 lacks expression of PNPase and therefore, lacks phosphorylytic exoribonuclease activity. This means that our mutant should behave like the mutant MC71 described by Clements et al. which expresses a non-functional truncated PNPase, shows an increased intracellular replication rate, and is able to cause persistent infection in BALB/c mice (3). However, whereas our mutant AVD101 was not able to grow out as much as the wild-type strain in RAW264.7 macrophages, it was shown previously that MC71 showed increased intracellular growth (3). Increased intracellular growth could lead to increased cytotoxicity and cell lysis, and reduced intracellular bacterial numbers due to cell loss which might be interpreted as attenuation. For AVD101, however, the in vitro attenuation of AVD101 could not be explained by increased cytotoxicity and increased cell-loss since the in vitro challenged cells were intact after 24 h and the induced cytotoxicity was similar to that induced by the parental wild-type strain (data not shown). Therefore, we concluded that this strain lacks the ability to replicate within RAW264.7 macrophages. Strikingly, this mutant was more virulent in vivo upon subcutaneous infection of C3H/HeN mice. This is based on the observation that the bacterial numbers were higher in the livers and spleens of AVD101 infected mice. Most strikingly and opposite to mice injected with wild-type *S. enterica* serovar Typhimurium, these mice showed weight loss on day 6 to 12 after infection (Fig. 5A).

The question was what could be the mechanism explaining the diverse phenotype of the AVD101 strain. PNPase belongs to the family of the exoribonucleases (2) and in *E. coli* has been shown to be involved in adaptation to growth at low temperatures by degrading mRNA that encode cold shock proteins thereby allowing resumption of bacterial growth (23). The gene *pnp* in *S. enterica* serovar Typhimurium is homologous to the gene in E. coli and a mutant expressing a truncated and non-functional form of PNPase shows a restricted cold adaptation response as seen for *E. coli* mutants deficient in PNPase (3, 23). *S. enterica* serovar Typhimurium contains two important gene clusters in localized regions of the chromosome that are involved in the invasion of and survival within phagocytes. These regions are called *Salmonella* pathogenicity islands (SPI-1 and SPI-2) and they

contain several genes that are involved in the delivery of virulence proteins into the host cell (9, 15). The action of the proteins encoded by these genes leads to the uptake of the bacteria by epithelial cells and to intracellular survival and replication within macrophages, respectively. Recently, it has been shown that PNPase in *S. enterica* serovar Typhimurium is involved in the global regulation of the expression of genes encoded by SPI1 and SPI2 (3). MC71, the mutant lacking functional PNPase showed increased mRNA levels coded by genes of SPI1 and SPI2 leading to increased bacterial replication rates in mice and murine macrophages (3). Our mutant AVD101 lacks the expression of PNPase and resembles the MC71 mutant. It is therefore most likely that mRNA levels coded by SPI1 and SPI2 are increased in this mutant as well which could help explaining the increased in vivo virulence. In contrast to the MC71 mutant, the AVD101 mutant showed reduced intracellular survival within RAW264.7 macrophages that could not be explained by an increase in induced cytotoxicity as one would expect when bacteria show strongly increased intracellular growth as observed for the MC71 mutant (3). A possible explanation for the in vitro attenuation of AVD101 could be the extremely increased susceptibility to superoxide (Table 2). Superoxide plays a very important role in the host defense against *S. enterica* serovar Typhimurium as is shown by the susceptibility to infection of mice deficient in a functional superoxide generating NADPH oxidase system (16). *Salmonella*, on the other hand, has developed mechanisms to resist such killing by superoxide. It has been shown previously that the type III secretion system encoded by SPI2 is essential in this defense, as mutants deficient in one of these systems show reduced survival within macrophages (10, 22) but regain virulence in cells that cannot produce any superoxide (22). Expression of SPI2 genes needs to be regulated to allow the bacteria to survive and replicate intracellularly. Defects in functional PNPase resulted in increased levels of mRNA of SPI2 encoded genes and suggested a role for PNPase in SPI2 gene regulation (3). This, together with the role of SPI2-encoded genes in superoxide defense, suggests that the relatively decreased in vitro intracellular survival of AVD101 and MC71 compared to wild-type despite the increased growth rate (3) is due to the increased susceptibility to superoxide. This suggests a role for PNPase in superoxide resistance and thus for survival within macrophages.

The reason why the PNPase mutants display increased in vivo virulence might be explained by increased uptake of the bacteria since most of the mRNA regulated by PNPase are coded by genes of SPI1 and encode proteins involved in invasion of phagocytic as well as non-phagocytic cells. The *pnp* mutant might have ended up in a different compartment within the host allowing the bacterium to survive and replicate, and resulting in increased virulence. Based on these data, one would expect that the PNPase mutant invades the non-phagocytic cells at a higher rate than the wild-type strain and if it also displays increased intracellular growth within these non-phagocytic cells as it did in vitro within macrophages, then this would explain the enhanced in vivo virulence since these non-phagocytic cells are not equipped with the superoxide producing NADPH oxidase complex and therefore cannot produce toxic amounts of superoxide.



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# Isolation and characterization of *Salmonella enterica* serovar Typhimurium mutants with increased or decreased susceptibility to superoxide

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appendix one

## Abstract

One of the early host defense mechanisms against microorganisms like *Salmonella enterica* serovar Typhimurium is the production of superoxide radicals and derivatives (4, 8). *Salmonella*, however, has developed mechanisms to resist this killing mechanism by preventing the production of these toxic compounds or by neutralizing the oxidants. A range of genes necessary for bacterial survival during oxidative stress has been identified but the exact role of many of these genes in survival of *Salmonella* within macrophages is still not completely understood.

We have generated *S. enterica* serovar Typhimurium mutants by random MudJ transposon insertion and selected for those strains with increased susceptibility to redox cycling agents that release superoxide intracellularly and next tested these strains for their ability to replicate intracellularly within RAW264.7 macrophages. By inverse PCR we determined the position in the genome where the MudJ transposon had inserted. Combining the data from several mutants might yield insight into the survival mechanisms of *S. enterica* serovar Typhimurium during superoxide stress encountered outside and within macrophages.

We have isolated 24 mutants that displayed hypersusceptibility to menadione. Of these 13 mutants retained full virulence, whereas 11 mutants were attenuated in vitro in macrophages. We have also isolated 6 mutants that were less susceptible to menadione than the wild-type strain and 4 were attenuated in vitro. We concluded that the fate of replication within macrophages cannot be predicted on the basis of the in vitro susceptibility to the redox cycling agent releasing intracellular superoxide, as some of the superoxide-susceptible mutants appeared to be just as or even more virulent than the wild-type while in the group of superoxide-resistant strains four out of six mutants displayed an attenuated in vitro phenotype. Still, mutational analysis should be helpful to predict the in vitro and in vivo phenotype.

## Introduction

Research on mutant strains of *Salmonella* has led to the identification of a number of genes that are necessary for bacterial survival during oxidative stress but the exact contribution of many of these genes for survival of the bacteria within macrophages is still poorly understood (reviewed in (3)). To gain insight into strategies that are used by *Salmonella* to resist superoxide stress and to survive and replicate within macrophages, we generated *S. enterica* serovar Typhimurium mutants by random MudJ transposon insertion and selected for strains with increased susceptibility or resistance to superoxide. These strains were tested for their ability to replicate intracellularly within RAW264.7 macrophages and by inverse PCR we determined the position in the genome where the MudJ transposon had been inserted.

## Materials and Methods

**Cells and culture conditions.** RAW264.7 were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (50 µg/ml) at 37ºC and 5% $CO_2$.

**Bacterial strains.** The bacterial strain used in this study is *S. enterica* serovar Typhimurium 14028s (ATCC). Single colonies of the different strains were grown in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm).

**Generation and isolation of *S. enterica* serovar Typhimurium mutants.** Mutants were generated by random P22 MudJ transposon insertion in 14028s and selected for superoxide sensitivity by streaking single colonies onto series of M9 agar plates containing 0.5 mg/ml, 0.25 mg/ml, or no menadione (Sigma).

**Disk diffusion assay.** To determine susceptibility to superoxide, disk diffusion assays were performed as described by Bauer et al. (2). Briefly, overnight cultures of *Salmonellae* were spread on M9 plates. A cotton disk containing 30 mmol menadione was placed in the center. After overnight incubation at 37$^{o}$C the diameter of the bacterium-free zone was determined (mm) as a measure for susceptibility.

**Inverse PCR.** To identify the gene in which the MudJ transposon had inserted, the DNA flanking the left end of the transposon was amplified and sequenced by inverse PCR using primers: 5'-CCGGGAGGACATTGGATTAT-3' (sense) and 5'-CGTACTTCAAGTGAATCAATAC-3' (antisense). The PCR product was purified using the QIAquick PCR purification kit (Qiagen) and was sequenced.



**Replication within macrophages.** RAW264.7 cells ($2 \times 10^5$) were challenged with *S. enterica* serovar Typhimurium at a 10:1 multiplicity of infection (MOI) as described previously (7). At 0 and 24 hours the cells were lysed in 1 ml milliQ and serial dilutions were made to determine the number of intracellular CFU.

## Results and Discussion

By multiple random MudJ transposon insertions in wild-type *S. enterica* serovar Typhimurium 14028s a total of 306 mutants were selected from the series of M9 plates supplemented with 0, 0.25, or 0.5 mg/ml menadione. These mutants were assayed for superoxide sensitivity using disk diffusion assays with the redox cycling agent menadione. Diameters of bacteria-free zones showed that from the mutants tested, 24 showed an increased and six showed a decreased susceptibility to intracellular superoxide induced by menadione when compared to the wild-type strain 14028s (Table 1). These mutants were selected for further research and were next tested for the ability to survive within RAW264.7 macrophages (Fig. 1).

On the basis of the in vitro susceptibility to intracellular superoxide and in vitro replication potential within macrophages the mutants can be divided into four groups: susceptible/attenuated (group 1), susceptible/virulent (group 2), more resistant/attenuated (group 3), and more resistant/virulent (group 4). For some of the mutants in each group, we identified the gene in which the MudJ transposon had inserted by amplifying and sequencing the left flanking DNA by inverse PCR. For reasons at present unknown, we so far succeeded in getting inverse PCR products for 9 mutants (Table 1).

Group 1 consists of 11 mutants of which four (AVD101, MM4, DvD32, and DvD71) are extremely attenuated. For mutant MM4 the MudJ transposon had inserted into *hycE*, the gene encoding a protein that is similar to the *E. coli* large subunit of hydrogenase 3 (part of FHL complex) and for mutant DvD71 in *ybiT*, encoding a putative ATPase component of ABC transporter with duplicated ATPase domain. The other 7 mutants (MM11, MM18, MKS6.2, DvD46, and DvD66) were moderately attenuated compared to the wild-type strain. The position of the MudJ transposon is only known for mutant ND6, MM18 and MM11. The phenotype of mutant ND6 is caused by MudJ transposon insertion in *pabC*, the gene encoding PabC, a 4-amino-4-deoxychorismate lyase (ADC lyase) that converts ADC into p-aminobenzoate (PABA) which is a precursor of folate and component of folic acid (1). Folate cannot be taken up from the environment so endogenous synthesis is essential for growth and cell processes. For MM18 the transposon had inserted into *nupC*, the gene that encodes a protein similar to *E.coli* NUP family nucleoside transport protein. Mutant MM11 contains a MudJ transposon insertion in *sitC* that encodes a *fur* regulated *Salmonella* iron transporter. *SitC* is is part of the *sit* operon encoding an ABC transporter that is involved in the transport of a siderophore-iron complex across the inner membrane and that is regulated by *fur* (5). Fur is the regulator of genes involved in the uptake of iron from the iron-limiting environment such as within a host. Uptake of iron needs to be tightly

regulated since free Fe(II) is toxic to the bacteria as it reacts with hydrogen peroxide in the Fenton reaction. Chelation of intracellular iron is a method to protect the bacteria from killing by hydrogen peroxide (reviewed in (3)).

**Table 1. Susceptibility of *S. enterica* serovar Typimurium mutants to menadione**

| Mutant | bacterial-free zone (mm) | inactivated gene | in vitro phenotype |
|---|---|---|---|
| 14028s | 26 ± 1 | | V[b] |
| ND102 | 36 ± 1 | nd[a] | V |
| ND103 | 30 ± 1 | nd | V |
| MM3 | 37 ± 1 | *yhgE* | V |
| MKS2 | 34 ± 1 | *kup* | V |
| MKS6.2 | 36 ± 1 | nd | V |
| DvD1 | 29 ± 2 | nd | V |
| DvD50 | 30 ± 2 | *ybdM* | V |
| DvD51 | 31 ± 3 | nd | V |
| DvD54 | 31 ± 1 | nd | V |
| DvD69 | 31 ± 1 | nd | V |
| DvD70 | 32 ± 1 | nd | V |
| M30 | 34 ± 2 | nd | V |
| MdR2 | 37 ± 2 | nd | V |
| ND6 | 32 ± 2 | *pabC* | A[c] |
| AVD101 | 31 ± 1 | *pnp* | A |
| ND104 | 34 ± 2 | nd | A |
| MM11 | 31 ± 1 | *sitC* | A |
| MM18 | 32 ± 1 | *nupC* | A |
| MM4 | 40 ± 1 | *hycE* | A |
| MKS6.1 | 40 ± 2 | nd | A |
| DvD32 | 29 ± 1 | nd | A |
| DvD46 | 29 ± 2 | nd | A |
| DvD66 | 29 ± 1 | nd | A |
| DvD71 | 30 ± 1 | *ybiT* | A |
| | | | |
| ND23 | 21 ± 1 | nd | V |
| MdR23 | 19 ± 1 | nd | V |
| ND32 | 20 ± 1 | nd | A |
| ND34 | 20 ± 1 | nd | A |
| ND37 | 20 ± 1 | nd | A |
| ND38 | 21 ± 1 | nd | A |

[a]nd, not determined
[b]V, virulent
[c]A, attenuated



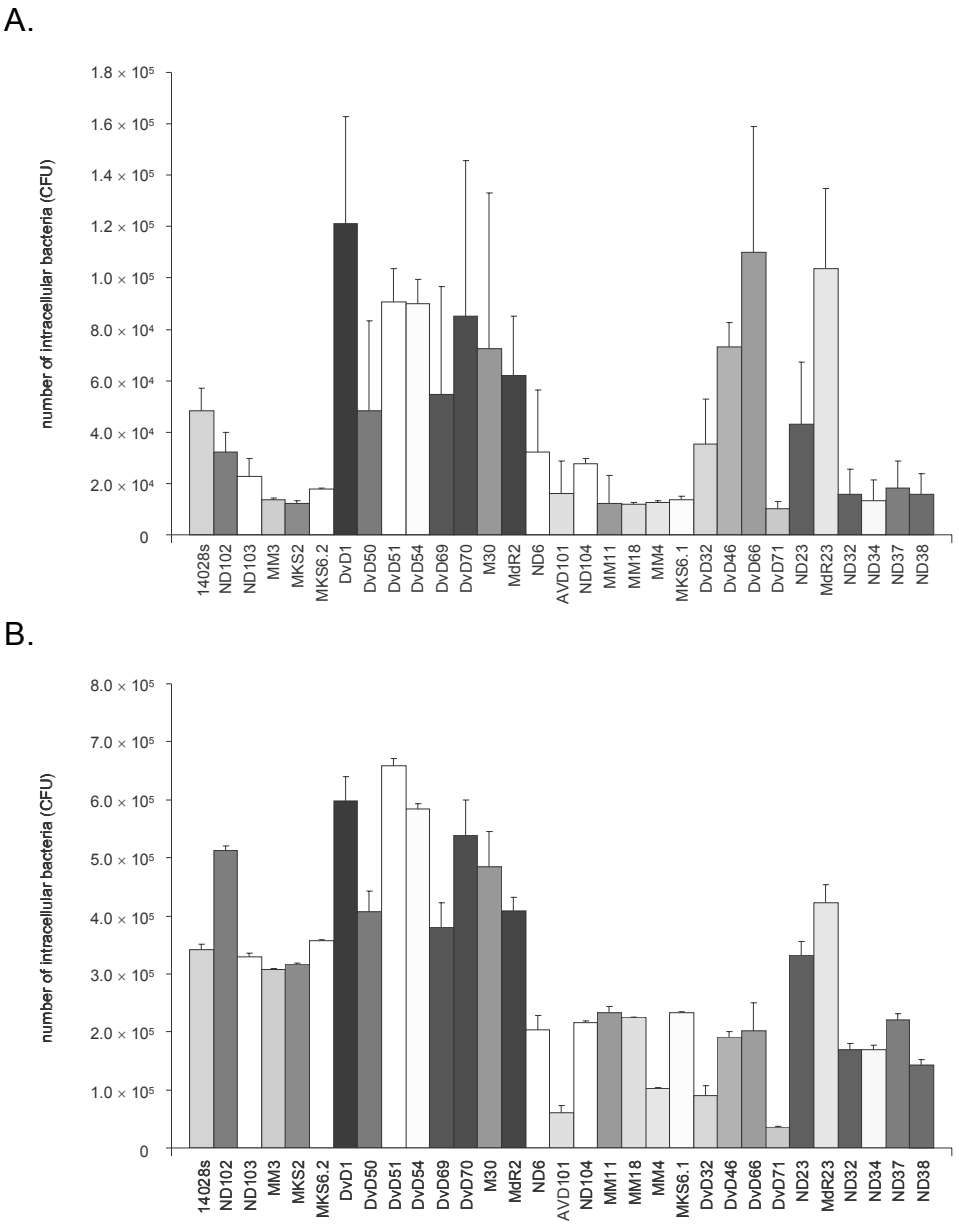


**Figure 1.** Number of intracellular bacteria in RAW264.7 murine macrophage-like cells infected with wild-type *S. enterica* serovar typhimurium 14028s and several random MudJ transposon insertion mutants with increased or decreased susceptibility to intracellular superoxide induced by menadione. At 0 (A) and 24 h (B) after infection of the cells the numbers of intracellular bacteria were determined bacteriologically. Average data ± SEM from 2-6 in vitro infection experiments is shown.

Seven strains in group 2 were as virulent as the wild-type (ND103, ND104, MKS2, MKS6.2, DvD50, DvD69, MdR2) and six mutants even seemed to be more virulent (ND102, DvD1, DvD51, DvD54, DvD70, M30). Of these mutants the MudJ transposon position is only known for MKS2 in which *kup*, encoding a protein similar to *E. coli* low affinity potassium transport system is inactivated and for DvD50 in which the putative transcriptional regulator of *ybdN* (= putative 3'-phosphoadenosine 5'-phosphosulphate sulfotransferase (PAPS reductase)/FAD synthetase encoded by *ybdM* is inactivated.

Group 3 contains four mutants (ND32, ND34, ND37, and ND38) that are moderately attenuated in RAW264.7 macrophages. For neither of these mutants we were able to get an inverse PCR product, so the position of the MudJ transposon in the genome is unknown.

Group 4 only consists of two mutants (ND23 and MdR23) that display an in vitro phenotype that is comparable to the wild-type strain. As for the mutants of group three no inverse PCR products were obtained.

The main conclusion that can be drawn from the data presented here is that the in vitro phenotype cannot be predicted from the in vitro susceptibility to intracellular superoxide generated intracellularly by the redox cycling agent menadione. The in vitro phenotypes of the selected mutants were all completely different and this strongly suggests that factors other than susceptibility to superoxide play a role in in vitro virulence. With respect to prediction of virulence in mice, the situation becomes even more complex since strain AVD101, as described in Chapter 5, is extremely susceptible to intracellular superoxide, is attenuated in macrophages, but is more virulent than the wild-type strain upon subcutaneous infection of C3H/HeN mice (6). The genetic defects caused by the MudJ transposon insertion leading to increased susceptibility to superoxide also appeared to be very diverse and did not directly explain the observed in vitro susceptibility to superoxide nor the in vitro intracellular phenotype.

To be able to predict the in vitro and in vivo phenotype on the basis of the in vitro susceptibility to superoxide in the future, more research is needed. Many more mutants need to be tested in in vitro infection experiments and for all mutants the inactivated gene should be identified. In addition, these mutants need to be tested in vivo as well, since even the in vivo phenotype cannot always be predicted from the intracellular behavior.

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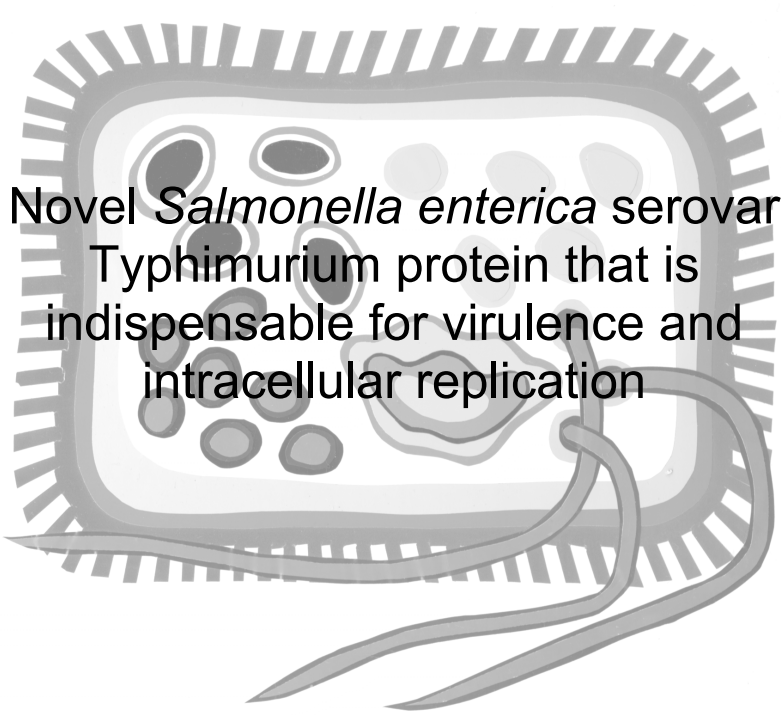
chapter six



# Novel *Salmonella enterica* serovar Typhimurium protein that is indispensable for virulence and intracellular replication

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## Abstract

Upon contact with host cells, the intracellular pathogen *Salmonella enterica* serovar Typhimurium promotes its uptake, targeting and survival in intracellular niches. In this process, the bacterium evades the microbicidal effector mechanisms of the macrophage, including oxygen intermediates. This study reports the phenotypic and genotypic characterization of an *S. enterica* serovar Typhimurium mutant that is hypersusceptible to superoxide. The susceptible phenotype is due to a MudJ insertion-inactivation of a previously undescribed *Salmonella* gene designated *sspJ* that is located between 54.4 and 64 minutes of the *Salmonella* chromosome, and encodes a 392 amino acid protein. In vivo, upon intraperitoneal injection of $10^4$ to $10^7$ bacteria in C3H/HeN and $10^1$ to $10^4$ bacteria in BALB/c mice, the mutant strain was less virulent than the wild type. Consistent with this finding, during the first hour after ingestion by macrophage-like J774 and RAW264.7 cells in vitro, the intracellular killing of strain *sspJ*::MudJ is enhanced 5-fold over that of wild-type microorganisms. Wild-type *Salmonellae* displayed significant intracellular replication during the first 24 hours after uptake but *sspJ*::MudJ mutants failed to do so. This phenotype could be restored to that of the wild type by *sspJ* complementation. The SspJ protein is found in the cytoplasmic membrane and periplasmic space. Amino acid sequence homology analysis did reveal a leader sequence and putative pyrroloquinoline quinone-binding domains, but no putative protein function. We excluded the possibility that SspJ is a scavenger of superoxide, or has superoxide-dismutase activity.

## Introduction

Intracellular pathogens like *Salmonella enterica* serovar Typhimurium respond to a specific host environment by selectively expressing appropriate factors which favor intracellular survival (10, 11, 14). *Salmonella* species predominantly invade the Peyer's patches and later during infection survive in mononuclear phagocytes. Salmonellae can prevent the induction or neutralize the action of antimicrobial effector mechanisms within the macrophage and can therefore survive and multiply within phagosomes (5, 10, 11, 14, 17). The ability of *S. enterica* serovar Typhimurium to enter and grow within epithelial cells and macrophages is essential for its survival, and mutants unable to do so are avirulent (9). Several genes involved in the intracellular survival of salmonellae have been identified. These genes include members of the *phoP/Q* regulon and housekeeping genes. In some cases, however, the function of the genes has yet to be determined (2); some of these genes are also found in *Escherichia coli,* making their relevance to the intracellular survival of salmonellae uncertain (13).

One of the major macrophage microbicidal effector molecules is reactive oxygen-intermediates, beginning with the production of superoxide by NADPH-oxidase. Since superoxide is a by-product of normal aerobic metabolism, both eukaryotic and prokaryotic cells have evolved ways to respond to superoxide stress by the activation of genes involved in a protective response (18). In *E. coli*, the *soxR/S* regulon is an important adaptive defense system against oxidative stress (19), and it is likely that the same holds for salmonellae. However, an *S. enterica* serovar Typhimurium *soxS* knockout strain is as virulent as the wild type, indicating that other systems can counteract the toxic effects of superoxide intermediates (8).

To neutralize superoxide, salmonellae produce four superoxide dismutases (SODs): an Fe-SOD, an Mn-SOD and two Cu,Zn-SODs (4, 7). The first two are produced in the cytoplasm, and although deletion of these genes increases in vitro susceptibility to superoxide generating agents, it does not alter virulence. The periplasmic Cu,Zn-SODs however, are important for *S. enterica* serovar Typhimurium, as mutants carrying mutations in both SODs are attenuated (7). Another protein that is necessary for survival under oxidative stress is the *zwf*-encoded glucose-6-phosphate dehydrogenase (G6PDH) (15). Recently, it was proposed that salmonellae might evade the NADPH-oxidase activity of phagocytes through a mechanism that depends on the function of genes located within pathogenicity island-2 (12). This pathogenicity island is notable for containing genes that are involved in the translocation of bacterial proteins into the host cell cytoplasm. Taken together, these findings indicate that numerous genes scattered over the *Salmonella* chromosome are necessary for combating oxidative stress.

In this study an *S. enterica* serovar Typhimurium mutant was identified that is hypersusceptible to superoxide due to disruption of a previously undescribed gene, designated *sspJ* (superoxide susceptibility protein). Based on protein sequence homology, conserved domains were identified, although no putative protein function could be predicted.



## Materials and Methods

**Bacterial strains, media and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) or minimal medium (M9) at 37°C. Where required, the medium was supplemented with kanamycin (50 µg/ml; Sigma) or ampicillin (50 µg/ml; Merck). Disk diffusion assays were performed on M9 agar plates of standardized volume.

**DNA manipulations.** Standard manipulations were performed as described by Maniatis et al (16). Restriction enzymes and other modifying enzymes were purchased from Gibco-BRL or Promega. Sequence analysis was performed using the Amersham T7 sequence kit.

**Table 1. Salmonella strains and plasmids used in this study**

| Strain or plasmid | Characteristics | Origin or reference |
|---|---|---|
| ***S. enterica* serovar Typhimurium** | | |
| ATCC 14028s | wild-type | ATCC |
| MD36 | Resistance to menadione | This study |
| MD36.12 | MudJ insertion in MD36 | This study |
| DLG294 | 14028s *sspJ*::MudJ | This study |
| DLG294-pWSK29 | DLG294 with plasmid | This study |
| DLG294-pWSK-*sspJ* | DLG294 complemented with *sspJ* | This study |
| **Plasmid** | | |
| pWSK29 | Low-copy-number plasmid | (24) |
| pTS175 | pWSK29 containing *sspJ* | This study |
| pBluescript | $SK^-$ | Stratagene |
| pTS125 | pBluescript containing *sspJ* | This study |
| pET19b | Prokaryotic expression vector | Novagene |

**Selection of superoxide-resistant and superoxide-hypersusceptible *Salmonella* mutants.** *S. enterica* serovar Typhimurium ATCC strain 14028s was used as the parental strain to isolate mutants that displayed increased resistance against superoxide, by an indirect strategy employing menadione and antibiotics as described for the isolation of *soxR/S* mutants in *E. coli* (5). Wild-type *S. enterica* serovar Typhimurium was subjected to random chemical mutagenesis by exposure to the alkylating agent nitrosoguanidine (MNNG). Briefly, late-log-phase *S. enterica* serovar Typhimurium was washed and exposed to MNNG (0.1 mg/ml) in citrate buffer for 90 minutes at 37°C. Next, the mutagen

was removed by spinning and washing the bacteria, followed by recovery in LB. Bacteria were plated on M9 supplemented with menadione in concentrations varying from 0.05 to 1.5 mg/ml. A concentration of 0.5 mg/ml menadione in M9 plates allowed the growth of only a few mutagenized bacteria.

One of the *S. enterica* serovar Typhimurium mutants that was resistant to menadione was arbitrarily chosen as the recipient of random *MudJ* insertional mutagenesis. Next, kanamycin-resistant colonies were screened for hypersusceptibility to menadione. One hypersusceptible mutant was taken for further analysis. P22 transduction was carried out to backcross the hypersusceptible phenotype into wild-type salmonellae, resulting in a kanamycin-resistant (MudJ) menadione-hypersusceptible strain.

**Disk diffusion assay.** To measure resistance against superoxide and antibiotics, disk diffusion assays were performed as described by Bauer et al. (1). Briefly, overnight and end-log-phase LB cultures of salmonellae were 1:10 diluted in phosphate-buffered saline (PBS) and spread on M9 plates. A cotton disk containing antibiotics (gentamicin, 100 μg; chloramphenicol, 30 μg) or redox cycling agents (menadione, 30 mmol; paraquat, 7.5 mg) was placed in the center. After overnight incubation at 37°C the diameter of the bacterium-free zone was determined (mm) as a measure for resistance.

**Mice and mortality of infection.** *Salmonella*-resistant ($Ity^r$) C3H/HeN and *Salmonella*-susceptible ($Ity^s$) BALB/c female mice were injected intraperitoneally with $10^4$ to $10^7$ (C3H/HeN) or $10^1$ to $10^4$ bacteria (BALB/c) and the course of infection followed (20). To this end, overnight bacterial cultures were pelleted, washed, and resuspended in PBS prior to intraperitoneal injection in 0.1 ml. The endpoints were percent mortality and the time to death.

**Intracellular killing of salmonellae.** Early killing of *Salmonella* by J774 or persistence of salmonellae in RAW264.7 macrophage-like cells was determined as follows (20). Cells were allowed to adhere to plastic wells at a density of $10^5$ cells/well during overnight incubation at 37°C in RPMI medium containing 10% (vol/vol) fetal calf serum. Bacteria grown overnight in LB were added to the wells at a macrophage-to-bacteria ratio of 1:10, and centrifuged (10 min at 1,200 rpm) onto the cells. Bacterial endocytosis was allowed to proceed for 30 min, and after three washes with PBS, the cells were reincubated at 37°C and 5% $CO_2$ in medium containing gentamicin.

For measurement of early killing by J774-cells, cells were lysed by water at 0, 1, and 2 h of incubation, starting immediately after the washing procedure. To determine persistence in RAW264.7 cells, gentamicin was added (100 μg/ml) for 1 h to kill any remaining extracellular bacteria. After washing, the cells were again incubated in medium containing gentamicin (10 μg/ml) for determination of persistence after 0, 3 and 24 h. The survival of intracellular bacteria over time was determined by plate counts following the removal of medium and hypotonic lysis of cells. Statistical analysis was done using Student's *t* test.



**Mapping of MudJ insertion.** To map the MudJ insertion, an F'::Mud-P22 insertion was transduced into DLG294, with selection for the donor $Cm^r$ marker, and next screened for homologous recombination by monitoring the loss of the $Km^r$ marker of MudJ, as described by Youderian et al (22). Mitomycin C-induced Mud-P22 lysates were mixed with tails obtained in strain PY 13579, and used for transduction of auxotrophic recipient strains with characterized deletions (at 0, 7, 23, 33, 42, 49, 62, 72, 83 and 89 minutes of the *Salmonella* chromosome, respectively; kindly provided by Stan Malloy). Following the identification of the gross location of the MudJ insertion-inactivated gene, Southern blots were obtained using the collection of 57 Mud-P22 lysates as a source of DNA (3) and the MudJ-inactivated gene as the DNA probe.

**Identification of the gene inactivated by the MudJ transposon.** MudJ-flanking DNA was cloned by inverse PCR using the following primers: 5'-GTCGTTTACGCGTTGGCGTATAATGG-3' and 5'-GCTTTACCACAACCGGCGT GGT-3' (2). The PCR product was cloned into the *Eco*RV site of pBluescript $SK^-$ (Stratagene) and sequenced using Amersham T7 sequence kit. A homologous gene of *E. coli* (ORF 392, coding for a protein of unknown function) was used to design a second set of primers for the isolation and sequencing of the whole ORF in *S.enterica* serovar Typhimurium (5'-CATCTAGAGGGACCCGATGC-3' and 5'-AACTCGAGTTTTCCTACGTTAGGGCG-3').

**Isolation of recombinant SspJ and preparation of rabbit hyper-immune serum.** The MudJ-inactivated gene was sub-cloned in pEt-19b, and the protein was expressed as fusion protein containing 10 histidine residues plus a 13-amino-acid linker attached to its N-terminus. Overproduction was achieved in *E. coli* BL21, in which the T7 RNA polymerase is put under the control of the *lac* promotor. At an optical density at 600 nm ($OD_{600}$) of 0.6, overproduction was induced with 1 mM IPTG (isopropylthiogalactopyranoside). After 5 h, bacteria were collected by centrifugation and the pellet was washed with 50 mM sodium phosphate (pH 8) and 300 mM NaCl. Pellets were stored at −20°C until purification affinity chromatography, according to the manufacturer's recommendations (Qiagen, Chatsworth, CA). The protein was purified to >99% homogeneity (based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and rabbit hyperimmune serum was obtained following weekly intramuscular injection of the protein in Freund's incomplete adjuvant into 2 New Zealand rabbits.

**Expression of SspJ in bacterial cell extracts.** To show expression of SspJ in wild-type salmonellae and absence in DLG294, total bacterial extracts were prepared and tested in a Western blot. To investigate whether SspJ is released from *S. enterica* serovar Typhimurium, the supernatants of end-log-phase liquid cultures were collected, and the proteins were concentrated by protein A-coated beads (Pharmacia), and assayed in a Western blot. To check for lysis of bacterial cells that could have caused the release of cytoplasmic proteins into the liquid cultures, Western blots were assayed with antiserum

raised against a nucleoid protein of salmonellae (Tahar van der Straaten, unpublished data).

**Scavenging of xanthine oxidase-mediated superoxide production.** Superoxide was generated in vitro using xanthine-oxidase (Sigma). Inhibition of superoxide formation was determined by using Stratagene's Lumimax kit. To a tube containing 2 μl of xanthine-oxidase (5 U/μl), 5 μl of 4 mM luminol and 93 μl of xanthine assay medium, 40 μl of various *Salmonella* strain lysates was added. Immediately prior to measuring the relative light units (RLU) by a luminometer, 50 μM xanthine in 100 μl of xanthine assay medium was added. The RLU were measured at 10-s intervals.

**SOD activity of bacterial lysates.** In order to determine whether lysates of *S. enterica* serovar Typhimurium wild-type bacteria have a higher SOD activity than the superoxide-sensitive mutant, bacterial lysates were run on a native 11% protein gel which was stained by Nitro Blue Tetrazolium (NBT), resulting in nonstained bands when SOD is active. The bacterial lysates were loaded on the protein gel; the gel was rinsed with water and incubated in 1-mg/ml NBT for 20 min. After washing the gel with water, the gel was incubated for 20 min in a solution consisting of 10 ml of 50 mM TEMED (*N,N,N'N'*-tetramethylethylenediamine), 56 μl of 10 mM riboflavine, and 7.4 ml 100 mM $K_3PO_4^-$.

## Results

**Isolation of *S. enterica* serovar Typhimurium mutants that display hypersusceptibility to superoxide.** Following mutagenesis of *S. enterica* serovar Typhimurium, 53 mutants were obtained from M9 plates containing 0.5 mg/ml menadione. These mutants were assayed twice for increased resistance against menadione. One of the menadione-resistant *Salmonella* mutants, designated MD36, was selected for analysis. MD36 was more resistant to the redox cycling agents menadione and paraquat compared to the parental strain, and less susceptible to antibiotics with disparate mechanisms of action (Table 2). MD36 was chosen to be the recipient of random MudJ insertions, and the resultant library was screened for mutants with a reverse phenotype, i.e., hypersusceptibility to menadione. Out of about 50,000 kanamycin-resistant colonies, one hypersusceptible mutant strain, designated MD36.12, was isolated and used for further analysis. The phenotype was repeatedly backcrossed into wild-type salmonellae using phage P22 transduction. Clearing of phage resulted in strain DLG294, which still exhibited the hypersusceptible phenotype (Table 2).

**Table 2. Susceptibility of *Salmonella* strains to oxidants and antibiotics in disk diffusion assay**

| | Mean zone of growth inhibition (mm) ± SD | | | |
|---|---|---|---|---|
| Strain | Menadione 10 μl | $H_2O_2$ 10 μl | Cholramphenicol 30 μg | Gentamicin 100 μg |
| 14028s | 30 ± 3 | 24 ± 4 | 27 ± 2 | 28 ± 1 |
| MD36 | 23 ± 2 | 39 ± 3 | 19 ± 2 | 28 ± 1 |
| MD36.12 | 34 ± 3 | 39 ± 4 | 30 ± 3 | 29 ± 1 |
| DLG294 | 41 ± 3 | 25 ± 4 | 31 ± 3 | 28 ± 1 |
| DLG294-pTS175 | 31 ± 4 | nd[a] | nd | 29 ± 1 |
| DLG294-pWSK29 | 41 ± 3 | nd | nd | 28 ± 1 |

[a] nd, not determined

**Mortality of *Salmonella* infection in resistant and susceptible mice.** To investigate whether the gene that was inactivated by the MudJ insertion and rendered DLG294 hypersusceptible to superoxide is relevant for the in vivo virulence of salmonellae, BALB/c and C3H/HeN mice were injected intraperitoneally with various numbers of DLG294 or the parental *S. enterica* serovar Typhimurium. DLG294 was less virulent than wild-type bacteria: in both strains of mice, about a 100-fold higher number of DLG294 than of wild-type bacteria was necessary to reach a similar mortality and time to death (Table 3a and 3b).

Of note, the rate of growth of DLG294 was identical to that of wild-type salmonellae when cultured in rich LB or minimal M9 liquid medium at 37° C under vigorous shaking (data not shown).

**Table 3a. Mortality in C3H/HeN and BALB/c mice**

| Mouse and | No. of mice dead/no. tested at inoculum | | | | | | |
|---|---|---|---|---|---|---|---|
| *Salmonella* strains | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| **C3H/HeN** | | | | | | | |
| 14028s | | | | 3/3 | 3/3 | 3/3 | 3/3 |
| DLG294 | | | | 0/3 | 1/3 | 2/3 | 3/3 |
| **BALB/c** | | | | | | | |
| 14028s | 4/4 | 4/4 | nd[a] | nd | | | |
| DLG294 | 1/4 | 4/4 | nd | nd | | | |

[a] nd, not done

**Table 3b. Time to death in C3H/HeN and BALB/c mice**

| Mouse and *Salmonella* strains | Median time to death (h) at inoculum | | | | | | |
|---|---|---|---|---|---|---|---|
| | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| **C3H/HeN** | | | | | | | |
| 14028s | | | | 216 | 144 | 84 | 48 |
| DLG294 | | | | ---[a] | --- | 168 | 48 |
| **BALB/c** | | | | | | | |
| 14028s | 154 | 120 | nd[b] | nd | | | |
| DLG294 | --- | --- | 132 | 96 | | | |

[a] ---, less than 50% of the mice died
[b] nd, not done

**In vitro intracellular killing of salmonellae by macrophages.** To investigate whether the gene that was inactivated by the MudJ insertion and rendered DLG294 hypersusceptible to superoxide is involved in bacterial resistance against the microbicidal effector mechanism of mononuclear phagocytes, the intracellular killing of DLG294 and wild-type *S. enterica* serovar Typhimurium 14028s by macrophage-like J774 and RAW264.7 cells was determined. During the first hours after uptake by J774 cells, the number of intracellular microorganisms (range $1.4 \times 10^5$ to $4.6 \times 10^5$ bacteria per $5 \times 10^5$ J774-cells) decreased exponentially (Fig. 1A). However, DLG294 was killed by J774 cells at twofold higher killing rates (killing rate, 0.031 ± 0.011/min; $n = 3$) than wild-type salmonellae (killing rate 0.014 ± 0.008/min; $n = 3$; $P < 0.025$). After 2 h, this difference in intracellular killing resulted in a 10-fold-lower number of intracellular DLG294 than for the wild type. Also in RAW264.7 cells, DLG294 was more easily contained than the parental strain: whereas the wild-type salmonellae replicated within RAW264.7 cells upon incubation over 24 h, DLG294 was unable to do so (Fig. 1B). To check for the ability of the cell lines to produce superoxide, NBT reduction was used as a measure of superoxide production. Both J774 and RAW264.7 were shown to produce superoxide during the uptake of inert particles and phorbol myristate acetate stimulation (data not shown).

Taken together, the *in vivo* and *in vitro* findings reveal a biologically relevant attenuation of virulence of DLG294 compared with that of parental, wild-type *S. typhimurium*, that is probably linked to hypersusceptibility of DLG294 to superoxide.



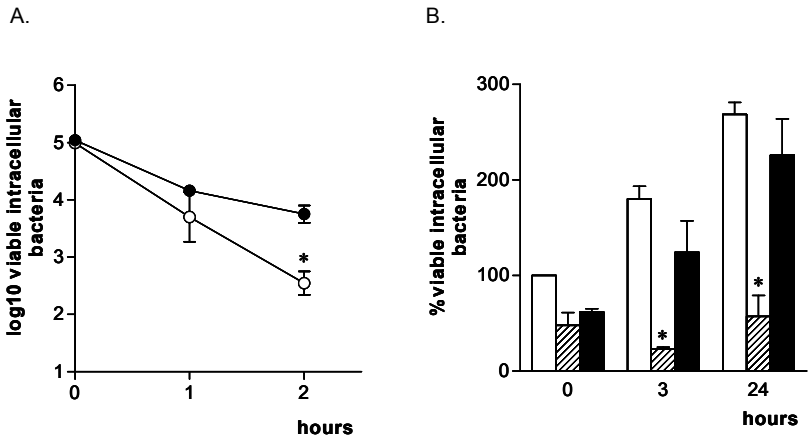


**Figure 1.** In vitro intracellular killing of DLG294 *(ssp*::MudJ; open circles) and wild-type *S. enterica* serovar Typhimurium 14028s (solid circles) by J774 macrophage-like cells (A). After uptake of the bacteria and removal of remaining extracellular microorganisms, at various time points the number of viable intracellular bacteria was determined microbiologically as a measure of intracellular killing, and expressed as percent viable intracellular bacteria left compared with the number present at the end of the uptake period. Data from a representative experiment are shown. After uptake by RAW264.7 cells (B), the changes in the number of intracellular wild-type *S. enterica* serovar Typhimurium 14028s (open bars), DLG294-pWSK29 (hatched bars) and DLG294- pTS175 (black bars) were determined at 0, 3, and 24 hours after infection. Data are the mean of three independently performed experiments. Asterisks indicate significant differences ($p < 0.05$).

**Mapping of the MudJ insertion.** Starting with transduction of MudJ in DLG294, multiple Mud-P22 Q but no Mud-P22 P $Cm^r$ and $Km^s$ convertants were obtained . Three different Mud-P22 Q lysates reverted the auxotrophic phenotype of MST 10 (mutation at 49 min) at very high efficiency (i.e. between $10^7$ to $10^8$ recombinants obtained; $n = 3$), that of MST 8 (mutation at 42 min) at moderate efficiency (i.e. $10^5$ to $10^6$; $n = 3$), and the other eight strains at low efficiency (less than $10^3$ recombinants; $n = 3$). Thus, consistent with the counterclockwise packaging of the Mud-P22 Q lysate, these findings indicate that the MudJ in DLG294 had inserted between 62 and 49 minutes of the *Salmonella* chromosome.

The exact location of the MudJ-inactivated gene of DLG294 was determined using a collection of 57 Mud-P22 lysates as the source of DNA. Hybridization with the MudJ-inactivated gene as the DNA probe revealed positive spots on Mud-P22 lysates *guaA*5641::MudQ and *purG*2149::MudQ, indicating that the MudJ-inactivated gene lies between 54.4 and 64 minutes of the *Salmonella* chromosome.

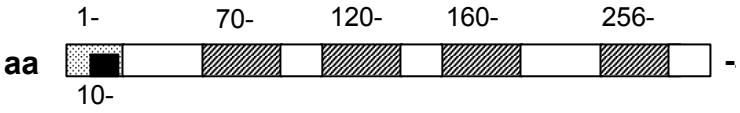


**Figure 2.** Schematic drawing of homologous domains within SspJ protein, 392 aminoacids. Depicted are a leader sequence from amino acids 1 to 22, lipid membrane attachment site from amino acids 10 to 21, and four PQQ domains from amino acids 70 to 107, 120 to 157, 160 to 197 and 256 to 293.

**Identification of the gene or gene cluster inactivated by the MudJ insertion.** By inverse PCR, part of the gene in which the MudJ had inserted was cloned and sequenced. A database search revealed homology with ORF392 of *E. coli* (a gene of unknown function, accession number: AAC75565). Using primers based on this homologous sequence, the whole ORF was cloned and sequenced from *S.* enterica serovar Typhimurium. The sequence was determined in DLG294 as well as wild-type *S.* enterica serovar Typhimurium and has been deposited in the NCBI database (accession number AF314961). The sequence revealed an open reading frame of 1,176 bp, encoding a 392-amino-acid protein with a predicted mass of 42.3 kDa. The gene was designated *sspJ* for superoxide susceptibility protein. Based on the predicted amino acid sequence from *sspJ*, a sequence homology search revealed the presence of a leader sequence and four putative pyrroloquinoline quinone (PQQ) domains thought to be specific for bacterial dehydrogenases (Fig.2) (6).

**Complementation of the superoxide hypersensitive phenotype.** After identification of the gene in which the MudJ transposon was inserted, the gene was isolated by PCR and ligated into low-copy-number plasmid pWSK29 (21). Complementation of DLG294 was achieved by electroporation with pTS175. Disk diffusion assays using complemented DLG294 (expressing the low-copy-number plasmid pWSK29 carrying an intact copy of *sspJ*) resulted in reversal of the menadione-hypersusceptible phenotype of DLG294 into wild-type susceptibility (Table 2).

Persistence in RAW264.7 was also restored to the wild type when SspJ was expressed on a low-copy-number plasmid in mutant DLG294. Transformation with the vector only did not affect the intracellular fate of DLG294 (Fig. 1B).

**Identification of SspJ in *Salmonella* cell extract and culture supernatant.** A Western blot using rabbit hyperimmune serum raised against purified SspJ revealed a protein of the predicted size in a total cell lysate of wild-type salmonellae. Since the protein has a signal sequence, it is probably present in the periplasm. There was a total absence of this protein in DLG294, and it was overexpressed constitutively in DLG294 carrying an SspJ-encoding multicopy plasmid (Fig. 3). Furthermore, the protein was identified in supernatant of end log-phase growth liquid cultures of wild-type *Salmonella* and DLG294 carrying an SspJ-encoding multicopy plasmid, but not in DLG294 (Fig. 3).

An antiserum raised against a *Salmonella* DNA-binding protein was used to check for nonspecific bacterial cell lysis. This protein could not be detected in the same culture supernatants. In addition, several stress conditions (pH5 to 9, osmolarity of 0.15 to 1.0 M NaCl, superoxide at 10 mM, and temperature of 30 to 42º) did not affect expression of SspJ in *Salmonella* wild type compared to normal growth conditions (LB and 37ºC) (data not shown).



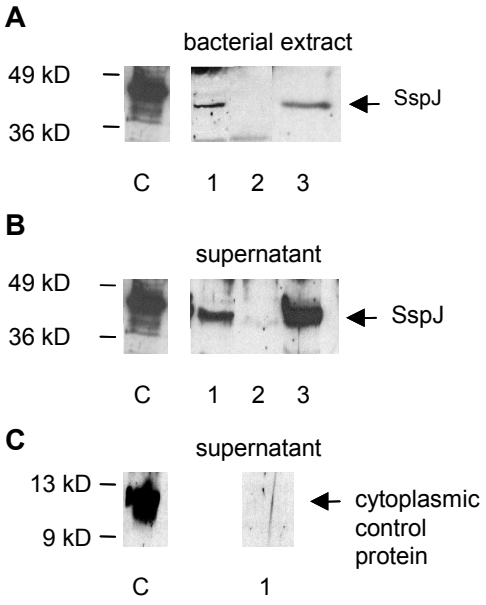


**Figure 3.** Expression of SspJ by *Salmonella* strains. Panel A shows expression of SspJ in a total extract of *S. enterica* serovar Typhimurium (wild type) and in DLG294(*sspJ*::MudJ) complemented by plasmid pBluescript carrying *sspJ* (DLG294-pTS125)) (lanes 1 and 3, respectively), but not in DLG294 (*sspJ*::MudJ) (lane 2). Panel B shows expression SspJ in culture supernatants, whereas panel C indicates that there is no expression of a cytoplasmic control protein. The first lane in all three panels is purified protein together with molecular weight markers.

**SspJ is not a superoxide scavenger.** To determine whether DLG294 is less able to inhibit superoxide production or scavenge superoxide, supernatants of overnight cultures of *Salmonella* wild-type, DLG294 and DLG294- pTS175 were assayed for presence of such activity in a xanthine oxidase assay. Addition of 10 units of SOD to xanthine oxidase decreased the amount of superoxide generated by almost 100% within 10 s. The addition of DLG294 supernatant to xanthine oxidase decreased the amount of superoxide generated by 71% ± 1% (*n* = 3) of the control, whereas the addition of supernatants from the wild type or *sspJ*-complemented DLG294 did decrease the amount of superoxide generated by 63% ± 15% (*n* = 3) and 70% ± 5% (*n* = 3) respectively This result indicate that the presence or absence of SspJ does not interfere with the production or scavenging of superoxide in this system.

**SspJ has no SOD activity**. Since disruption of SspJ expression resulted in the inability to resist increased intracellular superoxide levels, we tested whether DLG294 contains less SOD activity than the wild type and *sspJ*-complemented DLG294. Analysis of SOD activity in whole-cell bacterial lysates on non-denaturing gels showed no difference between wild type, the mutant and the complemented strain (data not shown).

## Discussion

Intracellular pathogens like *S. enterica* serovar Typhimurium are able to respond to the specific host environment by selectively expressing factors necessary for intracellular survival. Thus, despite the multitude of antimicrobial effector mechanisms of the host cells, the bacteria can multiply within spacious phagosomes of the macrophages.

To identify bacterial proteins that play a role in the ability of salmonellae to prevent the induction or neutralize the activity of the antimicrobial effector mechanism of phagocytes, we screened for genes of *S. enterica* serovar Typhimurium involved in bacterial defense against superoxide and the ability to survive within mononuclear phagocytes. A mutant of *S. enterica* serovar Typhimurium that was resistant to the redox cycling agent menadione was isolated following random chemical mutagenesis of wild-type salmonellae. Next, this mutant was used to isolate menadione-hypersusceptible mutants by obtaining random MudJ insertions. In this way, a hypersusceptible strain designated MD36.12 was obtained. This phenotype was backcrossed into wild-type *Salmonella*, resulting in DLG294. This *Salmonella* strain was hypersusceptible to menadione compared to wild-type parental *Salmonella* strain 14028s. Complementing the MudJ insertion-inactivated gene in DLG294 with the gene carried on a low-copy-number plasmid fully restored the phenotype back to wild type.

The biological relevance of the MudJ-inactivated gene was evident from the decreased virulence of DLG294 compared to wild-type *Salmonella* after intraperitoneal injection into *Salmonella*-resistant and *Salmonella*-susceptible mice, and the enhanced intracellular killing of this mutant strain within macrophage-like cells in vitro. Furthermore, within cells cultured for 24 h, wild-type salmonellae were able to multiply to about fivefold their initial numbers, whereas DLG294 was unable to replicate at all. That the MudJ-inactivated gene is essential for the survival and replication of *S. enterica* serovar Typhimurium within macrophages was confirmed by the finding that gene complementation could restore the wild-type phenotype.

The MudJ transposon was found inserted in a previously undescribed *Salmonella* locus, designated *sspJ (*for superoxide susceptibility protein*)*. Using MudP22 probe hybridization techniques and linkage analysis, the gene was mapped at 55 to 60 min on the *Salmonella* chromosome. SspJ displayed 78% sequence identity to a putative *E. coli* protein of unknown function that maps at 55.9 min in the *xse*A-*his*S intergenic region. Analysis of the protein sequence revealed the presence of a leader, suggesting that SspJ is transported to the periplasmic side of the inner cell membrane. This was confirmed by the results of the Western blot that revealed a protein of predicted size in the soluble and inner membrane fraction of wild-type salmonellae and the total absence of this protein in DLG294, as well as overexpression of this protein in DLG294 carrying an *sspJ*-encoding plasmid. Furthermore, the protein was identified in end-log-phase supernatants of wild-type *Salmonella* and DLG294 carrying an *sspJ*-encoding plasmid, but not in DLG294, suggesting that the protein may be released into the medium.



The mechanism by which SspJ contributes to protection from oxidative stress remains to be elucidated. However, we excluded that it acts as a scavenger of superoxide and, although the phenotype of the mutant appear very similar to that of *sod*C knockouts, that it has SOD activity. Based on protein homology analysis, four putative PQQ-binding domains are present in SspJ. PQQ domains are thought to be specific for NAD(P)-independent bacterial dehydrogenases located in the periplasmic space and bound to the inner cell membrane; a location that is consistent with the results for SspJ in the Western blot. However, SspJ lacks specific sequence characteristics of bacterial dehydrogenases and a hypothesis involving PQQ binding cannot explain our findings that both in rich LB medium and in minimal M9 culture medium that lacks PQQ, DLG294 is much more susceptible to the redox cycling agent menadione than wild-type salmonellae.

The homologue of SspJ in *E. coli*, ORF392, is 91% identical to *Salmonella* SspJ. It also contains the putative leadersequence and the PQQ domains. Based on this homology, it could be speculated that the SspJ homologue is functional in *E. coli*. We are currently investigating whether expression of ORF392 in DLG294 can also complement the superoxide-sensitive phenotype. The implications of the presence of this gene in *E. coli* however, are difficult to predict, since it is likely that *E. coli* killing is mediated by mechanisms other than oxidative stress, such as complement or low pH.

Currently we are investigating whether SspJ acts in a regulatory pathway that protects salmonellae against superoxide, either as a sensor or as an essential cofactor of SODs.

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chapter seven

# A superoxide-hypersusceptible *Salmonella enterica* serovar Typhimurium mutant strain is attenuated but regains virulence in $p47^{phox-/-}$ mice

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## Abstract

*Salmonella enterica* serovar Typhimurium is a gram-negative, facultative intracellular pathogen that predominantly invades mononuclear phagocytes and is able to establish persistent infections. One of the innate defense mechanisms of phagocytic cells is the production of reactive oxygen species, including superoxide. *S. enterica* serovar Typhimurium has evolved mechanisms to resist such radicals, and these mechanisms could be decisive in its ability to survive and replicate within macrophages. Recently, we described a superoxide-hypersusceptible *S. enterica* serovar Typhimurium mutant strain, DLG294, that carries a transposon in *sspJ*, resulting in the lack of expression of SspJ, which is necessary for resistance against superoxide and replication within macrophages. Here we show that DLG294, which is a 14028s derivative, hardly induced any granulomatous lesions in the livers upon subcutaneous infection of C3H/HeN ($Ity^r$) mice with $3 \times 10^4$ bacteria and that its bacterial counts were reduced by 3 log units compared to those of wild-type *S. enterica* serovar Typhimurium 14028s on day 5 after infection. In contrast, DLG294 replicated like wild-type *S. enterica* serovar Typhimurium 14028s and induced a phenotypically similar liver pathology in $p47^{phox-/-}$ mice, which are deficient in the $p47^{phox}$ subunit of the NADPH oxidase complex and which do not produce superoxide. Consistent with these results, DLG294 reached bacterial counts identical to those of wild-type *S. enterica* serovar Typhimurium 14028s in bone marrow-derived macrophages from $p47^{phox-/-}$ mice and in X-CGD PLB-985 cells at 24 h after challenge. These results indicate that SspJ plays a role in the bacterium's resistance to oxidative stress and in the survival and replication of *S. enterica* serovar Typhimurium both in vitro and in vivo.

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## Introduction

*Salmonella enterica* serovar Typhimurium is a gram-negative, facultative intracellular pathogen that predominantly invades mononuclear phagocytes and that is able to establish persistent infections by evasion or disturbance of the host defense (14). Despite the multitude of antimicrobial mechanisms present as part of the innate defense of phagocytic cells, *S. enterica* serovar Typhimurium is able to survive and replicate within phagosomes of macrophages. This ability of *S. enterica* serovar Typhimurium to enter and replicate within phagocytic cells is essential for its survival, as mutants unable to do so are avirulent (9). Although little is known of the exact mechanisms by which *S. enterica* serovar Typhimurium is able to survive after phagocytosis, *S. enterica* serovar Typhimurium responds to the specific host environment by expressing factors that are necessary for intracellular survival and for resistance against the defense systems of the host (5, 10, 11, 14, 18).

One of the major early defense mechanisms of macrophages against microorganisms is the production of toxic superoxide by the phagocyte NADPH oxidase and the subsequent generation of superoxide derivatives, both in vitro (17) and in vivo (22, 23, 27). The phagocyte NADPH oxidase is a multiprotein enzyme complex that comprises a heterodimeric, membrane-bound, flavocytochrome $b_{558}$ (composed of a 91-kDa glycoprotein and a 22-kDa protein) and four cytosolic factors (p47$^{phox}$, p67$^{phox}$, p40$^{phox}$, and Rac GTPase; phox for phagocyte oxidase) that translocate to the plasma membrane upon activation to interact with cytochrome $b_{558}$. This leads to the formation of an active NADPH oxidase complex that accepts electrons from NADPH and donates them to molecular oxygen, resulting in the generation of superoxide ($O_2^-$) (reviewed in (1)). Thus, upon stimulation of the phagocyte with opsonized microorganisms or other activating agents, the oxygen consumption increases dramatically (respiratory burst) and a large amount of superoxide is produced. This superoxide is converted into other, more potent, reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, and hypochlorous acid.

Many microorganisms, including *Salmonella*, have evolved a multitude of defense mechanisms to resist this superoxide-mediated killing by phagocytes. These mechanisms include avoidance of encounters with phagocyte-derived oxidants, neutralization of such oxidants, or prevention of their production. *S. enterica* serovar Typhimurium is highly dependent on such mechanisms as they help the pathogen to survive within the phagosome. Examples of such defense mechanisms used by *S. enterica* serovar Typhimurium include inhibition of protein kinase C activity in macrophages, production of antioxidant scavengers, formation of heat shock proteins, and production of superoxide dismutase (SOD) (reviewed in (18))

More recently, it was shown that *S. enterica* serovar Typhimurium could also prevent reactive oxygen species production by exclusion of NADPH oxidase from the phagosome in which *Salmonella* resides (12). The relative importance of each of these mechanisms for *S. enterica* serovar Typhimurium survival has not been elucidated; however, the periplasmic Cu,Zn-SOD and the type III secretion system encoded by *Salmonella*



pathogenicity island 2 (SPI2) are very important in this defense, as mutants deficient in one of these systems show reduced survival within macrophages (4, 12, 28).

We have recently described the isolation of a mutant *S. enterica* serovar Typhimurium strain (DLG294) that showed decreased resistance to oxidative stress induced by menadione in vitro, due to the inactivation of a gene designated *sspJ* (for *Salmonella* superoxide protection) (25). In this report we describe the further investigation of superoxide-sensitive mutant strain DLG294 and show that the gene encoding SspJ is essential for resistance to oxidative stress and thus for survival and replication of *S. enterica* serovar Typhimurium both in vitro and in vivo.

## Materials and Methods

**Animals.** Six- to 8-week-old female *Salmonella*-resistant ($Ity^r$) C3H/HeN mice and *Salmonella*-sensitive ($Ity^s$) C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) and Charles River Laboratories, respectively. Mice were maintained under standard conditions according to the institutional guidelines. Water and food were given ad libitum. Female mice homozygous for a targeted mutation in the $p47^{phox}$ subunit of the NADPH oxidase on a C57BL/6 background ($p47^{phox-/-}$) were generated as described previously (13) and obtained from Taconic Laboratory (Germantown, N.Y.). All experiments were approved by the local Animal Ethical Committee.

**Bacterial strains and plasmids.** Single colonies of wild-type *S. enterica* serovar Typhimurium 14028s (50% lethal dose after intraperitoneal injection: $5 \times 10^3$ bacteria for $Ity^r$ mice and $<10^2$ for $Ity^s$ mice) and superoxide-sensitive derivative DLG294 (25) were grown overnight in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37ºC while being shaken (225 rpm). In addition, DLG294 supplemented with low-copy-number plasmid pWSK29 expressing the *sspJ* gene, which is inactivated in DLG294 (DLG294-pTS175), and with its vector control (DLG294-pWSK29) were used (25). These bacteria were grown in LB medium supplemented with 50 µg of ampicillin (Merck)/ml. For in vivo infection experiments, bacteria were grown to the end of log phase and were then washed and diluted in sterile phosphate-buffered saline (PBS). The CFU in the inoculum were determined by plating serial dilutions.

**Cells and culture conditions.** PLB-985 and X-CGD PLB-985 cells (29) were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum, penicillin (1,000 units/ml), and streptomycin (50 µg/ml) at 37ºC and 5% $CO_2$. For granulocytic differentiation, the cells were exposed to 0.5% dimethylformamide for 5 to 6 days. Under these conditions, the PLB-985 cells acquire respiratory-burst activity, while the X-CGD PLB-985 cells do not (29).

Bone marrow-derived macrophages from C57BL/6 and $p47^{phox-/-}$ mice were obtained and cultured as described previously (2) with a few modifications. Briefly, mice were killed

by cervical dislocation, and the femurs were isolated under sterile conditions. The bone marrow was flushed from the femurs in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum. The cells were resuspended in DMEM supplemented with 20% horse serum and seeded in 24-wells tissue culture plates (Greiner) at a concentration of $2 \times 10^5$ nucleated cells per well and were allowed to adhere for 2 h. Nonadherent cells were removed, and the remaining adherent cells were cultured for 1 week in DMEM supplemented with 20% horse serum and 20% embryonic mouse fibroblast-conditioned medium as the source of colony-stimulating factor.

**Replication of *S. enterica* serovar Typhimurium within macrophages.** Macrophages ($2 \times 10^5$) were challenged with *S. enterica* serovar Typhimurium at a 10:1 multiplicity of infection. To promote the uptake of the bacteria, the bacteria were spun onto the macrophages by centrifugation at 300 × g for 10 min and the cells were allowed to internalize the bacteria for 30 min at 37ºC, 5% $CO_2$. For determination of the replication of *S. enterica* serovar Typhimurium within macrophages and PLB-985 cells, we used the bacterial infection protocol described by Rathman et al. (20) with a few modifications. The cells were washed with PBS and incubated for 1 h in medium supplemented with 100 µg of gentamicin/ml to kill the extracellular bacteria and were then washed again. This time point is designated time point zero. Medium supplemented with 10 µg of gentamicin/ml was added to the cells to kill any remaining extracellular bacteria and to prevent reinfection. It has been reported that this concentration of gentamicin does not affect growth of intracellular bacteria (26). At 24 h, the cells were washed with PBS to remove the gentamicin and were lysed in 1 ml of distilled water. Serial dilutions of the lysate were plated for determination of the number of intracellular CFU. For examination of our *S. enterica* serovar Typhimurium strains in an in vitro model of NADPH oxidase deficiency, we used X-CGD PLB-985, a cell line in which the gp91$^{phox}$ gene was disrupted by homologous recombination in leukemic cell line PLB-985. As a consequence, these cells fail to produce superoxide mediated by NADPH oxidase (29). For in vitro replication experiments using the nonadherent PLB-895 and X-CGD PLB-985 cells, the bacteria were not spun onto the cells but were incubated together with the cells while rotating. Like the adherent bone marrow-derived macrophages, these cells were treated with 100 µg of gentamicin/ml to kill the extracellular bacteria. After the cells were washed with PBS, medium supplemented with 10 µg of gentamicin/ml was added to the cells to kill any remaining bacteria and to prevent reinfection. At 24 h, the cells were washed with PBS to remove the gentamicin and were lysed in 1 ml of distilled water. Serial dilutions of the lysate were plated for determination of the number of intracellular CFU.

**In vivo *Salmonella* infections.** Mice were inoculated subcutaneously in the flanks with 0.1 ml bacterial suspension in PBS. Per group, four mice were used for each time point. Mice were sacrificed at several time points by carbon dioxide inhalation or by cervical dislocation. Blood was taken by cardiac puncture, and livers, spleens, and inguinal lymph nodes were aseptically removed. To determine the bacterial load within these



organs, single-cell suspensions were prepared using sterile 70-µm-mesh-size cell strainers (Falcon). Cells were pelleted by centrifugation for 10 min and were lysed in distilled water. The bacterial number per organ was determined bacteriologically by plating serial dilutions.

**Histopathology.** Livers from infected mice were harvested, and a segment of tissue was fixed in 10% buffered formalin solution for histopathology. Tissues were embedded in paraffin and 3-µm-thick sections were prepared and stained with hematoxylin and eosin. The pathology in the livers was examined and judged microscopically.

**Statistics.** Statistical analysis was performed using Student's *t* tests and a *P* value <0.05 was considered significant.

## Results

**In vivo growth of 14028s and DLG294 in *Salmonella*-resistant C3H/HeN mice.** Because DLG294 had been shown to be unable to multiply within the macrophage-like cell line RAW264.7 and caused less deaths among intraperitoneally infected C3H/HeN and C57BL/6 mice than strain 14028s (25), we examined the course of infection for both strains in mice by determining the CFU counts and examining the induced pathology in the livers, spleens, and lymph nodes. C3H/HeN mice were infected subcutaneously in the flanks with ~30,000 CFU of *S.enterica* serovar Typhimurium 14028S or DLG294. The inoculum was given subcutaneously to establish a reservoir in the lymph nodes, whence *Salmonella* readily spreads throughout the body via the lymph stream and becomes systemic, finally reaching the liver and the spleen (3). The mice challenged with wild-type *S. enterica* serovar Typhimurium 14028s showed a decrease in total body weight (Fig. 1A), while the DLG294-challenged mice did not. *S. enterica* serovar Typhimurium 14028s induced intense hepatosplenomegaly, while these organs of the DLG294-infected mice were only modestly enlarged, indicating that DLG294 caused less pathology than the wild-type 14028s strain (Fig. 1A). This was confirmed by the bacterial counts and the macroscopic appearance of these organs. *S. enterica* serovar Typhimurium 14028s was able to multiply within the livers, spleens, and lymph nodes and reached a peak between days 5 and 12. In contrast, DLG294 was not able to replicate and the number of bacteria in the livers, spleens, and lymph nodes was reduced by 3 log units compared to the number of 14028s bacteria (Fig. 1B). Also the macroscopic lesions in the organs were less severe in mice infected with DLG294 than in mice infected with 14028s. Taken together, these data indicate that DLG294 is severely attenuated *in vivo*. This low virulence of DLG294 could be fully reversed to that of wild-type *S. enterica* serovar Typhimurium 14028s by the expression of *sspJ* on low-copy-number plasmid pWSK29 (DLG294-pTS175) (Fig. 1C). This, together with the previous results obtained in vitro, indicate that the SspJ protein is important in the in vivo replication of *S. enterica* serovar Typhimurium.

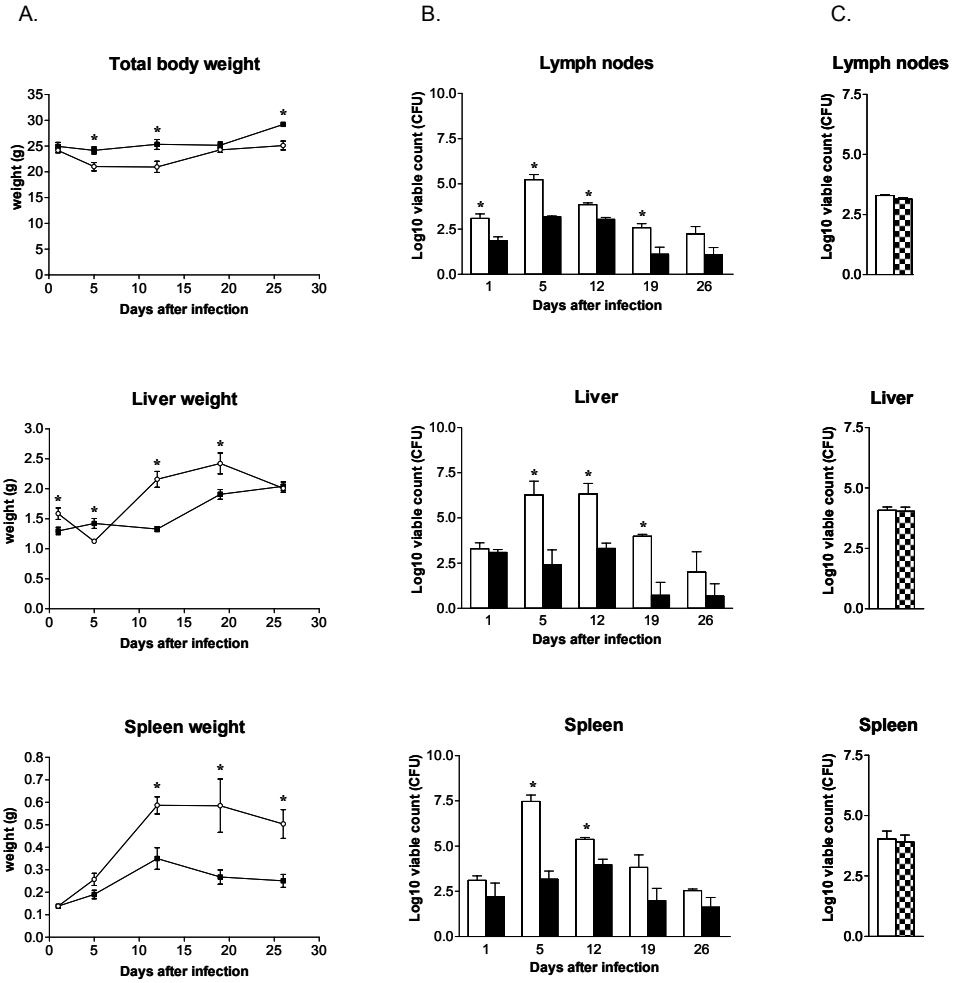


**Figure 1.** Total body weights and liver and spleen weights of (A) and bacterial counts in livers, spleens, and lymph nodes (B) of C3H/HeN mice infected with *S. enterica* serovar Typhimurium 14028s (open circles and white bars) and DLG294 (black squares and black bars) at several time points after infection. (C) Bacterial counts of C3H/HeN mice infected with *S. enterica* serovar Typhimurium 14028s (white bars) and DLG294 complemented by the low-copy-number plasmid pWSK29 carrying *sspJ* (DLG294-pTS175; checkered bars) on day 6 after infection. C3H/HeN mice were infected subcutaneously in the flanks with ~30,000 CFU of *S. enterica* serovar Typhimurium 14028s, DLG294, or DLG294-pTS175. The actual dosage was confirmed by plating serial dilutions of the inoculum. At the indicated time points, livers, spleens, and lymph nodes were aseptically removed and weighed, and the viable counts within these organs were determined by plating serial dilutions (n=4 per group). The results are expressed as $\log_{10}$ viable counts (means ± standard errors of the means). Asterisks indicate that the number of bacteria of wild-type 14028s is statistically different from that of DLG294 (Student's *t* test).

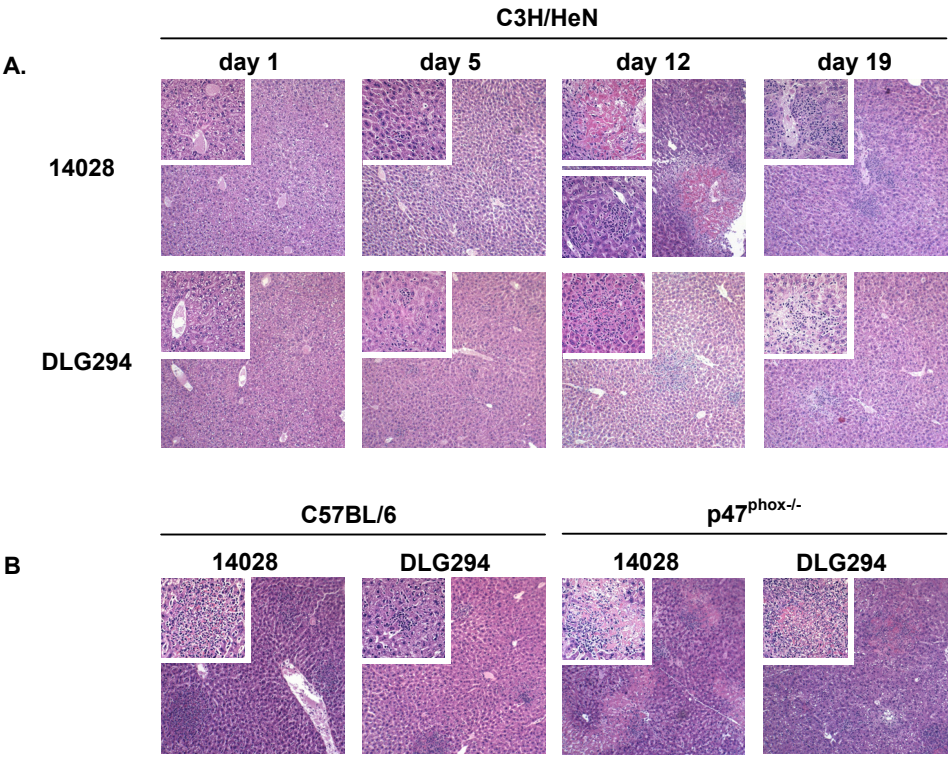


**Figure 2.** Microscopic appearance of livers from C3H/HeN mice infected with *S. enterica* serovar Typhimurium 14028s and DLG294 at several time points after infection (A) and C57BL/6 and p47$^{phox-/-}$ mice at 4 days after infection with *S. enterica* serovar Typhimurium 14028s and DLG294 (B). Mice were infected subcutaneously in the flanks as described in Materials and Methods. Hematoxylin- and eosin-stained sections were prepared from livers that were isolated at different time points after infection.

**Histopathology of *S. enterica* serovar Typhimurium 14028s and DLG294 infection in C3H/HeN mice.** Microscopic examination revealed that the livers of mice infected with wild-type *S. enterica* serovar Typhimurium 14028s and DLG294 had a normal appearance on day 1. Microabscesses started to appear in the livers in 14028s-infected mice on day 5 and to a greater extent on day 12, when the highest numbers of bacteria were observed in the livers. Representative tissue sections of the livers are shown in Fig. 2A. The microabscesses were still present on day 19, but the number was less than the number on day 12. Granulomatous lesions were first observed on day 5, but were more pronounced on day 12 of infection. On day 19, when the bacterial counts in the livers were reduced, the number of lesions was also reduced. Some microabscesses were present in livers of DLG294-infected mice on days 5, 12, and 19, but pathology was not as severe as in wild-type *S. enterica* serovar Typhimurium 14028s-infected mice. A few granulomas were also observed in the livers of DLG294-infected mice on days 5 and 12, but again the pathology was much less extensive as in 14028s-infected mice (Fig. 2A).

**In vitro intracellular replication of *S. enterica* serovar Typhimurium in bone marrow-derived macrophages.** The protein encoded by the *sspJ* gene (SspJ) is essential for replication within macrophages and is crucial for resistance to oxidative stress induced by menadione (25), and its absence results in decreased virulence in vivo (Fig. 1 and 2A). This suggested a role for SspJ in preventing the induction of, or in neutralizing the activity, of superoxide-mediated antimicrobial effector mechanisms of phagocytes, such as the NADPH oxidase system. We hypothesized that if this protein is involved in escaping oxidative stress, the superoxide-sensitive mutant strain DLG294 would be able to survive and replicate within cells that do not produce any superoxide. This hypothesis was tested in a macrophage intracellular replication assay using bone marrow-derived macrophages from p47$^{phox-/-}$ mice in a *Salmonella*-susceptible C57BL/6 (Ity$^{s}$) genetic background, as these mice have been shown to produce no superoxide (13).

There was no difference between the *S. enterica* serovar Typhimurium strains in replication in the bone marrow-derived macrophages of p47$^{phox-/-}$ mice (Fig. 3), indicating that the superoxide-sensitive mutant strain DLG294 was as virulent as 14028s in cells that do not produce any phagocyte superoxide. The numbers of intracellular bacteria of 14028s in the macrophages of the control C57BL/6 mice, which produce superoxide upon bacterial challenge, were higher than those of DLG294. The ability of DLG294 to replicate intracellularly was restored to wild type when *sspJ* was expressed on a low-copy-number plasmid (DLG294-pTS175), whereas introduction of the vector pWSK29 did not affect the intracellular count. For confirmation of superoxide deficiency in the macrophages of p47$^{phox-/-}$ mice, nitroblue tetrazolium reduction was determined. The macrophages of wild-type C57BL/6 mice were shown to be able to produce superoxide, while the macrophages of the p47$^{phox-/-}$ mice were not (data not shown).

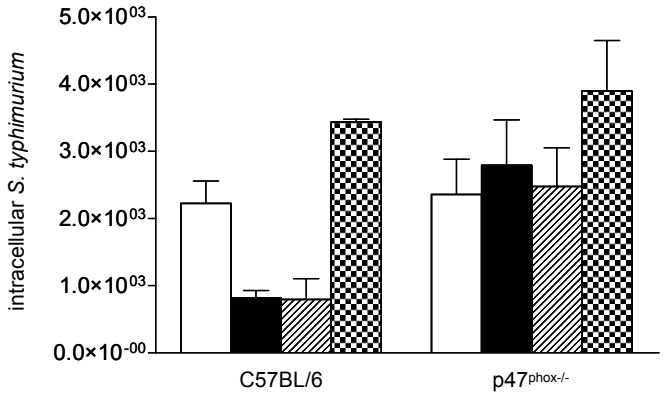


**Figure 3.** Intracellular *S. enterica* serovar Typhimurium in mouse bone marrow-derived macrophages from C57BL/6 and p47$^{phox-/-}$ mice. The cells were challenged with *S. enterica* serovar Typhimurium 14028s (white bars), DLG294 (black bars), DLG294-pWSK29 (hatched bars), and DLG294-pTS175 (checkered bars) as described in Materials and Methods. The numbers of intracellular bacteria were determined at 24 h after challenge. Asterisks indicate that the number of intracellular bacteria is significantly different from that of wild-type *S. enterica* serovar Typhimurium 14028s. Mean data of two independently performed experiments ± standard errors of the means are shown.

***S. enterica* serovar Typhimurium infection of wild-type and p47$^{phox-/-}$ mice.** The in vitro infection assay showed that DLG294 was able to replicate intracellularly in bone marrow-derived macrophages from mice that do not produce any superoxide due to a homozygous mutation in the p47$^{phox}$ subunit of the NADPH oxidase (p47$^{phox-/-}$ mice). In these cells, DLG294 reached bacterial counts comparable to those of wild-type *S. enterica* serovar Typhimurium 14028s. To analyze whether both strains are also equally virulent in vivo under conditions when no superoxide is produced, we used the *Salmonella* infection model in these p47$^{phox-/-}$ mice and their superoxide-producing controls C57BL/6 (Ity$^{s}$). Since Ity$^{s}$ mice are very sensitive to *S. enterica* serovar Typhimurium infection, the dose was decreased compared to the dose given to Ity$^{r}$ C3H/HeN mice. The p47$^{phox-/-}$ mice were expected to be even more sensitive to infection, and therefore, infection was only studied at early time points.

The infected C57BL/6 mice showed a slight reduction in body weight, and there was hardly any difference between 14028s- and DLG294-infected mice at such early time points after infection. However, the liver and spleen weights of 14028s-infected wild-type mice strongly increased compared to those of DLG294-infected wild-type mice, but not until 6 days after infection (data not shown). The bacterial counts in the different organs show that DLG294 was less virulent than 14028s in the wild-type (Ity$^{s}$) mice (Fig. 4), which was in concordance with the results obtained with the (Ity$^{r}$) C3H/HeN mice (Fig. 1).

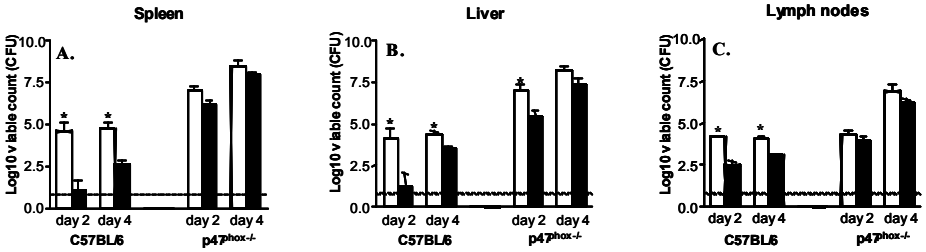


**Figure 4.** Bacterial counts in livers, spleens, and lymph nodes of p47$^{phox-/-}$ and wild-type C57BL/6 (Ity$^{s}$) mice infected with *S. enterica* serovar Typhimurium 14028s (white bars) and DLG294 (black bars). The p47$^{phox-/-}$ mice and the wild-type C57BL/6 mice were infected subcutaneously in the flanks with ~10,000 CFU of *S. enterica* serovar Typhimurium 14028s or DLG294. The actual dosage was confirmed by plating serial dilutions of the inoculum. Bacterial counts in livers, spleens, and lymph nodes were determined at days 2 and 4. The results are expressed as $\log_{10}$ viable counts (means ± standard errors of the means) obtained from groups of four mice per time point. Asterisks indicate that the number of wild-type 14028s bacteria is statistically different from that of DLG294 bacteria (Student's *t* test). All *Salmonella*-infected p47$^{phox-/-}$ mice were dead by day 5. Dashed lines, detection limit.

So, even in *Salmonella*-sensitive (Ity$^{s}$) mice, the superoxide-sensitive mutant strain DLG294 is attenuated. In p47$^{phox-/-}$ mice, however, 14028s induced no increase in liver weight within 4 days after infection, but did cause an increase in spleen weight. DLG294 did not seem to cause hepatosplenomegaly in the p47$^{phox-/-}$ mice, as the liver weight declined and the spleen weight remained the same at the early time points after infection (data not shown). As expected, due to the defect in innate defense, the bacterial loads

were higher in the p47$^{phox-/-}$ mice than in the wild-type mice (Fig. 4), and after 5 days of infection all the p47$^{phox-/-}$ mice died from the infection. At days 2 and 4 after infection, with the exception of the livers at day 2, there was no difference in bacterial loads between DLG294- and 14028s-infected mice, indicating that the replication of the superoxide-sensitive mutant strain was not hampered in mice that do not produce any superoxide.

**Histopathology of *S. enterica* serovar Typhimurium 14028s and DLG294 infection in wild-type and p47$^{phox-/-}$ C57BL/6 mice.** Microscopic examination of the organs of the C57BL/6 mice revealed that the livers appeared normal on day 1 for both the 14028s- and the DLG294-infected mice. Infiltrating cells started to appear in the livers of 14028s-infected wild-type mice on day 2, and large amounts of microabscesses and granulomatous lesions were present on day 4. Representative tissue sections are shown in Fig. 2B. Infiltrating cells and microabscesses were also observed in DLG294-infected wild-type mice on days 2 and 4, but to a much lesser extent and with only a very few beginning lesions on day 4. In the livers of the p47$^{phox-/-}$ mice, on the other hand, infiltrating cells were already observed in the livers on day 1 after infection in both the 14028s- and the DLG294-infected mice. Microabscesses started to appear on day 2. On day 4, the structure of the livers was destroyed in both groups of p47$^{phox-/-}$ mice, the microabscesses became very abundant, and many necrotic granulomatous lesions were present (Fig. 2B). Thus, the pathology induced by DLG294 was less severe in wild-type mice but was as severe as that induced by 14028s in the p47$^{phox-/-}$ mice, which do not produce any superoxide. Thus, when no superoxide was produced, superoxide-sensitive mutant strain DLG294 appeared to be as virulent and as pathogenic as wild-type *S. enterica* serovar Typhimurium 14028s. This strongly suggests a role for SspJ in the defense mechanisms of *S. enterica* serovar Typhimurium against superoxide produced by the NADPH oxidase.

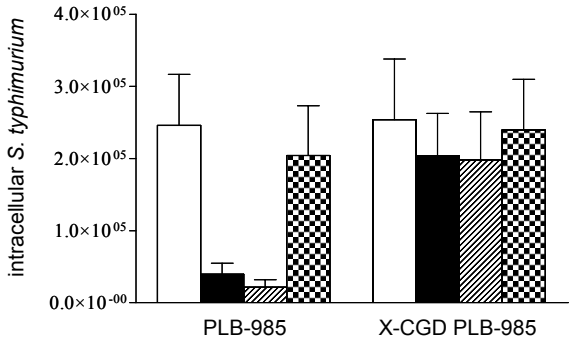


**Figure 5.** Intracellular *S. enterica* serovar Typhimurium in PLB-985 and X-CGD PLB-985 cells. The cells were challenged with *S. ente*rica serovar Typhimurium 14028s (white bars), DLG294 (black bars), DLG294-pWSK29 (hatched bars), and DLG294-pTS175 (checkered bars) as described in Materials and Methods. The numbers of intracellular bacteria were determined at 24 h after challenge. Asterisks indicate that the number of intracellular bacteria is significantly different from that of wild-type *S. enterica* serovar Typhimurium 14028s. Mean data of two independently performed experiments ± standard errors of the means are shown.

**In vitro intracellular growth of *S. enterica* serovar Typhimurium in PLB-985 cells.** The p47$^{phox-/-}$ mice showed an autosomal recessive deficiency in the p47$^{phox}$ subunit of the NADPH oxidase complex and were therefore unable to produce any superoxide. To exclude the possibility that the observed effect in the bone marrow-derived macrophages from p47$^{phox-/-}$ mice was due to the deficiency in the p47$^{phox}$ protein itself and not to the inactivation of the NADPH oxidase complex, we wanted to examine our *S. enterica* serovar Typhimurium strains in another in vitro model for NADPH oxidase deficiency. Therefore, we used X-CGD PLB-985, a cell line in which the gp91$^{phox}$ gene was disrupted by homologous recombination in the human leukemic cell line PLB-985 and which, as a consequence, fails to produce any superoxide (29). Cells were infected with *S. enterica* serovar Typhimurium 14028s, DLG294, DLG294-pWSK29, and DLG294-pTS175 as described for the replication assay in macrophages. Mean data from two independently performed experiments are shown in Fig. 5. At 24 h after infection, bacterial loads of wild-type *S. enterica* serovar Typhimurium 14028s within wild-type PLB-985 cells were higher than those of mutant strain DLG294. In the X-CGD PLB-985 cells, however, there was no difference in the intracellular CFU count after 24 h between the *S. enterica* serovar Typhimurium strains. The diminished intracellular replication of DLG294 could be fully reversed to that of wild-type *S. enterica* serovar Typhimurium 14028s by the expression of *sspJ* on the low-copy-number plasmid pWSK29 (DLG294-pTS175), whereas introduction of the vector (DLG294-pWSK29) did not affect the intracellular count. These data are in concordance with the data obtained with the macrophages of wild-type and p47$^{phox-/-}$ C57BL/6 mice (Fig. 3).

## Discussion

The main findings of this study are that the superoxide-sensitive *S. enterica* serovar Typhimurium mutant strain DLG294 (25) is attenuated in vivo and causes less pathology than wild-type *S. enterica* serovar Typhimurium 14028s but that it regains virulence in X-CGD PLB-985 cells and in p47$^{phox-/-}$ mice that show a deficiency in the phagocyte NADPH oxidase and therefore do not produce any superoxide. In addition, the decreased virulence of DLG294 is a direct cause of the defect in *sspJ* and is not a polar effect caused by diminished transcription of genes directly downstream of *sspJ*, since the virulence can be fully restored to that of the wild type by the expression of *sspJ* on a low-copy-number plasmid (DLG294-pTS175). These results suggest that the protein encoded by *sspJ* (SspJ) is necessary for virulence and plays a very important role in the defense mechanisms of *S. enterica* serovar Typhimurium against superoxide produced by the phagocyte NADPH oxidase.

We examined the in vivo course of infection of *S. enterica* serovar Typhimurium 14028s and DLG294 in the typhoid mouse model by infecting C3H/HeN mice (Ity$^{r}$) subcutaneously in the inguinal region to establish a reservoir near the lymph nodes; *Salmonella* readily spreads from this reservoir via the lymph stream, and the infection

becomes systemic, finally reaching the liver and spleen (3). This model gives rise to a more subtle chronic infection than the intraperitoneal, intravenous, and oral infection models that give rise to peracute and overwhelming systemic infections. As shown with this model, DLG294 is highly attenuated both in the relatively resistant Ity$^{r}$ mice and in *S. enterica* serovar Typhimurium-sensitive Ity$^{s}$ mice. This is remarkable, since Ity$^{s}$ C57BL/6 mice have somewhat reduced phagocyte production of reactive nitrogen and oxygen species (6, 19, 21) and yet our mutant strain DLG294 still was attenuated. Apparently, Ity$^{s}$ C57BL/6 mice produce sufficient superoxide to prevent DLG294 replication. In contrast, the virulence of DLG294 was completely restored in p47$^{phox-/-}$ mice, which do not produce any superoxide. These results were confirmed by in vitro replication experiments with bone marrow-derived macrophages from the wild-type (superoxide-positive) and p47$^{phox-/-}$ (superoxide-negative) mice and with X-CGD PLB-985 cells (29). Thus, when no superoxide was produced, superoxide-sensitive mutant *S. enterica* serovar Typhimurium strain DLG294 was able to replicate independent of the underlying genetic defect in the genes encoding the NADPH oxidase, which causes the defect in superoxide production.

We observed severe pathology in the livers of C3H/HeN and C57BL/6 mice infected with wild-type *S. enterica* serovar Typhimurium 14028s and in p47$^{phox-/-}$ mice infected with 14028s or DLG294. Liver pathology can be induced by superoxide produced upon activation of macrophages, as shown in mice chronically treated with alcohol (15). In our experiments, however, the liver pathology is induced by *S. enterica* serovar Typhimurium itself and is not caused by superoxide produced upon infection, as the p47$^{phox-/-}$ mice produce no superoxide but do develop severe liver damage upon infection with *S. enterica* serovar Typhimurium 14028s and DLG294. In addition, the pathology is most severe when bacterial numbers are highest.

A mutant that has a phenotype similar to that of DLG294 is a *zwf* mutant that is deficient in glucose-6-phosphate dehydrogenase (G6PD) (16). G6PD catalyzes the first step in the pentose phosphate cycle, which provides ribose for nucleoside synthesis and reducing equivalents in the form of NADPH. G6PD is very important in defense against oxidative and nitrosative stress because it provides NADPH, thereby maintaining the cellular redox state, regenerating reduced thiols, and repairing oxidative and nitrosative damage (8, 24). Mutants deficient in *zwf* show increased susceptibility to reactive oxygen and nitrogen intermediates and are attenuated in mice (16). Also the in vivo virulence of these mutants can be fully restored to that of the wild type by elimination of the phagocyte respiratory-burst oxidase (gp91$^{phox-/-}$ mice) (16). However, the *zwf* mutant is also more sensitive to hydrogen peroxide (16), whereas DLG294 is not (25), suggesting that SspJ is not a dehydrogenase.

It has previously been reported that periplasmic Cu,Zn-SOD (SodC) is very important in the defense of *S. enterica* serovar Typhimurium against oxidative stress, as was evident from *sodC* mutants, which showed reduced survival within macrophages and attenuated virulence in mice (4, 7). *S. enterica* serovar Typhimurium contains two periplasmic SODs, SodCI and SodCII (7). When one of these SODs is deleted, *S. enterica* serovar Typhimurium is slightly attenuated, but when both SODs are deleted, *S. enterica* serovar



Typhimurium is highly attenuated (7). The phenotype of DLG294 resembles that of this SodC double mutant and suggests a role for SspJ as an SOD. However, we have previously shown that SOD activities of DLG294 and wild-type *S. enterica* serovar Typhimurium 14028s are the same (25). In addition, SodCII expression is upregulated in stationary phase (7), while SspJ is constitutively expressed, and so far no inducing conditions have been found (25). These results indicate that the SspJ protein is not an SOD. However, SspJ does play a crucial role in the defense against superoxide. It could act in a regulatory pathway as a sensor or cofactor for SOD, it could be involved in neutralizing the toxic effects of superoxide, or it could even prevent the production of superoxide.

The last mechanism has been described for mutants deficient in SPI2, which have the same phenotype as DLG294 (12, 28). It is proposed that the proteins encoded by the genes located within this SPI2 defend the bacterium against superoxide by preventing the NADPH oxidase from trafficking toward the *Salmonella*-containing vacuoles (28) or by preventing the assembly of the NADPH oxidase at the phagosomal membrane (12). We are currently investigating whether *sspJ* has a role in this mechanism of preventing superoxide production.

The *sodCI* and *-CII*, the *zwf*, and the type III secretion mutants descibed above have diverse genetic defects, but they all have the same phenotype. Apparently, the interplay between all these distinct factors determines resistance to superoxide. It is clear that *sspJ is* important in this resistance, but its exact function and mechanism of action remain to be elucidated.

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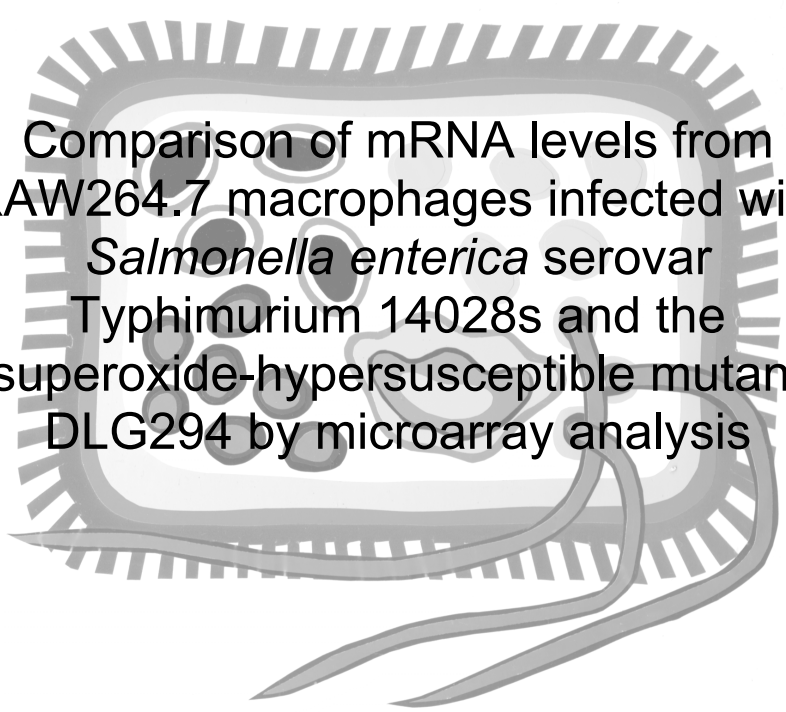
chapter eight



# Comparison of mRNA levels from RAW264.7 macrophages infected with *Salmonella enterica* serovar Typhimurium 14028s and the superoxide-hypersusceptible mutant DLG294 by microarray analysis

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*Manuscript in preparation*

## Abstract

Upon activation, macrophages initiate the transcription of genes coding for the expression of proteins and enzymes that participate in mounting the host response against pathogens such as *Salmonella*. DLG294 is an *S. enterica* serovar Typhimurium mutant strain that is hypersusceptible to superoxide and is attenuated due to its lack of expression of *sspJ*. To further characterize DLG294 we evaluated whether macrophages responded differently upon challenge with the virulent wild type strain 14028s than upon challenge with this attenuated superoxide-hypersusceptible strain DLG294 by defining the transcript profiles of RAW264.7 cells exposed to either on of these strains.

Infection with either one of these strains resulted in altered expression of a lot of genes involved in all kinds of cellular processes and defense mechanisms. By comparing the transcript profiles of RAW264.7 macrophages exposed to wild-type *S. enterica* serovar Typhimurium 14028s or DLG294 for 4 h, however, no great differences could be observed, indicating that the intracellular presence of either of these two strains does not elicit a different host-cell response. Therefore, the differences in outgrowth between wild-type *S. enterica* serovar Typhimurium and DLG294 could only be explained by the lack of expression of *sspJ* in DLG294, although the exact function and mechanism of action of the protein encoded by *sspJ* remain unknown.

## Introduction

*Salmonellae* are gram-negative, facultative intracellular pathogens that can cause a variety of diseases in animals and man, ranging from mild gastroenteritis to severe systemic infections like typhoid fever. *Salmonella enterica* serovar Typhimurium may cause gastroenteritis in man, but causes systemic infection in mice comparable to typhoid fever in man (15). *S. enteri*ca serovar Typhimurium predominantly invades mononuclear phagocytes and is able to cause persistent infections by evasion or disturbance of the host immune system (13). Despite the fact that these cells contain a multitude of antimicrobial defense mechanisms as part of the innate immune defense system, *S. enterica* serovar Typhimurium is able to enter, survive, and even replicate within these phagocytes. The exact mechanisms by which *S. enterica* serovar Typhimurium is able to survive after phagocytosis are unknown, but *S. enterica* serovar Typhimurium responds to the specific host environment by expressing factors that are necessary for intracellular survival and for resistance against the defense systems of the host (4, 8, 9, 13, 17). Like for most intracellular bacteria, this ability of *S. enterica* serovar Typhimurium to enter and replicate within phagocytic cells is essential for survival and pathogenesis, as mutants unable to do so are avirulent (7). In vitro studies have shown that *S. enterica* serovar Typhimurium is able to survive and replicate within non-activated macrophages and that this can also lead to the induction of apoptosis (18). The outcome of infection, however, strongly depends upon the interaction between the pathogen and its host.

Upon infection, the host will develop an immune response that limits bacterial growth and eventually kills and eliminates the pathogen. Although *Salmonella* resides and replicates within macrophages, these macrophages do play an important role in the host defense against *Salmonella*. In fact, interactions between the macrophage and *Salmonell*a are necessary for the early control of infection, and subsequently, for the induction of acquired immunity (11, 16). Also in immune mice that developed a *Salmonella*-specific response including antigen-specific antibodies, macrophages played a major role in restricting early bacterial growth since in vivo depletion of macrophages in these mice resulted in increased susceptibility and mortality (25). In vivo, macrophages are mainly activated through T lymphocyte-dependent responses, but are also activated upon infection with live bacteria or upon contact with several bacterial components called Pathogen Associated Molecular Patterns (PAMPs) (including LPS, porins and outer membrane proteins, fimbrial proteins, flagella, lipoproteins, glycoproteins, and peptidoglycan) (10). Macrophages have evolved mechanisms to recognize such PAMPs by expressing pattern recognition receptors that recognize the PAMPs and then initiate the innate immune response to clear the infection (20). Upon activation, macrophages induce the transcription of genes coding for the expression of proteins and enzymes that participate in mounting the host response against the pathogens. The induced gene expression profile seems to be dependent upon the activation state of the macrophages (19) and might therefore give an indication on how the macrophages respond to infection with a certain pathogen.



Recently, we have described the isolation and characterization of a superoxide hypersusceptible *S. enterica* serovar Typhimurium mutant strain DLG294 (21). This mutant strain DLG294 lacks the expression of *sspJ* and is highly attenuated in vivo in C3H/HeN mice and in vitro in macrophages, but is able to grow out as much as the wild-type strain in cells and mice that cannot produce any superoxide due to a non-functional NADPH oxidase complex (21, 24). These studies showed that expression of *sspJ* in *S. enterica* serovar Typhimurium plays an important role in resistance against superoxide, but its exact function and mechanism of action remained to be elucidated. Additional experiments have shown that DLG294 is not only hyper-sensitive to superoxide, but is also more susceptible to certain antibiotics (Tahar van der Straaten, unpublished data). Although hypersusceptibility to superoxide could be the major cause of attenuated virulence of DLG294, it cannot be excluded that other factors might also play a role. Since a lot of strains that are highly susceptible to menadione, i.e. intracellular superoxide, are not attenuated in mice it is clear that superoxide sensitivity alone does not determine virulence. For example, we have recently described *S. enterica* serovar Typhimurium mutants that are much more susceptible to menadione, yet are not attenuated at all (22, 23). These data suggest that other factors play a role. For instance the macrophage response to Salmonella, which determines the level of macrophage activation, may be an important factor. Therefore we decided to study whether the macrophage responds differently to DLG294 than to wild-type *S. enterica* serovar Typhimurium 14028s. A possible difference in the activation status of the macrophages might explain the differences in virulence of DLG294 and the wild-type strain and might clarify whether attenuation of DLG294 is solely due to its hypersusceptibility to superoxide produced by the macrophages or that additional mechanisms play a role.

## Materials and Methods

**Bacterial strains.** Single colonies of wild-type *S. enterica* serovar Typhimurium strain 14028s and superoxide-sensitive derivative DLG294 (21) were grown overnight in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37ºC while being shaken (225 rpm).

**Cells and cell culture conditions.** The mouse macrophage cell line RAW264.7 (ATCC TIB71) and the human granulocyte-like cell lines PLB-985 and X-CGD PLB-985 (26) were maintained at 37ºC with 5% $CO_2$ in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (50 µg/ml). For granulocytic differentiation, the PLB-985 and X-CGD PLB-985 cells were exposed to 0.5% dimethylformamide for 5-6 days. Under these conditions, the PLB-985 cells acquire respiratory burst activity, while the X-CGD PLB-985 cells do not (26).

**Replication of *S. enterica* serovar Typhimurium within RAW264.7 macrophages and PLB985 cells.** One day before challenge RAW264.7 cells were seeded in 6-wells plates in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal calf serum, but without antibiotics at $1 \times 10^6$ cells per well. The cells were challenged with *S. enterica* serovar Typhimurium at a 10:1 multiplicity of infection. To promote the uptake of the bacteria, the bacteria were spun onto the macrophages by centrifugation at 300 × g for 10 minutes and the cells were allowed to internalize the bacteria for 30 minutes at 37ºC with 5% $CO_2$. The cells were washed with phosphate-buffered saline (PBS) and were treated with 100 μg/ml gentamicin for 10 minutes to kill the extracellular bacteria and were then washed again. Medium supplemented with 10 μg/ml gentamicin was added to the cells to prevent reinfection and to kill any remaining bacteria. At 4 hours after infection, the cells were washed thoroughly with PBS and total RNA was isolated from 4 of the wells. As a control for infection, cells from duplicate wells were lysed with water and the number of intracellular bacteria was determined by plating serial dilutions. To obtain RNA from uninfected cells, the cells were treated exactly like the infected cells, but no bacteria were added. For infection of the non-adherent (X-CGD) PLB-985 cells, $1 \times 10^5$ cells were infected by incubating the cells together with $1 \times 10^6$ bacteria while rotating for 30 min at 37°C. The cells were then treated with 100 μg/ml gentamicin for another 60 min to kill the extracellular bacteria. After washing with PBS, the cells were lysed in 1 ml of distilled water. Serial dilutons of the lysate were made and plated for determination of the number of intracellular CFU.

**In vivo *Salmonella* infection.** Mice were inoculated subcutaneously with $3 \times 10^4$ CFU in the flanks with 0.1 ml bacterial suspension in PBS. Per group 4 mice were used to determine the bacterial load within the organs. On day 1 after infection, mice were sacrificed by carbon dioxide inhalation and the inguinal lymph nodes, livers and spleens were aseptically removed. The bacterial load within these organs was determined by preparing single-cell suspensions using 70-μm-mesh-size cell strainers (Falcon). The cells were pelleted by centrifugation for 10 min and the cells were lysed in distilled water. Serial dilutions of the lysate were made to determine the bacterial loads within the organs.

**RNA sample preparation.** Samples were prepared according to the protocols in the Affymetrix Gene Chip Expression Analysis technical manual (Affymetrix, Inc., Santa Clara, California) and were obtained from two independently performed in vitro replication experiments. Total RNA was isolated from the *S. enterica* serovar Typhimurium infected RAW264.7 cells at 4 hours after infection using TRIzol Reagent (Gibco BRL Life Technologies) according to the manufacturer's protocol. The RNA obtained from the uninfected cells was pooled, while the RNA samples from the infected cells were individually processed. After the ethanol precipitation in this TRIzol extraction procedure, the total RNA was cleaned up using the Qiagen RNeasy Total isolation Kit according to the manufacturer's protocol. The RNA was checked by gel electrophoresis before proceeding. Double stranded cDNA was prepared using the Gibco BRL SuperScript Choice System



and T7-$(dT)_{24}$ primer (Genset Corp., La Jolla, California). The cDNA was purified by phenol/chloroform extraction and ethanol precipitation. Biotin-labeled cRNA was synthesized in an in vitro transcription reaction using the ENZO Bioarray™ HighYield™ RNA Transcript Labeling Kit (ENZO Diagnostics, Inc. Farmingdale, New York) according to the manufacturer's recommendations. Finally, the biotin-labeled cRNA was purified using the Qiagen RNeasy Total Isolation Kit and was fragmented in 40 mM Tris-acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc at 94°C for 35 minutes.

**Microarray.** Microarray analysis was performed at the Leiden Genome Technology Center by Eveline Mank (Leiden, The Netherlands). The fragmented labeled cRNA (15 μg) was hybridized to GeneChip murine genome U74Av2 oligonucleotide arrays (Affymetrix, Santa Clara, CA). The chips were washed and stained with streptavidin-phycoerythrin in a GeneChip Fluidics station 400 (Affymetrix) and were then scanned using an Affymetrix GeneArray. Affymetrix Microarray Suite 5.0 (MAS5.0, Affymetrix) was used to analyze the data. The chips that were hybridized with cRNA from RAW264.7 cells infected with either *S. enterica* serovar Typhimurium 14028s or DLG294 were compared to a chip that was hybridized with pooled cRNA from uninfected cells to analyze which gene expression was changed by the infection. Difference calls were assigned as described previously (6): increased, 2; marginally increased, 1; nor changed, 0; marginally decreased, -1; decreased, -2. The sum of difference calls was calculated and a sum of $\geq 3$ or $\leq -3$ was the cut off value for increase or decrease, respectively. We have converted the signal log ratio output of increased and decreased expression into fold change for convenience using the formula recommended by Affymetrix:

$$\text{Fold increase} = 2^{\text{signal log ratio}}, \text{ if signal log ratio} > 0$$
$$\text{Fold decrease} = 2^{-\text{signal log ratio}}, \text{ if signal log ratio} < 0$$

To ensure that the data are reliable, genes were considered to be differentially expressed if $P < 0.01$ (Student *t*-test) and fold increase $\geq 2.0$ and fold decrease $\geq 1.75$.

**Cytometric bead array.** At 4 h after infection, supernatant from RAW264.7 cells infected with wild-type *S. enterica* serovar Typhimurium or DLG294 or from uninfected cells was taken, diluted 10 times, and used in a Mouse Inflammation Cytometric Bead Array™ (CBA; BD Biosciences, San Jose, CA) according to the manufacturer's recommendations to compare the type and amount of cytokines produced by these cells and is schematically depicted in Fig. 1. The CBA assay contains six populations of polysterene beads that have been coated with capture antibodies directed against either IL-6, IL-10, MCP-1, IFNγ, TNFα, or IL-12p70. These beads are of equal size but differ in fluorescence intensities and can be discriminated by detection in the FL3 channel of a flow cytometer. The six bead populations are mixed and 50 μl of this mixed suspension, together with 50 μl phycoerythrin(PE)-labeled detection antibodies are added to 50 μl of the test samples or cytokine standards and were incubated for 2 h at room temperature to

form the sandwich complexes. After washing the bead-cytokine-antibody-PE complexes, FACS analysis was performed on a BD FACSCalibur™ flow cytometer and data were acquired and analyzed using Becton Dickinson (BD) Cytometric Bead Array (CBA) software. Forward vs side scatter were used to gate on the beads. The fluorescence intensity detected in the FL-3 channel discriminates the six different bead populations and the mean fluorescence intensity measured with PE in the FL-2 channel is proportional to the cytokine concentration in the sample. By two-color dot plotting the FL-2 vs FL-3 the change in fluorescence intensity measured with PE for each of the six bead populations could be compared. The cytokine standards were used to make standard curves from which cytokine concentrations in the test samples could be calculated.

## Results and Discussion

**Early in vitro intracellular growth of *S. enterica* serovar Typhimurium.** DLG294 is an *S. enterica* serovar Typhimurium mutant strain that is hypersusceptible to superoxide due to its lack of expression of *sspJ* (21). This mutant strain is attenuated in vivo in C3H/HeN mice and in vitro in RAW264.7 macrophages, but regains virulence in cells and mice that do not produce any superoxide due to a non-functional NADPH oxidase complex (21, 24). These previous studies showed that *sspJ* is involved in the defense against superoxide produced by macrophages, but its exact function and mechanism of action were still unknown. From this we concluded that attenuation of DLG294 is due to its hypersusceptibility to superoxide. However, when we examined at bacterial counts very early after infection of X-CGD PLB985 cells we observed that bacterial loads of DLG294 were lower than those of the wild-type strain both in the wild-type, superoxide producing PLB985 cells as well as in the X-CGD PLB985 cells that cannot produce any superoxide due to disruption of the $gp91^{phox}$ gene (26) (Fig. 2A). Despite the fact that these X-CGD PLB-985 cells cannot produce any superoxide, DLG294, although not statistically significant, still showed lower bacterial numbers that the wild-type strain. When the cells were cultured for another 24 hours, DLG294 and the wild-type strain reached equal bacterial numbers in the X-CGD PLB985 cells while DLG294 is attenuated in PLB985 cells as described before (24). Similar data were observed in vivo, i.e. even though DLG294 reached similar bacterial numbers to wild-type 4 days after infection of mice (22), we observed that at 1 day after infection, bacterial numbers were lower than for the wild-type suggesting that early killing is different for wild-type and DLG294 (Fig. 2B and 2C). This suggests that attenuation of DLG294 is not solely due to increased susceptibility to superoxide and that additional factors such as the activation status of the cells play a role in the increased susceptibility and attenuation of DLG294 very early after infection. Once these early additional factors are overcome, DLG294 is able to grow out as much as the wild-type strain reaching comparable bacterial numbers in cells and mice that cannot produce any superoxide (24).

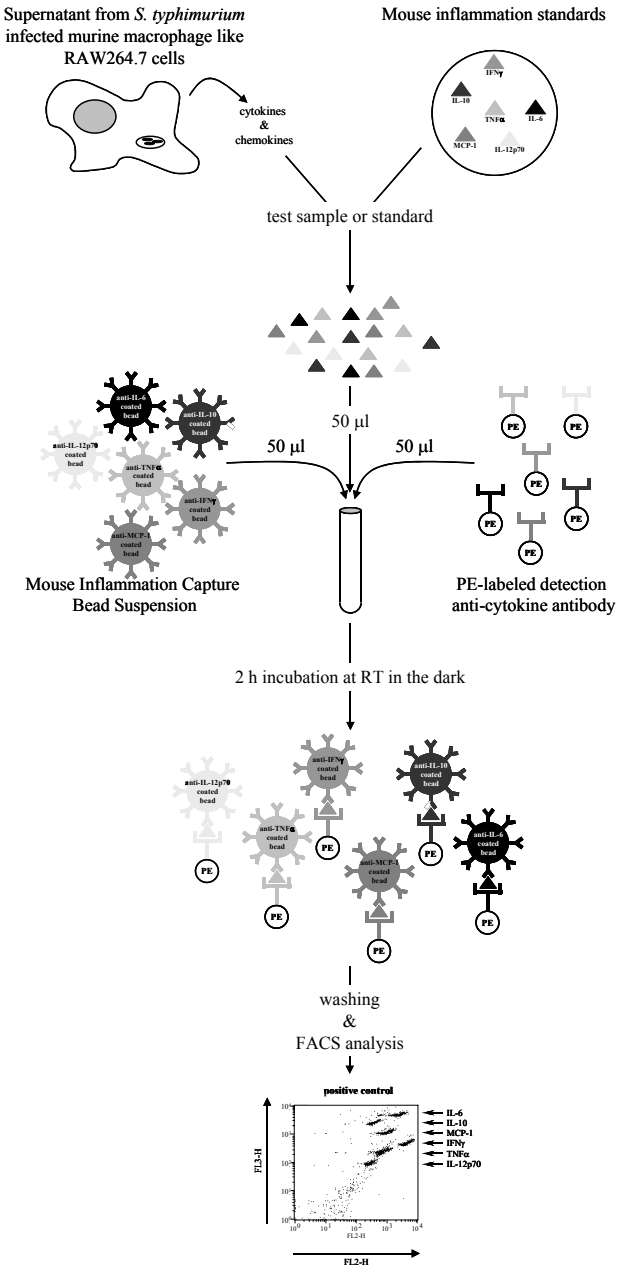


**Figure 1.** Schematic overview of the Cytometric Bead Array. Supernatant from RAW264.7 cells infected with wild-type *S. enterica* serovar Typhimurium or DLG294 or from uninfected cells was taken, diluted 10 times, and used according to the manufacturer's recommendations to compare the type and amount of cytokines produced by these cells.

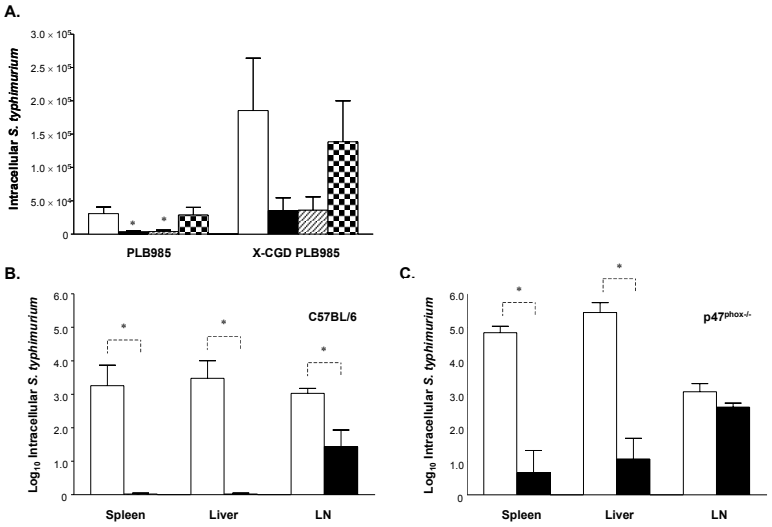


**Figure 2.** Bacterial numbers in (X-CGD) PLB985 cells after incubation with bacteria (MOI 10) (A) and in livers, spleens, and inguinal lymph nodes of C57BL/6 (B) or p47$^{phox-/-}$ mice (C) at 1 day after infection. Cells were incubated or mice were infected with wild-type *S. enterica* serovar Typhimurium 14028s (white bars), DLG294 (black bars), DLG294-pWSK29 (dashed bars), or DLG294-pTS175 (checkered bars). Asterisks indicate that the number of intracellular bacteria is significantly different (P<0.05) from that of wild-type *S. enterica* serovar Typhimurium 14028s.

**Comparison of gene expression profiles of uninfected, wild-type, and DLG294 infected RAW264.7 cells.** Next we studied the activation status of RAW264.7 macrophages infected with wild-type or DLG294 by determining gene expression profiles. Fig. 3 shows that the number of intracellular DLG294 was much lower than wild-type *S. enterica* serovar Typhimurium 14028s at 4 h after infection as shown previously (21). Bacterial numbers were equal between wild-type and DLG294 at 30 minutes after infection (data not shown), but the wild-type reached much higher bacterial counts after 24 h than DLG294, indicating that the uptake of DLG294 by the macrophages is similar to that of the wild-type strain, but its replication is severely impaired.

Infection with wild-type or DLG294 resulted in the induction of 174 genes. Only 9 of these genes were induced to a different extent in DLG294 and wild-type infected macrophages (Table 4). We will discuss genes that are highly induced, moderately induced, or that are repressed by infection and highlight the differences between wild-type and DLG294 infected cells. Genes showing down-regulated expression upon infection are shown in Table 3 that shows that the gene-expression was only moderate decreased compared to uninfected cells. The fold decreases do not exceed 3.9, while for the upregulated genes the expression increased up to more than a 100-fold for *Cxcl2* (Table 1). Genes showing decreased expression were mainly involved in cell cycle processes, signal transduction, and transcription.

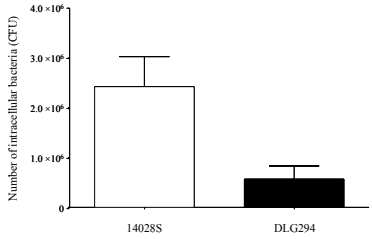


**Figure 3.** Number of intracellular bacteria in RAW264.7 macrophages at 4 h after infection (A). The cells were challenged with S. enterica serovar Typhimurium 14028s (white bars) or DLG294 (black bars) as described in Materials and Methods. Asterisks indicate that the number of intracellular bacteria is significantly different (P<0.05) from that of wild-type *S. enterica* serovar Typhimurium 14028s.

**Strongly induced genes.** A lot of the genes that are up-regulated by the infection are involved in inflammatory processes and apoptosis, but also in signal transduction and transcription (Tables 1 and 2). The genes showing the most pronounced increase in expression are genes involved in inflammation and chemotaxis and was highest for *Cxcl2*, a gene encoding macrophage inflammatory protein 2 that is involved in the chemotaxis of leukocytes, but that does not induce chemokinesis or an oxidative burst.

Other genes showing highly increased expression are genes involved in the defense response and include cytokines, chemokines, MHC Class II, activation markers, and IFNγ or LPS induced genes involved in the immune response.

**Table 1. Macrophage gene expression strongly induced by *S. enterica* serovar Typhimurium infection**

| Accession Nr. | Fold Increase | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Inflammation, Cytokines,and Chemokines** | | | | |
| M13926 | 12.06 | 14.06 | **Csf3** | Colony-stimulating factor 3 (granulocyte) |
| M14639 | 8.21 | 10.57 | *Il1a* | Interleukin 1 alpha |
| AF065947 | 75.41 | 59.18 | *Ccl5* | Chemokine (C-C motif), ligand 5 |
| U16985 | 29.27 | 34.63 | *Ltb* | Lymphotoxin B |
| M33266 | 25.24 | 19.09 | *Cxcl10* | Chemokine (C-X-C- motif), ligand 10 |
| X53798 | 115.43 | 124.31 | *Cxcl2* | Chemokine (C-X-C- motif), ligand 2 |
| X62502 | 5.47 | 5.10 | *Ccl4* | Chemokine (C-C motif), ligand 4 |
| X70058 | 8.49 | 8.29 | *Ccl7* | Chemokine (C-C motif), ligand 7 |
| D84196 | 9.52 | 10.93 | *Tnf* | Tumor necrosis factor |
| **Transportation and Binding Proteins** | | | | |
| AI844128 | 6.75 | 7.56 | *Ehd1* | EH-domain containing 1 |
| AI747899 | 2.32 | 2.94 | *Pitpnb* | Phosphatidylinositol transfer protein, beta |
| AF006467 | 12.62 | 6.15 | *Pitpnm* | Phosphatidylinositol membrane-associated |
| **Apoptosis** | | | | |
| U44088 | 36.38 | 21.39 | *Phlda1* | Pleckstrin homology-like domain, family A, member 1 |
| **Cell Cycle, Differentation, and Proliferation** | | | | |
| M64849 | 3.45 | 5.15 | *Pdgfb* | Platelet-derived growth factor chain B precursor (sis) |
| AF099973 | 6.56 | 6.83 | *Slfn2* | Schlafen 2 |
| **Biosynthesis** | | | | |
| U00978 | 12.29 | 11.75 | *Impdh1* | Inosine-5'-monophosphate dehydrogenase 1 |
| X07888 | 16.00 | 19.75 | *Hmgcr* | 3-hydroxy-3-methylglutaryl coenzyme A reductase |
| **Defense Response** | | | | |
| AF002719 | 6.19 | 6.59 | *Slpi* | Secretory leukoprotease inhibitor |
| X54149 | 6.08 | 8.76 | *Gadd45b* | Growth arrest and DNA damage-inducible protein GADD45 be (myeloid differentiation primary-response protein Myd118) |
| **Protein Degradation and Processing** | | | | |
| U66873 | 5.73 | 6.12 | *Pla2g5* | Phospholipase A2, group V |

**Table 1. -continued-**

| Accession Nr. | Fold Increase | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Immune Response** | | | | |
| X56602 | 41.79 | 39.05 | *Isg15* | Interferon stimulated protein |
| U43084 | 62.02 | 41.79 | *Ifit1* | Interferon-induced protein with tetratricopeptide repeats 1 |
| AV152244 | 18.02 | 13.02 | *G1p2* | Interferon, alpha-inducible protein |
| AI323667 | 93.76 | 91.38 | *Irg1* | Immunoresponsive gene 1 |
| L38281 | 76.36 | 57.99 | *Irg1* | Immunoresponsive gene 1 |
| **Antigen Presentation** | | | | |
| X52914 | 10.23 | 10.50 | *H2-K* | Histocompatibility 2, K region |
| D90146 | 22.06 | 32.48 | *H2-Q7* | Histocompatibility 2, Q region locus 7 |
| M27134 | 23.12 | 25.85 | *H2-K2* | Histocompatibility 2, K region locus 2 |
| **Signalling Receptors** | | | | |
| U65747 | 7.07 | 4.77 | *Il13ra2* | IL-13 receptor, alpha 2 |
| **Transcription** | | | | |
| X53654 | 6.30 | 6.64 | *Pou2f2* | POU domain, class 2, transcription factor 2 |
| Y11245 | 5.28 | 5.33 | *Foxm1* | Forkhead box M1 |
| X95316 | 5.48 | 4.04 | *Usf1* | Upstream transcription factor 1 |
| L00039 | 6.30 | 6.74 | *Myc* | Myelocytomastosis oncogene |
| **Regulatory** | | | | |
| AJ222800 | 8.00 | 8.49 | *Smpd2* | Shingomyelin phosphodiesterase 2, neutral |
| M89800 | 10.20 | 11.31 | *Wnt6* | Wingless-related MMTV integration site 6 |
| U57524 | 4.64 | 5.33 | *Nfkbia* | Nuclear factor of kappa light chain enhancer in B-cells inhibitor, alpha |
| **Signal Transduction** | | | | |
| AB016589 | 13.50 | 21.11 | *Ikbke* | Inhibitor of kappa-B kinase epsilon |
| M63659 | 17.93 | 21.93 | *Gna12* | Guanine nucleotide binding protein, alpha 12 |
| L35302 | 57.99 | 51.70 | *Traf1* | TNF receptor-associated factor 1 |
| AF053974 | 5.47 | 3.35 | *Swap70* | Swap complex protein, 70 kDa |
| U58203 | 4.87 | 5.48 | *Arhgef1* | Rho guanine nucleotide exchange factor (GEF) 1 |
| U34960 | 4.09 | 5.28 | *Gnb2* | Guanine nucleotide binding protein, beta 2 |
| X61399 | 7.25 | 7.12 | *Mlp* | MARCKS-like protein |
| AW120722 | 5.63 | 6.56 | *Mapkapk2* | MAP kinase-activated protein kinase 2 |
| AV374868 | 6.75 | 6.19 | *Socs3* | Suppressor of cytokine signalling 3 |
| M83380 | 5.78 | 6.08 | *Relb* | Avian reticuloendotheliosis viral (v-rel) oncogene related B |
| U88328 | 32.48 | 28.08 | *Socs3* | Suppressor of cytokine signalling 3 |
| **Other** | | | | |
| AF084480 | 6.14 | 8.30 | *Baz1b* | Bromodomain adjacent to zinc finger domain, 1B |
| AF037437 | 4.15 | 7.56 | *Psap* | Prosaposin |
| AB002136 | 7.32 | 7.84 | *Gpaa1* | Glycosylphosphatidylinositol anchor attachment protein 1 |
| U82610 | 16.24 | 27.00 | *Lcp1* | Lymphocyte cytosolic protein 1 |
| **Unknown Gene Function** | | | | |
| AF064447 | 3.61 | 5.47 | *Fem1a* | Feminization 1 homolog a |
| U50384 | 4.48 | 9.55 | *Smyd5* | SET and MYND domain containing 5 |
| AF073882 | 14.64 | 14.50 | *Mtmr7* | Myotubularin related protein 7 |
| U89434 | 10.06 | 16.15 | *Tbgr4* | Transforming growth factor beta regulated gene 4 |
| X67644 | 7.73 | 8.57 | *Ier3* | Immediate early response 3 |
| AA822413 | 5.89 | 5.86 | *Fbxw5* | F-box and WD-40 domain protein 5 |
| AW060657 | 7.73 | 8.59 | *Pmf1* | Polyamine-modulated factor 1 |
| AI837006 | 14.23 | 15.46 | *Cotl1* | Coactosin-like 1 |

The production of cytokines and chemokines and the induction of activation markers are all processes involved in innate defense against *Salmonella*. Cytokines are small soluble proteins that mediate and regulate the (anti-) inflammatory responses and can have local or systemic effects on several components of the immune system. Upon infection of RAW264.7 macrophages with *S. enterica* serovar Typhimurium, expression of genes encoding cytokines involved in innate defense are induced, such as granulocyte colony-stimulating factor 3, Interleukin 1 $\alpha$, lymphotoxin $\beta$, Tumor necrosis factor (encoded by *Csf3*, *Il1a*, *Ltb*, and *tnf*, respectively) (Table 1), and to a lesser extent, Interleukin 1



receptor antagonist (encoded by *Il1rn*) which regulates the expression and bioactivity of IL-1 (Table 2). Also genes encoding receptors involved in cytokine signaling such as TNF receptor, superfamily, members 5 and 1b (*tnfrsf5* and *tnfrsf1b*) and IL-13 receptor, alpha 2 (*Il13ra2*) are upregulated upon infection (Tables 1 and 2, signaling receptors).

**Table 2. Macrophage gene expression moderately induced by *S. enterica* serovar Typhimurium**

| Accession Nr. | Fold Increase | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Inflammation, Cytokines,and Chemokines** | | | | |
| X03505 | 2.74 | 4.15 | *Saa3* | Serum amyloid A3 |
| L32838 | 2.66 | 1.69 | *Il1rn* | IL-1 receptor antagonist |
| M88242 | 4.78 | 4.00 | *Ptgs2* | Prostaglandin-endoperoxide synthase 2 |
| **Transportation and Binding Proteins** | | | | |
| U95145 | 3.37 | 3.01 | *Akap1* | A kinase (PRKA) anchor protein 1 |
| X57349 | 3.37 | 3.81 | *Trfr* | Transferrin receptor |
| U15976 | 1.68 | 2.55 | *Slc27al* | Solute carrier family 27 (fatty acid transporter, member 1 |
| L23755 | 2.25 | 2.93 | *Slc19a1* | Solute carrier family 19 (sodium/hydrogen exchanger), member 1 |
| D21207 | 2.50 | 3.06 | *Bzrp* | Benzodiazepine receptor, peripheral |
| AI852578 | 4.01 | 3.87 | *Slc11a2* | Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 |
| AI747899 | 2.32 | 2.94 | *Pitpnb* | Phosphatidylinositol transfer protein, beta |
| **Extracellular Matrix and Adhesion** | | | | |
| U91513 | 3.37 | 3.19 | *Ninj1* | Ninjurin 1 |
| M90551 | 2.55 | 3.01 | *Icam1* | Intercellular adhesion molecule 1 |
| X79003 | 4.45 | 3.87 | *Itga5* | Integrin alpha-5 (fibronectin receptor alpha) |
| **Apoptosis** | | | | |
| X67914 | 2.86 | 2.47 | *Pdcd1* | Programmed cell death 1 |
| AF032459 | 4.59 | 3.61 | *Bcl2l11* | BCL2-like 11 (apoptosis facilitator) |
| U59758 | 3.28 | 3.32 | *Trp53* | Transformation related protein 53 |
| L37296 | 2.89 | 2.93 | *Bad* | Bcl-associated death promotor |
| M83649 | 4.83 | 4.69 | *Tnfrsf6* | Tumor necrosis factor receptor superfamily, member 6 (Fas antigen) |
| AJ242778 | 4.04 | 4.30 | *Tnip1* | TNFAIP3 interacting protein 1 |
| **Cell Cycle, Differentation, and Proliferation** | | | | |
| D29678 | 2.52 | 2.75 | *Cdk5* | Cyclin-dependent kinase 5 |
| M95200 | 2.70 | 2.80 | *Vegfa* | Vascular endothelial growth factor A |
| M64849 | 3.45 | 5.15 | *Pdgfb* | Platelet-derived growth factor chain B precursor |
| AJ009862 | 3.66 | 3.62 | *Tgfb1* | Transforming growth factor, beta 1 |
| U09507 | 3.17 | 3.23 | *Cdkn1a* | Cyclin-dependent kinase inhibitor 1A (p21) |
| AI849928 | 2.65 | 2.38 | *Ccnd1* | Cyclin D1 |
| AW047032 | 3.19 | 4.16 | *Pin1* | Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 |
| AW045530 | 2.02 | 2.49 | *Incenp* | Inner centromere protein |
| **DNA replication** | | | | |
| AW213225 | 2.61 | 3.77 | *Ddx18* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 18 |
| **RNA processing** | | | | |
| AW120557 | 3.25 | 4.59 | *Lsm4* | LSM4 homolog,U6 small nuclear RNA associated |
| **Biosynthesis** | | | | |
| AB005623 | 2.55 | 2.55 | *Agpat1* | 1-acylglycerol-3-phosphate O-acetyltransferase (lysophosphatidic acid acyltransferase, alpha) |
| AW122653 | 3.26 | 2.65 | *Mvk* | Mevalonate kinase |
| **Cytoskeleton and Membrane Proteins** | | | | |
| J04181 | 2.83 | 3.88 | *Actb* | Actin, beta, cytoplasmic |
| AJ249706 | 3.70 | 4.26 | *Myo10* | Myosin X |
| AB024717 | 2.83 | 2.73 | *Clecsf9* | C-type lectin (calcium-dependent, carbohydrate recognition domai lectin, superfamily member 9 |
| D14883 | 2.56 | 3.16 | *Kai1* | Kangai 1 (suppression of tumorigenicity 6, prostate) |
| D00472 | 2.75 | 2.55 | *Cfl1* | Cofilin 1, non-muscle |
| X56123 | 2.45 | 2.59 | *Tln* | Talin |
| AI837100 | 3.62 | 3.77 | *Cd83* | CD83 antigen |
| AI842889 | 2.49 | 2.75 | *Atp6v0b* | ATPase, $H^+$ transporting, V0 subunit B |

**Table 2. -continued-**

| Accession Nr. | Fold Increase | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Protein Synthesis and Modification** | | | | |
| M76131 | 2.55 | 2.55 | *Eef2* | Eukaryotic translation elongation factor 2 |
| X69656 | 2.39 | 2.65 | *Wars* | Tryptophanyl-tRNA synthetase |
| X05021 | 3.00 | 3.96 | *Rpl27a* | Ribosomal protein L27a |
| AI265655 | 2.59 | 2.86 | *Ppil2* | Peptidylprolyl isomerase (cyclophilin)-like 2 |
| AV380793 | 2.39 | 2.93 | *Eif4g1* | Eukaryotic translation initiation factor 4, gamma 1 |
| **Mitochondrion** | | | | |
| U85089 | 2.30 | 2.83 | *Txn2* | Thioredoxin 2 |
| AF043249 | 3.19 | 2.97 | *Tomm40* | Translocase of outer mitochondrial membrane 40 homolog |
| D17571 | 2.23 | 2.73 | *Por* | P450 (cytochrome) oxidoreductase |
| AI849904 | 2.17 | 2.74 | *Dlst* | Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxoglutarate complex) |
| **Lipid Catabolism** | | | | |
| AA408341 | 3.10 | 3.52 | *Pla2g5* | Phospholipase A2, group V |
| **Metabolism** | | | | |
| AF032466 | 2.56 | 2.49 | *Arg2* | Arginase type II |
| Z84471 | 2.30 | 2.75 | *G6pdx* | Glucose-6-phosphate dehydrogenase, X-linked |
| AI843795 | 2.56 | 3.28 | *Pgls* | 6-phosphogluconolactonase |
| AW047185 | 2.56 | 3.38 | *Thop1* | Thimet oligopeptidase 1 |
| AI060798 | 3.38 | 4.16 | *Ptges* | Prostaglandin E synthase |
| AI852592 | 2.18 | 3.07 | *Ndufb2* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2 |
| **Defense Response** | | | | |
| AV090497 | 4.44 | 3.66 | *Solpi* | Secretory leukoprotease inhibitor |
| **Superoxide Production** | | | | |
| AB002663 | 2.28 | 2.52 | *Ncf1* | Neutrophil cytosolic factor 1 ($p47^{phox}$) |
| U43384 | 2.55 | 2.93 | *Cybb* | Cytochrome b-245, beta polypeptide |
| M31775 | 3.66 | 4.64 | *Cyba* | Cytochrome b-245, alpha polypeptide |
| **Protein Degradation and Processing** | | | | |
| M25149 | 2.49 | 2.89 | *Psmd3* | Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3 |
| AW124386 | 2.61 | 3.15 | *Ubl5* | Ubiquitin-like 5 |
| AI850365 | 2.38 | 2.65 | *Ubc-rs2* | Ubiquitin C, related sequence 2 |
| **Regulatory** | | | | |
| X70764 | 3.46 | 4.09 | *Mark2* | MAP/microtubule affinity-regulating kinase 2 |
| AF018262 | 2.46 | 2.93 | *Ppp5c* | Protein phosphatase 5, catalytic subunit |
| U20857 | 3.15 | 4.38 | *Rangap1* | RAN GTPase activating protein 1 |
| J02935 | 3.29 | 4.34 | *Prkar2a* | Protein kinase, cAMP-dependent regulatory, type II-alpha |
| AF043070 | 2.30 | 3.04 | *Bckdk* | Branched chain ketoacid dehydrogenase kinase |
| AW049387 | 2.56 | 2.86 | *Arl2* | ADP-ribosylation factor-like 2 |
| **Intracellular Trafficking** | | | | |
| D87900 | 1.62 | 2.50 | *Arf3* | ADP-ribosilation factor 3 |
| Y13361 | 2.27 | 2.86 | *Rab7* | RAB7, member RAS oncogene family |
| **Signalling Receptors** | | | | |
| U05673 | 3.74 | 3.38 | *Adora2b* | Adenosine A2b receptor |
| M83312 | 3.37 | 3.48 | *Tnfrsf5* | TNF receptor superfamily, member 5 |
| X62700 | 4.15 | 4.51 | *Plaur* | Urokinase plasminogen activator receptor |
| X87128 | 4.38 | 4.87 | *Tnfrsf1b* | TNF receptor superfamily, member 1b |
| AA608277 | 2.65 | 2.75 | *Adora2b* | Adenosine A2b receptor |
| AI838195 | 3.46 | 4.26 | *Ogfr* | Opioid growth factor receptor |
| **Signal Transduction** | | | | |
| X95761 | 2.62 | 3.08 | *Lbcl1* | Lymphoid blast crisis-like 1 |
| X76850 | 3.98 | 2.62 | *Mapkapk2* | MAP kinase-activated protein kinase 2 |
| X84797 | 3.46 | 4.20 | *Hcls1* | Hematopoietic cell specific Lyn substrate 1 |
| X80638 | 3.77 | 4.46 | *Arhc* | Ras homolog gene family , member C |
| Y17808 | 2.59 | 3.49 | *Ptk91* | PTK9 protein typrotein tyrosine kinase 9-like (A6-related protein) |
| U20159 | 2.64 | 2.73 | *Lcp2* | Lymphocyte cytosolic protein 2 |
| U42383 | 2.73 | 2.47 | *Ppm1g* | Protein phosphatase 1G (formarly 2C), magnesium-dependent, gamma isoform |
| AI642662 | 2.55 | 2.22 | *Dusp16* | Dual specific phosphatase 16 |
| AA153773 | 2.74 | 3.53 | *Tbl3* | Transducin (beta)-like 3 |
| AA764261 | 2.77 | 2.97 | *Pim1* | Proviral integration site 1 |
| AW124934 | 3.46 | 3.06 | *Peli1* | Pellino 1 |
| AA762522 | 4.38 | 4.59 | *Dtx2* | Deltex 2 homolog |



**Table 2. -continued-**

| Accession Nr. | Fold Increase | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Transcription** | | | | |
| U09419 | 2.93 | 4.09 | *Nr1h2* | Nuclear receptor subfamily 1, group H, member 2 |
| AF015881 | 2.93 | 3.15 | *Nfe2l1* | Nuclear factor, erythroid-derived 2, -like 1 |
| AF043220 | 3.06 | 3.98 | *Gtf2i* | Genereal transcription factor II I |
| AB009693 | 4.51 | 4.94 | *MafG* | V-maf musculoaponeurotic fibrosarcoma oncogene family, protein G |
| AF060076 | 2.62 | 2.66 | *Phc2* | Polyhomeotic-like 2 |
| X55038 | 2.77 | 3.74 | *Cenpb* | Major centromere autoantigen B |
| U20735 | 2.66 | 2.77 | *JunB* | Jun-B oncogene |
| X14678 | 4.05 | 2.33 | *Zfp36* | Zinc finger protein 36 |
| U47543 | 2.45 | 2.87 | *Nab2* | Ngfi-A binding protein 2 |
| M61007 | 2.93 | 3.23 | *Cebpb* | CCAAT/enhancer binding protein (C/EBP), beta |
| L03215 | 2.59 | 2.84 | *Sfpi1* | SFFV proviral integration 1 |
| AF017085 | 3.10 | 3.88 | *Gtf2i* | General transcription factor II 1 |
| J04103 | 2.73 | 2.55 | *Ets2* | E26 avian leukemia oncogene 2, 3'domain |
| AI846152 | 2.74 | 2.70 | *Dscr1* | Down syndrome critical region homolog 1 |
| AW047899 | 2.97 | 2.75 | *Nfkb2* | Nuclear factor of kappa light polypeptide gene enhancer in B cells 2, p49/p100 |
| AI850881 | 2.47 | 2.65 | *Gtf2h4* | General transcription factor II H, polypeptide 4 |
| **Other** | | | | |
| L24118 | 2.93 | 3.14 | *Tnfaip2* | Tumor necrosis factor, alpha-induced protein 2 |
| U60884 | 2.62 | 2.77 | *Bin1* | Bridging integrator 1 |
| U32197 | 2.86 | 3.62 | *Fpgs* | Folylpolyglytamyl synthetase |
| U05837 | 2.83 | 3.26 | *Hexa* | Hexoaminidase A |
| **Unknown Gene Function** | | | | |
| AF061346 | 2.22 | 2.86 | *Tnfaip2* | TNF$\alpha$ induced protein 1 |
| U87965 | 2.32 | 2.65 | *Gtpbp1* | GTP-binding protein 1 |
| AF033201 | 2.65 | 2.84 | *Cpsf4* | Cleavage and polyadenylation specific factor 4 |
| M59821 | 4.30 | 4.29 | *Ier2* | Immediate early response 2 |
| AW210320 | 2.62 | 2.59 | *Ptov1* | Prostate tumor overexpressed gene1 |
| AW125378 | 2.66 | 2.94 | *Aamp* | Angio-associated migratory protein |
| AW122679 | 2.59 | 2.47 | *Prrg2* | *Proline-rich Gla (G-carboxyglutamic acid) polypeptide 2* |
| AW122052 | 2.16 | 2.86 | *Nans* | N-acetylneuraminic acid synthase (sialic acid synthase) |
| AW125157 | 2.45 | 2.63 | *Fbxw1b* | F-box and WD-40 domain protein 1B |
| AI837492 | 2.39 | 3.14 | *Orf61* | Open reading frame 61 |

These genes are upregulated upon infection with wild-type *S. enterica* serovar Typhimurium 14028s as well as by the superoxide-hypersusceptible mutant DLG294 and only the expression of *Il1rn* is statistical significantly different being more expressed in wild-type 14028s-infected cells (Table 4) indicating that the inflammatory response induced by DLG294 is only slightly lower than that induced by the wild-type.

Chemokines are small molecules that are involved in chemotaxis and activation of leukocytes at the site of inflammation. These chemotactic cytokines can be divided into four subfamilies, designated C, CC, CXC, and CX3C chemokine ligands, based on the positions of their cysteine residues (2, 3). Genes encoding chemokines are induced upon infection with wild-type *S. enterica* serovar Typhimurium as well as with DLG294. The gene expression of *Ccl5*, *Cxcl10*, *Cxcl2*, *Ccl4*, and *Ccl7* are all highly induced (Table 1). Only the expression of *Cxcl10* differs significantly between 14018s and DLG294 infected cells being more expressed in 14028s infected cells (Table 4).

*Isgl5*, *Ifit1*, *Glp2*, and *Irg1* are genes involved in immune responses and the expression of these four genes was highly induced upon infection of RAW264.7 cells with either wild-type or DLG294 *Isgl5*, *Ifit1*, and *Glp2* are genes that are induced upon stimulation with IFN$\gamma$ while *Irg1* is an LPS inducible gene (14). The expression of all four

genes seemed slightly lower for the DLG294 infected cells (Table 1). Gene expression of *Ifit1* appeared to be significantly lower for DLG294 infected cells compared to the wild-type infected cells (Table 4) suggesting that the IFNγ response may be slightly lower in DLG infected cells. Also the expression of the *H2* genes involved in antigen presentation were highly induced in the infected cells (Table 1), although no differences could be observed between the wild-type and DLG294 infected cells.

**Table 3. Macrophage gene expression reduced by *S. enterica* serovar Typhimurium**

| Accession nr. | Fold Decrease | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Transportation and Binding Proteins** | | | | |
| AW227545 | 2.02 | 2.30 | *Strn* | Striatin, calmodulin binding protein |
| **Extracellular Matrix and Adhesion** | | | | |
| D50086 | 3.25 | 3.25 | *Nrp* | Neuropilin |
| U47323 | 2.00 | 2.46 | *Stim1* | Stromal interaction molecule 1 |
| **Apoptosis** | | | | |
| AI643420 | 1.52 | 1.81 | *Bag3* | Bcl2-associated athanogene 3 |
| **Cell Cycle, Proliferation, and Differentiation** | | | | |
| AF003000 | 1.86 | 1.62 | *Terf2* | Telomeric repeat binding factor 2 |
| U95826 | 3.88 | 3.16 | *Ccng2* | Cyclin G2 |
| D78382 | 2.02 | 2.10 | *Tob1* | Transducer of ErbB-2.1 |
| D87326 | 1.81 | 2.30 | *Gsg2* | Germ cell-specific gene 2 |
| U42384 | 1.88 | 1.74 | *Fin15* | Fibroblast growth factor inducible 15 |
| AF086905 | 1.93 | 1.94 | *Chek2* | CHK2 checkpoint homolog |
| M36033 | 1.69 | 1.76 | *Ptpra* | Protein tyrosine phosphatase, receptor type A |
| Z35294 | 1.53 | 2.00 | *Mtcp1* | Mature T-cell proliferation 1 |
| M57647 | 2.64 | 3.62 | *Kitl* | Kit ligand |
| AB033921 | 1.80 | 1.96 | *Ndr2* | N-myc downstream regulated 2 |
| Y12474 | 1.76 | 1.88 | *Cetn3* | Centrin 3 |
| AW121600 | 2.10 | 2.25 | *Ndr4* | N-myc downstream regulated 4 |
| AW209238 | 1.74 | 1.93 | *Tacc3* | Transforming, acidic coiled-coil containing protein 2 |
| AV349686 | 1.87 | 1.96 | *Ndr2* | N-myc downstream regulated 2 |
| **Cytoskeleton and Membrane Proteins** | | | | |
| X98471 | 1.93 | 1.93 | *Emp1* | Epithelial membrane protein 1 |
| M58661 | 1.78 | 1.81 | *Cd24a* | CD24a antigen |
| U38967 | 1.83 | 1.91 | *Tmsb4x* | Thymosin, beta 4, X chromosome |
| AW121972 | 2.15 | 2.00 | *Waspip* | Wiskott-Aldrich syndrome protein interacting protein |
| AI505453 | 1.63 | 1.87 | *Myhg* | Myosin heavy chain IX |
| AW121840 | 1.99 | 2.23 | *Sel1h* | Sel1 (suppressor of Lin-12) 1 homolog |
| **DNA replication** | | | | |
| AI447783 | 2.22 | 2.46 | *Helb* | Helicase (DNA) B |
| AA681520 | 1.81 | 2.02 | *Gmnn* | Geminin |
| AW060791 | 1.76 | 1.69 | *Pole4* | Polymerase (DNA-directed), epsilon 4 (p12 subunit) |
| **RNA Processing** | | | | |
| U22262 | 2.75 | 2.66 | *Apobec1* | Apolipoprotein B editing complex 1 |
| **Protein Synthesis** | | | | |
| M61215 | 1.64 | 1.93 | *Fech* | Ferrochelatase |
| AF076681 | 1.93 | 1.74 | *Eif2ak3* | Eukaryotic translation initiation factor 2 α kinase 3 |
| AB004789 | 1.63 | 1.75 | *Dpm1* | Dolichol-phosphate (beta-D-mannosyltransferase 1 |
| **Mitochondrion** | | | | |
| X51941 | 1.81 | 1.80 | *Mut* | Methylmalonyl-Coenzyme A mutase |
| AF017175 | 2.16 | 2.07 | *Cpt1a* | Carnitine palmitoyltransferase 1 (liver) |
| U07159 | 1.80 | 1.81 | *Acadm* | Acetyl-Coenzyme A dehydrogenase, medium chain |
| AI842835 | 1.76 | 1.69 | *Uqcrc2* | RTKubiquinol cytochrome c reductase core protein 2 |
| **Metabolism** | | | | |
| X77731 | 2.15 | 2.56 | *Dck* | Deoxycytidine kinase |
| AI851983 | 1.66 | 1.81 | *Gsr* | Glutathione reductase 1 |
| AI842808 | 1.76 | 1.68 | *Ndufb5* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5 |



**Table 3. -continued-**

| Accession Nr. | Fold Decrease | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Biosynthesis** | | | | |
| X86000 | 2.50 | 3.30 | *Siat8d* | Sialyltransferase 8 (alpha-2, 8-sialyltransferase) D |
| U85414 | 2.55 | 2.30 | *Gclc* | Glutamate-cysteine ligase, catalytic subunit |
| M26270 | 1.78 | 1.73 | *Scd2* | Steaoryl-Coenzyme A desaturase 2 |
| AW060843 | 1.78 | 1.96 | *Lias* | Lypoic acid synthetase |
| D16333 | 1.46 | 1.75 | *Cpo* | Coproporphyrinogen oxidase |
| **DefenseResponse** | | | | |
| M29394 | 1.57 | 2.07 | *Cat* | Catalase |
| U77461 | 1.63 | 1.81 | *C3ar1* | Complement component 3a receptor 1 |
| **Protein Degradation and Processing** | | | | |
| AB007139 | 1.52 | 1.81 | *Psme3* | Proteasome (prosome, macropain) 28, subunit 3 |
| AF079565 | 3.01 | 2.65 | *Usp2* | Ubiquitin-specific protease 2 |
| AW122823 | 2.08 | 2.00 | *Ube2r2* | Ubiquitin-conjugating enzyme E2R2 |
| AI844932 | 1.94 | 2.23 | *Fbxo8* | F-box only protein 8 |
| **Immune Response** | | | | |
| AB007599 | 1.87 | 1.93 | *Ly86* | Lymphocyte antigen 86 |
| U15635 | 1.91 | 2.10 | *Samhd1* | SAM domain and HD domain, 1 |
| **Intracellular Trafficking** | | | | |
| D49544 | 2.16 | 2.39 | *Kifc1* | Kinesin family membe C1 |
| AI847561 | 1.81 | 1.87 | *Ap4s1* | Adaptor-related protein complex AP-4, sigma 1 |
| AV059766 | 1.71 | 1.86 | *Kif20a* | Kinesin family member 20a |
| **Signalling Receptors** | | | | |
| D13458 | 2.55 | 2.08 | *Ptger4* | Prostaglandin E receptor 4 (subtype EP4) |
| AF031127 | 1.57 | 1.78 | *Itpr5* | Inositol 1,4,5-triphosphate receptor 5 |
| AV012229 | 1.78 | 2.00 | *Fcer1g* | Fc receptor, IgE, high affinity 1, gamma polypeptide |
| **Regulatory** | | | | |
| D86344 | 2.47 | 2.47 | *Pdcd4* | Programmed cell death 4 |
| U20238 | 2.94 | 2.47 | *Rasa3* | RAS p21 protein activator 3 |
| AW122931 | 1.81 | 1.81 | *Ikbkg* | Inhibitor of kappaB kinase gamma |
| AI835963 | 2.15 | 2.02 | *Pias3* | Protein inhibitor of activated STAT3 |
| AI851250 | 1.75 | 1.81 | *Spred2* | Sprouty protein with EVH-1 domain 2, related sequence |
| AV335997 | 2.08 | 2.07 | *Rgs10* | Regulator of G-protein signalling 10 |
| **Signal Transduction** | | | | |
| U37465 | 2.93 | 2.46 | *Ptpro* | Protein tyrosine phosphatase, receptor type O |
| L11316 | 1.76 | 1.86 | *Ect2* | Ect2 oncogene |
| AF068182 | 2.30 | 2.30 | *Blnk* | B-cell linker |
| L21671 | 1.46 | 1.93 | *Eps8* | Epidermal growth factor receptor pathway substrate 8 |
| U67187 | 1.57 | 1.76 | *Rgs2* | Regulator of G-protein signalling 2 |
| AF079528 | 3.03 | 2.39 | *Ier5* | Immediate early response 5 |
| AF020313 | 1.68 | 1.75 | *Apbb1ip* | Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein |
| AA981154 | 1.53 | 1.96 | *Srpk2* | Serine/Arginine-rich protein specific kinase 2 |
| AI317205 | 2.30 | 2.47 | *Map3k1* | Mitogen activated protein kinase kinase kinase 1 |
| AW124633 | 1.69 | 2.07 | *Nek7* | NIMA (never expressed in mitosis gene-a)-related expressed kinase 7 |
| AI849416 | 2.00 | 1.87 | *Lats2* | Large tumor suppressor 2 |
| AI847399 | 1.87 | 1.68 | *Rgs10* | Regulator of G-protein signalling 10 |
| AI835968 | 2.65 | 2.46 | *Rin2* | Ras and Rab interactor 2 |
| AI846534 | 1.62 | 1.94 | *Nek6* | NIMA (never in mitosis gene-a)-related expressed kinase 6 |
| **Transcription** | | | | |
| M22115 | 1.88 | 1.80 | *Hoxa1* | Homeo box A1 |
| AF020200 | 1.88 | 2.35 | *Pbx3* | Pre B-cell leukemia transcription factor |
| AF062567 | 1.58 | 1.83 | *Sp3* | Trans-acting transcription factor 3 |
| M32057 | 1.68 | 1.75 | *Zfp239* | Zinc finger protein 239 |
| AF000581 | 2.08 | 1.99 | *Ncoa3* | Nuclear receptor coactivator 3 |
| AF064088 | 2.00 | 2.07 | *Tieg1* | TGFB inducible early growth response 1 |
| AF064088 | 1.74 | 1.80 | *Tieg1* | TGFB inducible early growth response 1 |
| X64840 | 1.75 | 1.75 | *Tcf12* | Transcription factor 12 |
| AF077861 | 2.14 | 2.39 | *Idb2* | Inhibitor of DNA binding 2 |
| AI449034 | 2.23 | 1.86 | *Rest* | RE1-silencing transcription factor |
| AI847906 | 1.80 | 1.75 | *Tcf20* | Transcription factor 20 |

**Table 3. -continued-**

| Accession Nr. | Fold Decrease | | Gene | Gene or Protein |
|---|---|---|---|---|
| | 14028s | DLG294 | | |
| **Other** | | | | |
| L27439 | 1.53 | 2.19 | *Pros1* | Protein S (alpha) |
| AF040252 | 2.02 | 1.75 | *Fkbp7* | FK506 binding protein 7 |
| U70674 | 2.38 | 2.22 | *Numb* | Numb gene homolog |
| X74351 | 1.83 | 1.75 | *Xpa* | Xeroderma pigmentosum, complementation group A |
| U64450 | 1.57 | 2.15 | *Npm3* | Nucleoplasmin 3 |
| AF004326 | 2.46 | 2.89 | *Agpt2* | Angiopoietin 2 |
| AF041472 | 1.62 | 1.81 | *Sca2* | Spinocerebellar ataxia 2 homolog |
| AI987985 | 2.86 | 3.26 | *Zfp288* | Zinc finger protin 288 |
| AW060819 | 1.80 | 1.80 | *Twsg1* | Twisted gastrulation homolog 1 |
| AA733664 | 2.15 | 2.52 | *Cpeb2* | Cytoplasmic polyadenylation element binding protein 2 |
| AW125218 | 1.53 | 1.76 | *Hat1* | Histidine aminotransferase |
| **Unknown Gene Function** | | | | |
| U73039 | 1.94 | 1.88 | *Nbr1* | Neighbor of Brca1 gene 1 |
| M18070 | 1.69 | 1.75 | *Prnp* | Prion protein |
| AF058797 | 1.51 | 1.68 | *Ywhab* | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide |
| AW120605 | 1.83 | 1.81 | *Mllt3* | Myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog |
| AI842472 | 3.19 | 2.49 | *Zdhhc14* | Zinc finger, DHHC domain containing 14 |
| AA930526 | 1.81 | 2.04 | *Mtmr13* | Myotubularin related protein 13 |
| AA968123 | 1.96 | 2.04 | *Nav1* | Neuron navigator 1 |
| AW120725 | 1.81 | 1.81 | *Ubl3* | Ubiquitin-like 3 |
| AW060827 | 2.02 | 2.00 | *Them2* | Thioesterase superfamily, member 2 |

**Moderately induced genes.** Genes showing only moderate induced expression are mostly genes involved in cell cycle and cell death and apoptosis (Table 2). For all genes, no differences could be observed between wild-type and DLG294 infected cells. *Pdcd1*, *Bcl2l11*, *Trp53*, *Bad*, *Tnfrsf6* (Fas antigen), and *Tnip1* are all genes encoding proteins that are involved in apoptosis and all genes show a 2-4 fold induction in expression. Also the expression of some genes involved in cell cycle (regulation) such as *Cdk5*, *Cdkn1a*, *Ccnd1* are induced upon infection. Again, these genes show only a slight increase in expression.

**Direct comparison of gene expression profiles of 14028s and DLG294.** Direct comparison of the gene expression profiles of RAW264.7 cells infected with either wild-type *S. enterica* serovar Typhimurium or the superoxide hypersusceptible mutant strain DLG294 revealed only small differences in the expression in only a few genes (Table 4). Nine genes showed increased expression in wild-type infected cells compared to DLG294 infected cells encoding proteins that are involved in inflammation and immune responses, RNA processing, transcription, and regulation. Most of these genes play a role in innate defense of the macrophages against pathogens such as *Salmonella*. *Cxcl10* encodes a chemokine (C-X-C motif) that is also known as IFN$\gamma$-induced protein 10. For *Mycobacterium tuberculosis* infected cells it has been shown that optimal expression of *cxcl10* is dependent upon TNF$\alpha$ (1). *Tnf* expression is induced in both wild-type and DLG294 infected cells (Table 1). Although not statistical significantly different in the direct comparison, from Table 1 it seems that *tnf* is induced even more in the DLG294 infected cells, while the induction of *cxcl10* expression is lower than for the wild-type infected cells.



**Table 4. Fold change in macrophage gene expression 14028s vs DLG294**

| Accession Nr. | Fold Change | Gene | Gene or Protein | Function |
|---|---|---|---|---|
| M33266 | 1.4 | *Cxcl10* | Chemokine (C-X-C- motif), ligand 10 | Inflammation, Cytokines, and Chemokines |
| L32838 | 1.6 | *Il-1rn* | IL-1 receptor antagonist | Inflammation, Cytokines, and Chemokines |
| AF037437 | -1.5 | *Gtrgeo22* | Gene trap ROSA b-geo 22 | Cytoskeleton and Membrane Proteins |
| AW213225 | -1.4 | *Ddx18* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 8 | DNA Replication |
| L17076 | 1.6 | *Raly* | HnRNP-associated with lethal yellow | RNA Processing |
| AI852592 | -1.5 | *Ndufb2* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2 | Metabolism |
| U43084 | 1.5 | *Ifit1* | Interferon induced protein with tetratricopeptide repeats 1 | Immune Response |
| X14678 | 1.4 | *Zfp36* | Zinc finger protein 36 | Transcription |
| M31418 | 1.5 | *Ifi202a* | Interferon activated gene 202A | Transcription |
| L20450 | 1.6 | *Zfp97* | Zinc finger protein 97 | Transcription |
| AA981581 | 1.9 | *Hnrpu* | Heterogeneous nuclear ribonucleoprotein U | Regulatory |
| AF037437 | -1.8 | *Psap* | Prosaposin | Other |
| U82610 | -1.5 | *Lcp1* | Lymphocyte cytosolic protein 1 | Other |
| X05546 | -1.7 | *Iap2* | Intracisternal A particle 2 | Other |
| AI645561 | 1.4 | *Narg1* | NMDA receptor-regulated gene 1 | Other |

**Protein Assay as Confirmation of Microarray Analysis.** One way to validate the results form the mRNA expression levels by microarray analysis is to determine the protein levels of certain genes involved in defense against *S. enterica* serovar Typhimurium. We decided to assess proteins secreted into the supernatant of infected RAW264.7 cells at 4 h after infection. This was done by a Mouse Inflammation Cytometric Bead Array, which allows the analysis of six proteins simultaneously. As shown in Figure 3, we observed that uninfected RAW264.7 cells secrete no detectable amounts of IL-6, IL-10, IFN$\gamma$, and IL12-p70, but do secrete low amounts of MCP-1 and TNF$\alpha$.

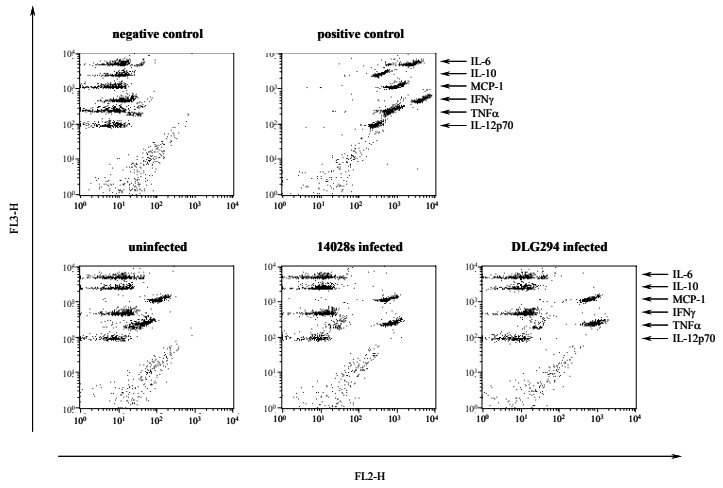


**Figure 4.** Mouse Inflammation Cytometric Bead Array analysis of supernatant from RAW264.7 cells that were left untreated or that were infected with S. enterica serovar Typhimurium 14028s or DLG294. Cells were challenged as described in Materials and Methods. At 4 h after challenge, the supernatant was taken and used for analysis in the Cytometric Bead Array according to the manufacturer's recommendations

The secretion of MCP-1 and TNFα is induced strongly upon infection of the cells with either wild-type *S. enterica* serovar Typhimurium 14028s or the mutant strain DLG294. MCP-1 is a chemotactic factor encoded by *Ccl2* that is secreted by the macrophages to attract monocytes, but not neutrophils. Upon infection of the cells with wild-type or DLG294 the production of MCP-1 increased 3-fold to 60.3 and 64.5 ng respectively (Fig. 4). Both strains induced the production and secretion of MCP-1 to a similar extent, which corresponds to the relative increases in mRNA expression of the gene encoding MCP-1 (*Ccl2*) in the microarray analysis (2.10 fold for wild-type and 1.94 fold for DLG294 infected cells; data not shown). Expression levels of mRNA for TNFα, however, increased 9.5 and 10.9 fold for wild-type- and DLG294-infected cells respectively, while the secreted TNFα levels were induced 22.9 fold to 100.6 ng for wild-type and 33.6 fold to 147.9 ng respectively. These results show that the increase in secreted amounts of the protein does not correspond exactly to the increase in mRNA expression, but they do show that the relative induction of mRNA expression and secreted product by wild-type and DLG294-infected cells do correlate and confirms the data observed in the microarray analysis. The other four proteins were not detected within the supernatant of uninfected and infected RAW264.7 cells.

**Concluding remarks.** Since the gene expression profiles are dependent upon the activation status of the macrophages (19) and no clear differences in the gene expression profiles of RAW264.7 cells infected with wild-type *S. enterica* serovar Typhimurium or DLG294 were observed, it might be concluded that the activation status of the macrophages is not altered by infection with DLG294 compared to the wild-type strain. Apparently, lack of expression of *sspJ* in DLG294 does not result in stronger or lesser activation of the macrophages, indicating that both strains are equally capable of modulating the macrophages' response. This strongly suggests that attenuation of DLG294 is not due to the hosts' macrophages, but is due to the lack of expression of *sspJ* in DLG294 causing its pleiotropic phenotype, including hypersusceptibility to superoxide. This is supported by the fact that DLG294 is able to grow out in cells and mice that do not produce any superoxide (24), although the activation statuses of these cells are unknown.

Research on *S. enterica* serovar Typhimurium mutants, including DLG294, has led to the isolation and identification of mutants that are either more susceptible or more resistant to superoxide. From these studies it became clear that *Salmonella* has evolved many mechanisms to cope with superoxide stress that may either prevent production or limit the damage that is done by these compounds (reviewed in (12)). It also became clear that mutants might share the same in vitro phenotype according to superoxide sensitivity, but they might have different in vivo phenotypes and very diverse genetic defects, ranging from defects in SOD's, glucose-6-phosphate dehydrogenase, or DNA repair mechanisms. So from the in vitro phenotype it cannot be predicted what the consequences of this mutation are for the in vivo and in vitro virulence. And reversely, from the in vivo and in vitro phenotype it cannot be predicted what the underlying genetic and proteomic defects might be. One way to understand the differences in the in vitro and in vivo phenotype is to



do research on host genes induced upon infection with *Salmonella* as is done in this study. Another approach would be to look at in vivo-regulated genes of *S. enterica* serovar Typhimurium itself during infection of host cells (5). Direct comparison of gene expression profiles of intracellular DLG294 and wild-type *S. enterica* serovar Typhimurium might reveal the role of *sspJ* in defense against superoxide stress and in virulence of *S. enterica* serovar Typhimurium.

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chapter nine

# Intracellular gene expression profiling of *Salmonella enterica* serovar Typhimurium and mutant DLG294 that is hypersusceptible to the redox cycling agent menadione

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## Abstract

DLG294 is a *Salmonella enterica* serovar Typhimurium mutant that is hypersusceptible to intracellular superoxide that is highly attenuated in vivo in C3H/HeN mice and in vitro in macrophages. The altered virulence of DLG294 is not due to increased activation of the macrophage but must be due to the lack of expression of sspJ. With the study presented here, we tried to address the role of *sspJ* by checking the broad spectrum phenotypes of DLG294 and the wild-type strain using phenotype microarrays and by looking at in vivo-regulated genes of *S. enterica* serovar Typhimurium itself during infection of host cells.

In this study we show that DLG294 is more susceptible to acid, lixivium, and antibiotics that inhibit protein synthesis, suggesting that the interior defense against toxic compounds is wrongly regulated/disregulated in DLG294. Comparison of the gene expression profiles of intracellular DLG294 and wild-type 14028s revealed only a few small differences. This indicates that *sspJ* does not have an important role in regulating (virulence) gene expression but that it has a direct role in survival under stress conditions like those encountered inside the macrophage. We propose that the absence of SspJ alters the integrity of the *Salmonella* membrane leading to increased permeability and thus enhanced susceptibility to toxic compounds and antibiotics and to the attenuated in vivo phenotype of DLG294.

## Introduction

*Salmonellae* are Gram-negative facultative intracellular pathogens that can cause a variety of diseases in animals and man, ranging from mild gastroenteritis to severe systemic infections like typhoid fever. *Salmonella enterica* serovar Typhimurium may cause gastroenteritis in man, but causes systemic infection in mice comparable to typhoid fever in man (17). *Salmonella* predominantly invades mononuclear phagocytes and is able to cause persistent infections by evasion or disturbance of the host immune system (16). Upon infection with *Salmonella*, the host will try to develop an immune response to limit bacterial growth and to eventually kill and eliminate the pathogen. Despite the presence of a multitude of antimicrobial defense mechanisms as part of the innate immune defense system in phagocytes, *Salmonella* is able to enter, survive, and even replicate within these cells. *S. enterica* serovar Typhimurium responds to the specific host environment by expressing factors that are necessary for intracellular survival and for resistance against the defense systems of the host (6, 8, 9, 16, 20), but the exact mechanisms by which it is able to survive after phagocytosis are largely unknown.

One of the major early defense mechanisms against *Salmonella* is the production of reactive oxygen intermediates (ROI), both in vitro (18) and in vivo (24, 25, 33). Since superoxide is a by-product of normal aerobic metabolism, both eukaryotic and prokaryotic cells have evolved ways to respond to superoxide stress by the activation of genes involved in a protective response (23). Several genes and systems have been described that play a role in the defense response of *S. enterica* serovar Typhimurium against ROI, such as the SoxR/S regulon, the OxyR system, *katE* encoding cytosolic catalase, and the superoxide dismutases SocCI and SodCII (reviewed in (14)). The relative importance of each of the mechanisms involved in defense against oxidative stress for *S. enterica* serovar Typhimurium intracellular survival has not been elucidated. However, the periplasmic Cu,Zn-SOD and the type III secretion system encoded by *Salmonella* pathogenicity island 2 (SPI2) have been shown to be important in this defense, as mutants deficient in one of these systems show reduced survival within macrophages (5, 10, 34).

We have recently identified a superoxide hypersusceptible *S. enterica* serovar Typhimurium *sspJ* mutant strain DLG294 that is highly attenuated in vivo in C3H/HeN mice and in vitro in macrophages (27, 32). The attenuated phenotype is related to its hypersusceptibility to superoxide since DLG294 is able to grow out as much as the wild-type strain in cells and mice that lack one of the components of the NADPH-oxidase and as a result cannot produce superoxide (32). The exact function and mechanism of action of *sspJ*, however, are still unknown. Although hypersusceptibility to superoxide could be the major cause of attenuated virulence of DLG294, it cannot be excluded that other factors might also play a role. Virulence is not determined by superoxide sensitivity sec since many menadione-susceptible mutants are not attenuated at all (28, 29, 31). These data suggest that other factors play a role.

One way to address the differences in virulence of DLG294 and the wild-type strain is to study the macrophage activation status by comparing the gene expression profiles of



infected macrophages (22) as this might give an indication on how the macrophages respond to infection with a certain pathogen. We previously addressed the question if a possible difference in the activation status of the macrophages might explain and might clarify whether attenuation of DLG294 is solely due to its hypersusceptibility to superoxide produced by the macrophages or that additional mechanisms play a role. Since we observed no or only minor differences in gene expression profiles between DLG294 or wild-type-infected RAW264.7 macrophages (30) we have performed another study in which we checked the broad spectrum phenotypes of DLG294 and the wild-type strain using phenotype microarrays and have assessed in vivo-regulated genes of *S. enterica* serovar Typhimurium during infection of host cells as described by Eriksson et al. (7).(12)

## Materials and Methods

**Bacterial strains and culture conditions.** The bacterial strains used in this study are listed in Table 1. Bacteria were grown overnight (ON) on LB broth plates at 37°C. The day of infection, a large number of bacterial cells were resuspended in 10 ml PBS. The bacterial cells were pelleted and resuspended in 1 ml PBS. For opsonization, 10% mouse serum was added and bacteria were incubated at 37°C for 30 min.

**Table 1. *S. enterica* serovar typhimurium strains used in this study**

| Strain | characteristics | origin or reference |
|---|---|---|
| 14028s | wild-type | ATCC |
| DLG294 | 14028s sspJ::MudJ | (27) |
| SL1344 | *rpsL hisG* | (13) |

**In vitro acid challenge.** ON cultures of bacteria grown in LB medium pH7.0 were diluted 1:100 in LB medium + 0.4% glucose pH 7.0 (unadapted) of in LB medium + 0.4% glucose pH 4.5 (adapted) and were incubated at 37°C for 1 h. A sample was taken to determine the number of bacteria before challenge with acid (t=0 h) and the remaining bacteria were spun down and resuspended in LB medium + 0.4% glucose pH 3.0 and were incubated at 37°C. At 1 and 2 h after challenge, samples were taken to determine the number of bacteria that were still viable.

**Cell culture and in vitro infection model.** RAW264.7 cells were grown in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, L-Glutamine, 100 U/ml penicillin, and 50 mg/ml streptomycin. For each extraction of RNA $1 \times 10^8$ cells were seeded in four 225 $cm^2$ tissue culture flasks (Costar) in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, and L-Glutamine. Cells were infected at an MOI of 100 with bacteria that

were opsonized or non-opsonized for 1 h at 37°C and 5% $CO_2$ to allow uptake of the bacteria. Then extracellular bacteria were killed by adding medium containing 30 μg/ml gentamicin. After 1 h incubation at 37°C and 5% $CO_2$ the medium was replaced for medium containing 5 μg/ml gentamicin and were incubated for another 2 h.

**Phenotype Microarray (PM).** PM tests were performed in duplicate by Biolog Inc. (Hayward, California, U.S.A.) as described in (3). All PMs were incubated at 36°C in an OmniLog and monitored for color change in the wells. Kinetic data were analyzed with OmniLog-PM software. The phenotype of mutant DLG294 was compared to that of the wild-type 14028s.

**RNA extraction.** After 4h, the infected RAW264.7 macrophages were lysed on ice for 30 min in 0.1% SDS, 1% acidic phenol, 19% ethanol in water. Bacterial pellets were collected by centrifugation and RNA was extracted using the Promega SV total RNA purification kit. Approximately $10^8$ CFU were isolated on each time point and yielded 3-5 μg RNA. Size chromatography was done with an Agilent 2100 Bioanalyser.

**Microarray.** DNA microarray analysis was performed as described in (4), except that the arrays were printed on Corning CMT-GAPS™-coated slides and contained 666 extra genes from *S. enterica* serovar Typhimurium DT104 and SL1344, *S. enterica* serovar enteritidis PT4, and *S. enterica* serovar Galinarum 287/91 ("Salsa" microarray, http://www.ifr.bbsrc.ac.uk/Safety/Microarrays/default.html).

**Probe labeling and scanning.** RNA was first reverse transcribed into cDNA and was then labeled by random priming. For labeling protocols, see http://www.ifr.bbsrc.ac.uk/Safety/Microarrays/Protocols.html. Fluorescently labeled genomic DNA was used as a reference in each experiment. After hybridization, the slides were scanned using a GenePix 4000 A scanner (Axon Instruments). The fluorescent spots and the background signals were then quantified using GenePix Pro software (Axon Instruments). All RNA samples were hybridized to microarrays in duplicate.

**Data analysis.** Spots that showed a reference signal lower than the background signal plus two standard deviations were excluded from the analysis. The background signal was subtracted from each spot signal and fluorescence ratios were calculated. After performing data centring, data were analyzed using Genespring™ software (Silicon Genetics) as described previously (7). Only coding regions showing at least a two-fold difference in the mutant compared to the wild-type strain were regarded as being differentially expressed. Hierarchal clustering of gene expression profiles was performed using the Pearson correlation.



## Results and Discussion

**In vitro phenotypes lost by DLG294 vs 14028s.** In order to address the role of *sspJ* in *S. enterica* serovar Typhimurium, we compared the broad spectrum phenotypes of the *sspJ* mutant DLG294 and the wild-type strain using phenotype microarrays. In this assay, the ability of wild-type *S. enterica* serovar Typhimurium and DLG294 to grow in the presence of 2000 different nutrients, antibiotics, and toxic compounds was evaluated by determining metabolic rates. With this assay most known aspects of cell function can be monitored and the range of phenotypes include cell surface composition and transport, catabolism, biosynthesis, macromolecules, cellular machinery, respiratory functions, and stress and repair functions. Quite a few phenotypes were lost by the *sspJ* mutant compared to the wild-type strain (Table 2). DLG294 was more sensitive to an acidic or alkalic environment as its growth is impaired at pH9.5 and pH4.5 compared to that of the wild-type strain (Table 2). We have further tested the sensitivity to acid in an in vitro challenge assay in which we used logphase LB cultures of 14028s and DLG294 grown at pH 7.0 and challenged with LB medium pH 3.0. The numbers of DLG294 declined faster than those of the wild-type strain confirming the increased susceptibility to acid (Fig. 1). However, when DLG294 bacteria were allowed to adapt at intermediate pH (pH 4.5) and were then challenged with pH 3.0, DLG294 behaved like the wild-type strain (Fig. 1).

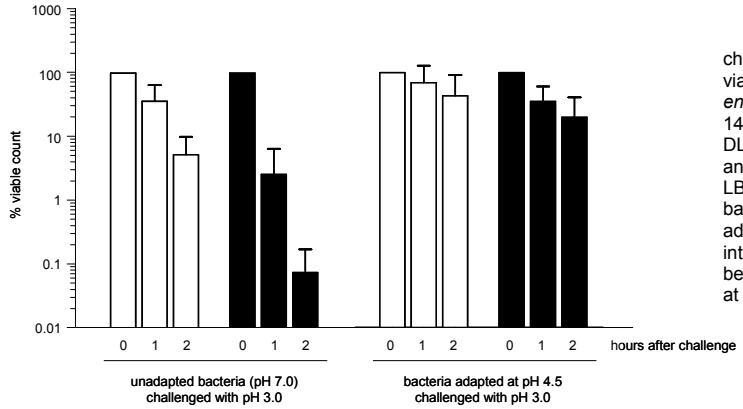


**Figure 1.** In vitro acid challenge assay. Percentage viable count of wild-type *S. enterica* serovar Typhimurium 14028s (white bars) and DLG294 (black bars) at 0, 1, and 2 h after challenge with LB medium pH 3.0. The bacteria were allowed to adapt in LB medium with intermediate pH 4.5 for 1 h before challenge or were left at pH 7.0 (i.e. unadapted).

DLG294 also has diminished resistance to protein synthesis inhibiting antibiotics. The macrolides inhibit bacterial protein synthesis by inhibiting the 50S ribosomal subunit. The other protein synthesis inhibitors have more diverse mechanisms of action such as inhibition of polymerization of glycoproteins (vancomycin), premature termination of chains during translation (puromycin), inhibition of translocation during protein synthesis (fusadic acid), or inhibition of peptide bound formation in the ribosomal machinery (blasticidin S). The resistance to the β-lactam type of antibiotics as well as the related cephalosporins was also impaired in the DLG294 mutant compared to the wild-type strain. These antibiotics inhibit bacterial wall synthesis and are therefore bactericidal for rapidly dividing cells.

**Table 2. Phenotypes lost by DLG294 compared to wild-type 14028s**

| test[a] | difference[b] | | mode of action |
|---|---|---|---|
| | min | max | |
| Ketoprofen | | -70 | anti-capsule, anti-inflammatory |
| Sanguinarine | | -102 | ATPase, $Na^+/K^+$ and $Mg^{2+}$ |
| sodium pyrophosphate | | -59 | chelator, hydrophilic |
| 2,2'-dipyridyl | | -108 | chelator, lipophilic |
| orphenadrine | | -108 | cholinergic antagonist |
| Prinidol | | -55 | cholinergic antagonist |
| promethazine | | -76 | cyclic nucleotide phosphodiesterase |
| 9-aminoacridine | | -75 | DNA interchelator |
| 4-hydroxycoumarin | | -89 | DNA interchelator |
| Acriflavine | | -44 | DNA interchelator |
| novobiocin | | -87 | DNA topoisomerase |
| domiphen bromide | | -78 | fungicide |
| D-serine | | -60 | inhibits 3PGA dehydogenase (L-serine and pantothenate synthesis) |
| trifluoperazine | | -94 | ion channel, $Ca^{2+}$ |
| dequalinium chloride | | -72 | ion channel, $K^+$ |
| benzothonium chloride | | -100 | membrane, detergent, cationic |
| poly-L-lysine | | -110 | membrane, detergent, cationic |
| lauryl sulfobetaine | | -73 | membrane, detergent, zwitterionic |
| amitriptyline | | -95 | membrane, transport |
| Lys-Gly | | -140 | N-source |
| Val-Lys | | -81 | N-source |
| 3% urea | | -86 | osmotic sensitivity, urea |
| plumbagin | | -121 | Oxidizing agent |
| potassium superoxide | | -112 | Oxidizing agent |
| D, L-thioctic acid | | -59 | Oxidizing agent |
| cysteamine-S-phosphate | | -118 | P-source |
| pH 9.5 + amino acids[c] | -150 | -66 | pH, deaminase |
| pH 4.5 + amino acids[d] | -100 | -62 | pH, decarboxylase |
| chlorpromazine | | -108 | phenothiazine |
| compound 48/80 | | -53 | phospholipase A, ADP ribosilation |
| antibiotics[e] | -223 | -68 | protein synthesis |
| respiration influencing agents[f] | -135 | -55 | respiration |
| rifamycin SV | | -189 | RNA polymerase |
| rifampicin | | -112 | RNA polymerase |
| transport influencing agents[g] | | -161 | transport, toxic anion |
| D, L-methionine hydroxamate | | -64 | tRNA synthetase |
| antibiotics[h] | -216 | -66 | wall, cephalosporin |

[a] chemicals were tested in 96-well PMs
[b] The OmniLog-PM software generates time course curves for respiration (tetrazolium color formation) and calculates differences in the areas for mutant and control cells. The units are arbitrarily.
[c] L-phenylalanine; L-tryptophan; L-leucine; L-isoleucine; L-norvaline; glycine; L-homoserine; L-methionine; agmatine; b-hydroxy glutamate
[d] urea; D, L diaminopimelic acid; L-lysine, g-hydroxy glutamic acid; L-ornithine
[e] vancomycin; tylosin; puromycin; fusidic acid; blasticidin S; spiramycin; oleandomycin; josamysin; troleandomycin; erythromycin
[f] tetrazoleum violet; thioridazine; crystal violet; iodonitro tetrazoleum violet; sorbic acid; FCCP; sodium caprylate, cinnamic acid; CCCP; ruthenium red
[g] sodium metasilicate; sodium cyanate; chromium chloride; lead (II) nitrate; manganese (II) chloride
[h] cefoxitin; cephaloridine; piperacillin; oxacillin; nafcillin; cloxacillin; pheneticillin; aztreonam

Another major difference in phenotypes is the increased sensitivity of DLG294 to agents that influence the respiratory chain such as the respiratory uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) a drug known to inhibit the efflux systems that use the proton motive force. CCCP, however, has no effect on resistance to menadione as has been shown previously for the wild-type strain 14028s (28).



Taken together, the phenotypes lost by DLG294 could suggest that the membrane integrity of DLG294 has changed compared to the wild-type strain and, as a result, has become more leaky resulting in enhanced susceptibility to certain toxic compounds such as the macrolides, β-lactams, and cephalosporins, to stress inducing conditions such as acidic and alkalic pH, and menadione.

**In vitro phenotypes gained by DLG294 vs 14028s.** The phenotype microarray analysis revealed that some phenotypes were gained (Table 2) by DLG294 compared to wild-type. Since DLG294 contains a kanamycin resistance cassette in the MudJ transposon that has inserted into *sspJ*, it is more resistant to kanamycin and some other aminoglycosides (Table 3). DLG294 also has increased ability to use nitrogen-sources for growth which is consistent with the hypothesis that the membrane integrity of DLG294 has changed compared to the wild-type strain and has become more permeable for nutrients and certain toxic compounds.

**Table 3. Phenotypes gained by DLG294 compared to wild-type 14028s**

| Test[a] | difference[b] | mode of action |
|---|---|---|
| L-arabinose | 62 | C-source |
| L-rhamnose | 58 | C-source |
| chloroxylenol | 140 | Fungicide |
| ethylamine | 117 | N-source |
| acetamide | 103 | N-source |
| g-D-Glu-Gly | 100 | N-source |
| Phe-Trp | 83 | N-source |
| Tyr-Ile | 79 | N-source |
| cytosine | 78 | N-source |
| b-Ala-Gly | 75 | N-source |
| b-Ala-Phe | 72 | N-source |
| D-Leu-D-Leu | 56 | N-source |
| D-Ala-Leu | 55 | N-source |
| D-lysine | 55 | N-source |
| nitrite | 52 | N-source |
| Gly-D-Val | 51 | N-source |
| adenosine | 136 | nutrient stimulation |
| 2'-deoxy-adenosine | 76 | nutrient stimulation |
| thymidine-5'-monophosphate | 52 | P-source |
| phosphono acetic acid | 51 | P-source |
| kanamycin | 212 | protein synthesis, aminoglycoside |
| paromomycin | 201 | protein synthesis, aminoglycoside |
| neomycin | 162 | protein synthesis, aminoglycoside |
| geneticin (G418) | 148 | protein synthesis, aminoglycoside |

[a] chemicals were tested in 96-well PMs
[b] The OmniLog-PM software generates time course curves for respiration (tetrazolium color formation) and calculates differences in the areas for mutant and control cells. The units are arbitrarily.

**Transcriptional profiling of intracellular 14028 and SL1344.** We used the recently described method for isolating *S. enterica* serovar Typhimurium RNA from intracellular bacteria (7) to compare the gene expression profiles of different strains that resided and replicated within RAW264.7 macrophages. RAW264.7 macrophages were infected as

described in Materials and Methods. At 4 h after infection, the cells were lysed and bacterial pellets were isolated and used for RNA extraction. Since the bacterial RNA was immediately stabilized, there is only minimal degradation (Fig. 2). The extracted bacterial RNA was labeled and hybridized to the *S. enterica* serovar Typhimurium "Salsa" array with labeled bacterial DNA from SL1344 grown to mid-logphase in LB medium pH 7.0 as a reference. The genes that are 2-fold differentially expressed in the intracellular bacteria compared to mid-logphase grown SL1344 in LB broth pH7.0 were equally distributed in the genome as shown for 14028s in Figure 3B. We first compared the gene expression profile of intracellular 14028s to that of SL1344 at 4 h. This was done to evaluate whether these profiles are comparable since most intracellular *S. enterica* serovar Typhimurium array data have been are generated with this SL1344 strain (7). The patterns of gene expression after normalization were only slightly different for certain genes of the 14028s strain compared to the SL1344 strain (Fig. 3C). The similarity in gene expression profiles of intracellular 14028s and SL1344 becomes even more apparent from the cluster diagram shown in Figure 3A.

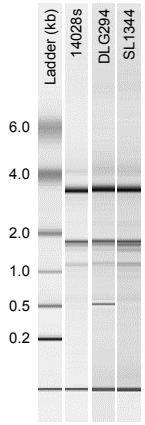


**Figure 2.** Size chromatographic separation of RNA. Total RNA was extracted from intracellular 14028s, DLG294 and SL1344 and analyzed on a Bioanalyser.

**Direct comparison of gene expression profiles of intracellular 14028s and DLG294.** We next evaluated the gene expression profile of DLG294 compared to the wild-type strain 14028s to look for genes that are differentially expressed. What becomes clear from Figure 4C is that only a few of the genes were differentially expressed and that they are located all over the *S. enterica* serovar Typhimurium genome with a few small clusters of genes showing altered gene expression. The genes that showed altered expression for DLG294 only show only a small difference in expression level as can be seen in the cluster diagram (Fig. 4B) and the relative gene expression profile of DLG294 versus the wild-type strain 14028s stays within the range of 0.3-5 fold change (Fig. 4A). For each of the genes showing altered gene expression, the relative expression was depicted in Figure 5. The expression of only 11 genes was increased for intracellular DLG294 compared to the wild-type strain and 19 genes showed decreased expression. None of the genes of the virulence gene clusters such as SPI-1 and SPI-2, nor genes encoding the superoxide dismutases were altered, indicating that the DLG294 mutant does not lack expression of the well-known virulence genes nor the defense mechanisms against superoxide.

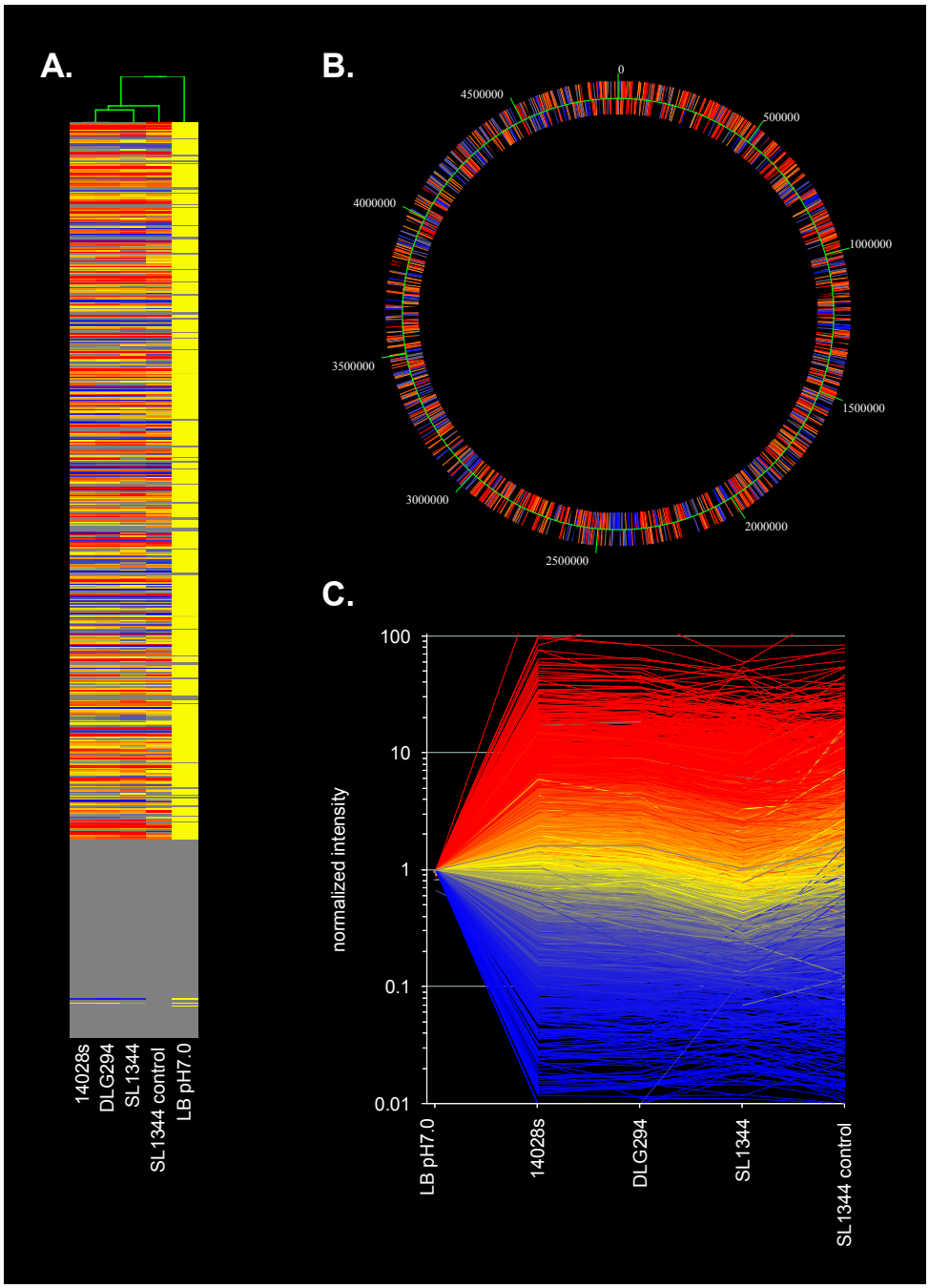


**Figure 3.** Cluster diagram (A), gene map (B), and gene expression profile (C) of the two-fold differentially expressed genes in intracellular 14028s, DLG294, SL1344 and SL1344 control compared to mid-logphase SL1344 grown in LB pH7.0. Each line represents one gene. Red indicates at least a twofold increase, yellow indicates no change, and blue indicates a minimum twofold decrease in expression.

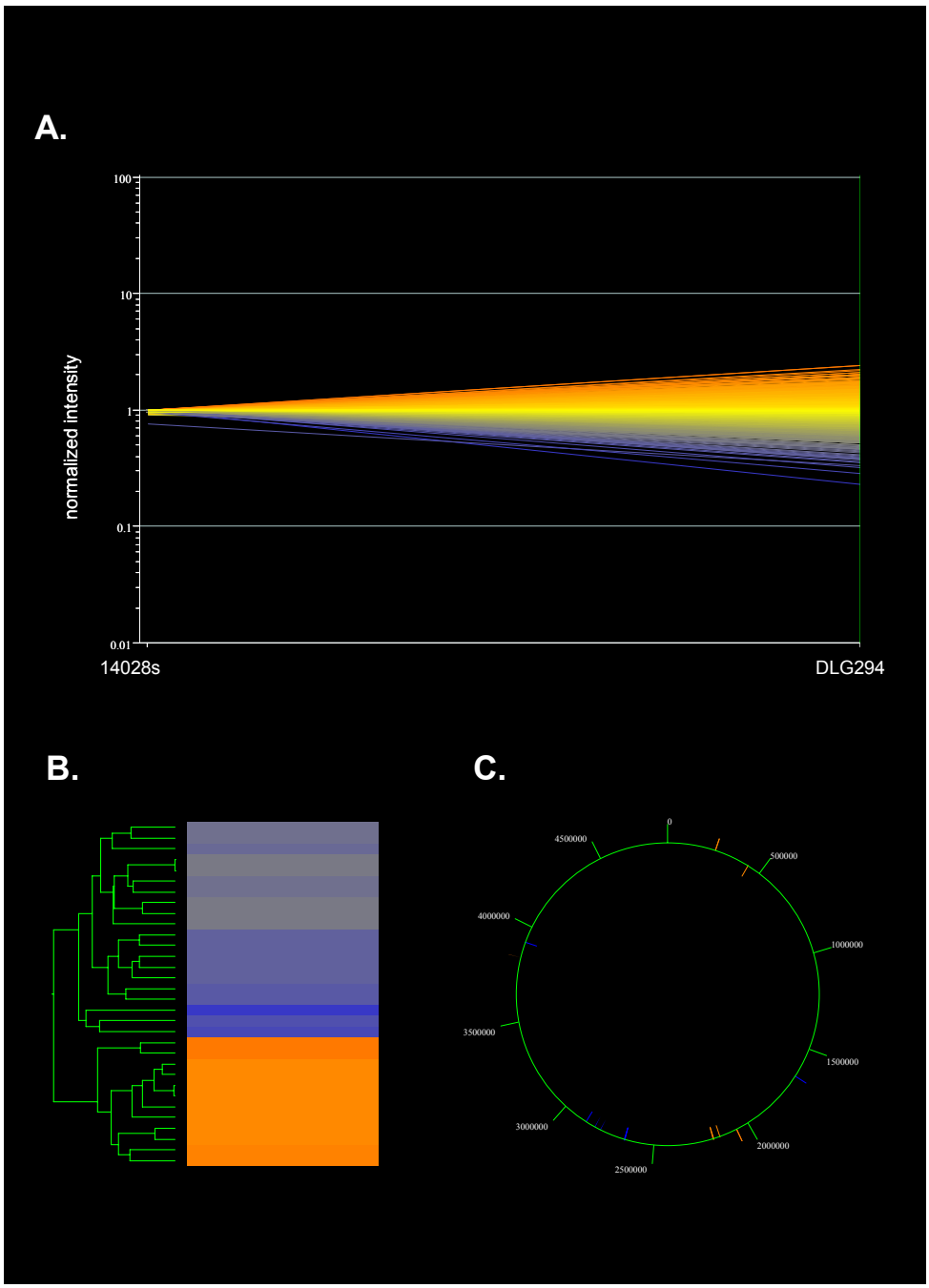


**Figure 4.** Relative gene expression profile (A), cluster diagram (B), and gene map (C) of two-fold differentially expressed genes in intracellular DLG294 compared to intracellular wild-type 14028s. Each line represents one gene. Red indicates at least a twofold increase, yellow indicates no change, and blue indicates a minimum twofold decrease in expression.

**Genes downregulated in intracellular DLG294.** The MudJ-inactivated gene *sspJ* was among the 19 down-regulated genes as well as the two genes that are located directly downstream of *sspJ,* i.e. *engA* and *yfgJ* (Fig. 5 and Table 4). These three genes, together with *yfgM*, are part of the *yfg-eng* locus in which the ORFs are all transcribed in the same direction (2, 19). The MudJ transposon did have a polar effect on the expression of *engA* and *yfgJ* since the expression of these genes was decreased in intracellular DLG294 compared to the wild-type. *EngA* encodes a GTP binding protein of which the physiolocical role is unknown and *yfgJ* encodes a putative cytoplasmic protein. The attenuated in vivo and in vitro phenotype of DLG294, however, cannot be explained by the polar effect of the MudJ transposon on these genes since complementation with a low-copy-number plasmid expressing only the *sspJ* gene completely restored the in vitro and in vivo phenotype to that of the wild-type strain and confirmed that the superoxide hypersusceptibility and attenuated phenotypes are due to the lack of expression of *sspJ* (27, 32). Recently, Amy et al. have described the attenuated in vivo phenotype of an *yfgL* (=*sspJ*) *S. enterica* serovar Enteritidis in chickens and suggested a role for the *yfg-eng* locus in colonisation of chickens (2). As for our *sspJ* mutant DLG294, this mutant showed lower bacterial numbers within macrophages after 3.5 h. The *S. enterica* serovar Enteritidis *yfgL* mutant, however, was shown to lack motility due to lack of production of the *fliC* and *fliD* encoded proteins flagellin and filament capping protein as well as the SPI-1 encoded protein SipA. For our mutant DLG294, however, no decrease in gene expression of *fliC* and *fliD* was observed nor did we observe differences in SPI-1 or SPI-2 encoded genes. DLG2294 even showed an increase in expression of genes involved in flagellar biosynthesis as the expression of the genes encoding the flagellar biosynthesis proteins FliP and FliR were increased for DLG294 compared to the wild-type strain (Fig. 5 and Table 4) and could suggest enhanced production of these inner membrane proteins involved in flagellar biosynthesis (19).

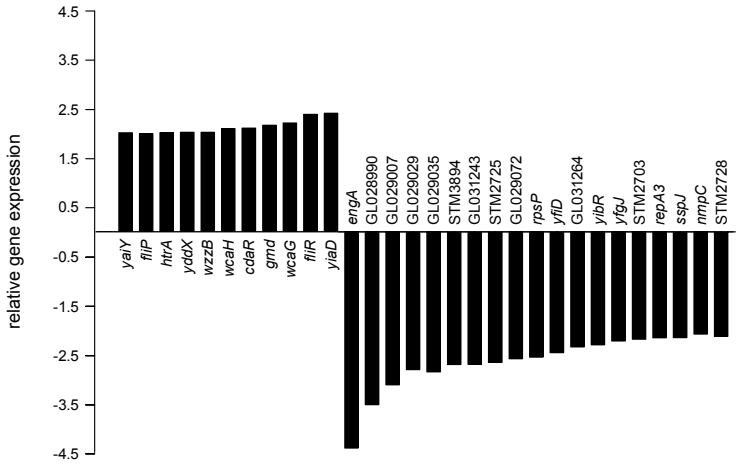


**Figure 5.** Fold change in gene expression of selected genes that were 2-fold differentially expressed in intracellular DLG294 compared to intracellular wild-type 14028s.

Other genes of which the expression was down-regulated in DLG294 are *rpsP* encoding 30S ribosomal subunit protein 16S, *yfiD* encoding a putative formate acetyltransferase, *yibR* encoding a putative inner membrane protein, *nmpC* encoding a predicted bacterial porin, and several other of which the gene name and function are unknown or that are orthologues to certain *E. coli* genes (Table 4). For most of these genes it is hard to predict their role in attenuated virulence of DLG294. *RepA3*, however, is a gene that is lower in DLG294 compared to the wild-type strain and that could have influenced the attenuation of DLG294 since this gene is present on the pSLT plasmid. This plasmid pSLT is the virulence plasmid of *S. enterica* strains (1) that enhances the growth rate of the bacterium during systemic phase of disease (11). The gene *repA3* is encoding a protein that is involved in the initiation of replication of this plasmid. Reduced plasmid replication might lead to reduction of virulence of the mutant strain. However, the expression of genes present on the plasmid are not altered compared to wild-type and are all upregulated to a similar extent as for the SL1344 strain in J774A.1 cells (7).

**Table 4. Up- and downregulated genes in intracellular DLG294 vs 14028s**

| Downregulated | | Upregulated | |
|---|---|---|---|
| gene | function | gene | function |
| *enA* | putative GTP-binding protein | *yaiY* | putative inner membrane protein |
| GL028990 | no orthologues | *fliP* | flagellar biosynthesis |
| GL029007 | orthologous to *pepA* | *htrA* | periplasmic serine protease |
| GL029029 | orthologous to *E. coli yjhP* | *yddX* | putative cytoplasmic protein |
| GL029035 | no orthologues | *wzzB* | putative regulator of length of O-antigen component of LPS chains |
| STM3894 | unknown | *wcaH* | GDP-mannose mannosyl hydrolase in colanic acid biosynthesis |
| GL031243 | putative flutathione S-transferase | *cdaR* | putative inner membrane protein |
| STM2725 | unknown | *gmd* | GDP-D-mannose dehydratase in colanic acid biosynthesis |
| GL029072 | putative PTS permease | *wcaG* | bifunctional GDP fucose synthetase |
| *rpsP* | 30S ribosomal subunit S16 | *fliR* | putative flagellar biosynthetic protein |
| *yfiD* | putative formate acetyltransferase | *yiaD* | putative outer membrane lipoprotein |
| GL031264 | orthologous to *E. coli yeiG* | | |
| *yibR* | outative inner membrane protein | | |
| *yfgJ* | putative cytoplasmic protein | | |
| STM2703 | Fels2 prophage; similar to invertase (pin) in phage E14 | | |
| *repA3* | DNA replication | | |
| *yfgL/sspJ* | putative serine/threonine kinase | | |
| *nmpC* | predicted bacterial porin (outer membrane protein) | | |
| STM2728 | Fels-2 prophage; hypothetical protein | | |

**Genes upregulated in intracellular DLG294.** Only 11 genes showed increased expression in intracellular DLG294 (Fig 5 and Table 4). Two genes (*fliP* and *fliR*) are involved in flagellar biosynthesis as mentioned above. Again, for four genes, i.e. *yaiY*, *yddX*, *cdaR*, and *yiaD* encoding putative inner membrane, outer membrane, and cytoplasmic proteins (Table 4), the exact function of the proteins is unknown. Three of the



induced genes (*wcaH*, *gmd*, and *wcaG*) are involved in colanic acid biosynthesis. *WcaH* encodes the GDP-mannose mannosyl transferase, *gmd* the GDP-D-mannose dehydratase, and *wcaG* the bifunctional GDP fucose synthetase. Colanic acid, or M(ucous) antigen, is a repeat unit polysaccharide that forms a capsule and that is produced by most enteric bacteria, presumably as a means of protection against desiccation. The gene expression profiles from 14028s and DLG294 in comparison with the reference showed that the expression of these three genes was induced in intracellular bacteria and that this was higher for the superoxide-hypersusceptible mutant DLG294. This could suggest that the mutant produces more colanic acid for protection against the influx of damaging agents that act inside the macrophage as a kind of compensatory mechanism.

The gene *htrA*, encoding a periplasmic serine protease, is induced in intracellular DLG294 compared to wild-type. It has been described previously that inactivation of *htrA* or inactivation of *rpoE,* the sigma factor identified in *E. coli* as being important for survival under extreme heat-stress and that controls *htrA*, leads to a phenotype that is identical to that of DLG294 (26). These mutants display increased susceptibility to superoxide and are attenuated in mice and that, just like the *sspJ* mutant DLG294, are able to cause fatal infections in mice deficient in NADPH oxidase as well (21).(15) One could explain sensitivity of the mutants to exogenous superoxide by assuming that exposure to superoxide results in direct damage to periplasmic proteins. Elimination of these damaged proteins by HtrA, the periplasmic protease which is under control of *rpoE*, would then protect against this damage. The increase in *htrA* expression in DLG294 could suggest that this mutant imagines that it is under extreme heat stress and starts producing this heat stress protein since the conditions inside the bacteria are not right as was suggested by the lost phenotypes in the in vitro phenotype array (Table 2). Increased expression of *htrA* could also suggest a mechanism to try and compensate for the lack of expression of *sspJ*, although this "compensatory" mechanism is not sufficient to protect against oxidative damage and to restore virulence since DLG294 is still hypersusceptible to superoxide and displays an attenuated in vitro and in vivo phenotype.

**Concluding remarks.** DLG294 has a completely different phenotype compared to the wild-type. It is hypersusceptible to intracellular superoxide generated by menadione. It is attenuated in macrophages and in mice and regains virulence in $p47^{phox-/-}$ mice. However, when we compared gene expression profiles of intracellular DLG294 and wild-type 14028s we found that the expression profiles were only slightly altered. This indicates that *sspJ* does not have an important role in regulating (virulence) gene expression but that it has a direct role in survival under stress conditions like those encountered inside the macrophage. We propose that the absence of SspJ alters the integrity of the *Salmonella* membrane leading to increased permeability and subsequent accumulation of toxic compounds. This in turn would lead to enhanced susceptibility to these toxic compounds (like menadione) and antibiotics and to the attenuated in vivo phenotype of DLG294. The small differences in gene expression probably reflect a difference in intracellular environment as a result of this increased membrane permeability.

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chapter ten

# Summarizing discussion

Partly adapted from:

**Riny Janssen, Tahar van der Straaten, Angela van Diepen, and Jaap T. van Dissel.** 2003. Responses to reactive oxygen intermediates and virulence of *Salmonella typhimurium*. Microbes. Infect. **5:**527-534.

## Summarizing discussion

*Salmonella* is a rod-shaped bacterium that occurs ubiquitous in nature. Most strains in the soil are not dangerous to human or animal life. However, some of the *Salmonellae* are able to survive within their host and can cause disease in humans and animals. *Salmonella* infections are one of the most common food-related illnesses in the world and form a major problem for people in developing countries. In industrialized countries, the microorganisms are a threat especially to people with an impaired immune system. Symptoms of disease may vary from asymptomatic carriage in which the bacteria reside within the intestines and are shedded via the stool, to life-threatening sepsis in which the bacteria enter the blood stream. The type of disease is dependent upon the *Salmonella* strain the person gets infected with and the defense system of the host. Many infections are due to *S. enterica* serovar Enterica or serovar Typhimurium via food or water that is contaminated with animal waste.

*Salmonella* infection occurs through the ingestion of contaminated food or drinks. The acid in the stomach kills most of the ingested bacteria. However, in particular when the pH in the stomach is slightly higher than normal, some of the bacteria will pass the stomach. In the intestines, *Salmonella* has to compete with other defense mechanisms such as a thick mucus layer and the natural flora of the gut. If *Salmonella* succeeds in this task and survives, it eventually reaches the M cells in the Peyers' patches of the small intestines. *Salmonella* invades these M cells and depending on the *Salmonella* strain, two types of disease can occur. During infection with the solely human typhoidal strains *S. enterica* serovar Typhi or serovar Paratyphi, the bacteria will spread to the underlying lymphoid tissues where they will infect macrophages. Next, the bacteria will enter the bloodstream and will reach the liver and spleen where *Salmonella* initiates a severe inflammatory response that is characteristic for typhoid fever and will be lethal in about 10-15% of the cases if not treated. However, when a person gets infected with the non-typhoidal strains such as *S. enterica* serovar Typhimurium, the bacteria usually stay within the Peyers' patches and induce a local inflammatory response that is mediated by cytokines, chemokines, and neutrophils. This type of disease is called gastroenteritis and is characterized by diarrhea and is usually self-limiting. In certain groups of patients such as immunocompromised individuals, however, infection with this "harmless" bacterium may lead to severe disease. Another problem in this group of patients is the reactivation of latent *S. enterica* serovar Typhimurium infection. Patients then suffer from recurring infections with the same strain, as has been described for instance for AIDS patients.

In many scientific studies on *Salmonella* an in vivo mouse model is used. Murine infection with *S. enterica* serovar typhimurium, causing gastroenteritis in humans, leads to disease in mice that is comparable to typhoid fever in man and therefore, serves a model for human infection with *S. enterica* Typhi or Paratyphi. The natural infection is the oral route, but experimental infections can also be induced by intravenous, intraperitoneal, or subcutaneous injection of bacteria. A soon as the bacteria have reached the blood, all these types of infections are similar and are very much alike that of the typhoid fever in



humans. The bacteria spread to the liver and spleen where they reside and multiply within macrophages. This, together with the influx of immune cells (macrophages and neutrophils) leads to enlargement of the liver and spleen (hepatosplenomegaly). Depending on *Salmonella* strain and dosage, an immune response is initiated that allows the mice to survive and that protects against secondary infection. Natural protection against *S. enterica* serovar Typhimurium infection is partly determined by the genes *Ity* and *lps*. *Ity* is the gene encoding Nramp1, a membrane phosphoglycoprotein that is expressed in macrophages of liver and spleen. Mice expressing the resistant allele (*Nramp1*$^{G169}$) can control the initial in vivo replication of *S. enterica* serovar typhimurium allowing time for the development of a T cell-mediated immune response needed for clearance of the bacteria. However, mice expressing the sensitive allele *Nramp1*$^{D169}$ cannot control the infection and will die. Expression of *Nramp1* is enhanced upon activation of the macrophages by IFN$\gamma$ and LPS. Another gene protecting against *Salmonella* infection is *lps*, the gene encoding Toll-like receptor 4 (TLR4). TLR4 is a receptor involved in the recognition of lipopolysaccharide (LPS) that is abundantly expressed by Gram-negative bacteria such as *Salmonella*. Upon activation, an intracellular signaling cascade is initiated leading to the transcription of genes encoding cytokines such as IFN$\gamma$, Il-18 en TNF$\alpha$. These cytokines play an important role in the activation of macrophages and thus in the defense against *S. enterica* serovar Typhimurium. Mice such as the C3H/HeJ strain that have a mutation in *lps*, cannot respond to LPS due to a non-functional TLR4 and as a consequence, are extremely sensitive to *S. enterica* serovar Typhimurium infections.

In defense against *S. enterica* serovar Typhimurium macrophages play a major role. Macrophages are phagocytic cells that contain a multitude of antimicrobial defense mechanisms. However, *S. enterica* serovar Typhimurium has evolved to survive and even replicate within this hostile environment. An important defense mechanism of macrophages against microorganisms is the production of oxygen radicals. These radicals are highly toxic and would probably result in bacterial death if *Salmonella* had not adapted. In the genome of Salmonella there are two Salmonella pathogenicity islands (SPI-1 and SPI-2) that encode type III secretion systems (TTSS). The first TTSS encoded by SPI-1 is involved in the uptake by the host cell and encodes a kind of syringe apparatus through which certain proteins can be injected into the host cell cytosol thereby inducing changes in the cell membrane that leads to the uptake of the bacterium, even by non-professional phagocytes. This process is called *Salmonella*-induced uptake. *Salmonella* enters a vacuole where it can survive due to the induction of the second TTSS encoded by SPI-2. In this process proteins are injected into the host cell cytosol and lead to the disturbance of essential antibacterial processes, allowing *Salmonella* to survive. For instance, SPI-2 encoded proteins prevent the translocation and assembly of the active NADPH oxidase complex and in this way, *Salmonella* can prevent the production of superoxide. Besides SPI-1 and SPI-2 encoded proteins, other genes such as *soxR/S* and *phoP/Q* play an important role in defense against superoxide and in survival within the host.



Chapter 1 gives an overview of what is currently known about *Salmonella*, the interaction with the host and systems that play a role in the defense against superoxide and in survival within macrophages.

In Chapter 2 the development is described of an in vivo model for reactivating *S. enterica* serovar Typhimurium infection through total body irradiation or $CD4^+$ T cell depletion. An important complication of *S. enterica* serovar Typhimurium infection in certain groups of patients is the recurrence of infection. The infection is cleared, but *Salmonella* may reside within the body despite the host immune system, and will strike again as soon as the immune system is impaired. It is unknown at which niche *Salmonella* hides and which processes play role in the suppression of growth during the phase of persistency. Reactivating *Salmonella* infections have been mainly described for patients who underwent irradiation or received glucocorticoids and for individuals with AIDS or other immune impairments. Our model shows that $CD4^+$ T cells play a role in the suppression of growth of *S. enterica* serovar Typhimurium during the phase of persistency.

Since a couple of years, patients suffering from rheumatoid arthritis or Crohn's disease are being treated with the tumor necrosis factor $\alpha$ (TNF$\alpha$) neutralizing antibodies Infliximab or Ethanercept. This type of treatment has proven to be of great benefit to these patients, but makes them susceptible to primary as well as reactivating infection with intracellular bacteria such as *Mycobacterium tuberculosis*. TNF$\alpha$ is a cytokine that plays an important role in the activation of macrophages and in defense against pathogens including *Salmonella*. For *Salmonella* infections it is known that neutralization of TNF$\alpha$ leads to increased risk of severe infections, but whether such treatment may also lead to reactivation of a latent *Salmonella* infection, as in latent *Mycobacterium tuberculosis* infection, is not clear. In Chapter 3 we investigated whether TNF$\alpha$ plays a role in the suppression of *S. enterica* serovar Typhimurium during the phase of persistency in mice. In the model used, we did not observe reactivation of the *S. enterica* serovar Typhimurium infection after treatment with antibodies to TNF$\alpha$. In addition, we observed that addition of anti-TNF$\alpha$ to IFN$\gamma$-stimulated mouse macrophages (RAW264.7) had no effect on the IFN$\gamma$ induced effect of reduced outgrowth of *S. enterica* serovar typhimurium. This could mean that TNF$\alpha$ plays a minor role compared to IFN$\gamma$ during infection with *S. enterica* serovar Typhimurium. As long as IFN$\gamma$ produced by $CD4^+$ T cells remains present, latent *S. enterica* serovar Typhimurium infection will be suppressed and reactivation will not occur.

In Chapter 4 we describe the research on bacterial mutants that are able to survive for a longer period of time within macrophages. We have created *S. enterica* serovar Typhimurium mutants by random P22 MudJ transposon insertion. In this way we have created several mutants that were selected for the ability to survive for a longer period of time than wild-type S. enterica serovar Typhimurium within mouse macrophages. Eventually, two mutants were selected that after inverse PCR and sequence analysis appeared to be the same. The MudJ transposon had inserted into *rmlC*, the gene encoding dTDP-4-deoxyrhamnose 3,5-epimerase, an enzyme involved in the formation of the O-antigen of the lipopolysaccharide (LPS). Analysis of the LPS showed that this mutant had truncated LPS and was very similar to an *S. enterica* serovar Typhimurium strain of the

rough Ra chemotype. This is a mutant lacking the O-antigen and contains only the lipid A and the core region of the LPS. Also the Ra mutant was able to survive for a longer period of time in RAW264.7 macrophages; even after 48 h high numbers of bacteria could still be detected. Despite this ability to survive longer, these mutants were not capable of inducing severe infection in mice. These LPS mutants are killed in vitro by rat or human complement, and likely, the attenuated phenotype in mice can be explained by this increased in vitro susceptibility to complement, although we could not confirm this with high numbers of bacteria in mouse serum that has low levels of complement.

By random MudJ transposon insertion in wild-type *S. enterica* serovar Typhimurium 14028s we have created mutants that were next selected for their susceptibility to intracellular superoxide, as described in Appendix 1. By inverse PCR and sequence analysis we determined the position in the genome where the MudJ transposon had been inserted and which gene might have been inactivated. One of the mutants obtained in this way was studied in further detail and has been described in Chapter 5. In this mutant, designated AVD101, the MudJ transposon had inserted into the promotor region of *pnp*, the gene encoding PNPase. This protein is involved in the degradation of mRNA and in the growth adaptation at low temperatures and it is considered a regulator of virulence and persistence of *S. enterica* serovar Typhimurium. We described an additional role for PNPase in the resistance to superoxide and for intracellular survival within macrophages.

In the research on superoxide-resistance genes we describe in Chapter 6 the isolation and characterization of DLG294, an *S. enterica* serovar Typhimurium mutant in which, through MudJ transposon insertion, an as yet unknown gene was inactivated. This gene was designated *sspJ*. The protein SspJ is no longer produced by this mutant resulting in increased susceptibility of this mutant to menadione, a redox cycling agent that generates superoxide radicals within the bacterium. DLG294 appeared to be attenuated in vitro in macrophages and in vivo in mice. By constitutive expression of *sspJ* on a plasmid the phenotype of DLG294 was restored to that of the wild-type strain. This confirmed the role of SspJ in the defense against superoxide and in virulence. However, the exact role and functioning of SspJ is not clear yet.

DLG294 was next studied in vivo in mice, as described in Chapter 7. DLG294 induced hardly any granulomatous lesions in the liver after subcutaneous infection of *Salmonella*-resistant ($Ity^r$) C3H/HeN mice with $3 \times 10^4$ CFU and the numbers of bacteria were 3 log units lower than those of the wild-type strain on day 5 after infection. However, DLG294 appeared to be as virulent as the wild-type strain and induced similar liver pathology in $p47^{phox-/-}$ mice. These mice lack a functional NADPH oxidase system because of a lack of $p47^{phox}$ and as a result cannot produce superoxide. Also in bonemarrow-derived macrophages of these $p47^{phox-/-}$ mice and in X-CGD PLB985 cells the bacterial numbers of DLG294 were as high as those of the wild-type strain. These results suggest that SspJ plays a role in resistance against oxidative stress and in survival and replication of *S. enterica* serovar Typhimurium both in vitro and in vivo.

Macrophages play an important role in *Salmonella* infections. They exert a dual role; that of a host cell possibly hiding the bacterium from the hostile exterior, and that of an



effector cell in acquired immunity. DLG294 is more sensitive to superoxide and attenuated in macrophages, and we hypothesized that hypersusceptibility to superoxide plays a causative role in its attenuated behavior. However, other processes might play a role as well leading to the reduced ability of DLG294 to survive within macrophages. Infection with *S. enterica* serovar Typhimurium leads to the activation of the macrophages to kill and eliminate the bacteria. Diverse mechanisms play a role in this activation process. In Chapter 8 we have investigated the effect of wild-type and DLG294 infection on the gene expression in macrophages using Affymetrix gene chips. Using these chips, the expression of 6,400 genes can be studied simultaneously. Wild-type *S. enterica* serovar Typhimurium and DLG294 appeared to influence the expression of many genes, however, no differences between the two types of infected cells were apparent. From this we concluded that the reduced outgrowth of DLG294 in macrophages must be attributed to the mutation in *sspJ* and not to a different, indirectly induced, activation status of the macrophages compared to that of wildtype-infected macrophages.

To further characterize DLG294, we have studied the in vitro phenotype of DLG294 further in Chapter 9 using a phenotypical array in which several hundreds of processes can be studied at the same time. This makes it possible to compare the in vitro phenotype of DLG294 to that of the wild-type strain. Also, we have looked at the intracellular gene expression profile of DLG294 in RAW264.7 macrophages and compared that to intracellular wild-type *S. enterica* serovar Typhimurium. The phenotypical array revealed that DLG294 gained the ability to use nitrogen sources for growth, has hampered resistance to several antibiotics, and shows increased susceptibility to acidic and alkalic pH. Comparison of the gene expression profile of intracellular DLG294 with that of the wild-type strain revealed only a few differences. Most likely, DLG294 has reduced membrane integrity that leads to increased uptake of toxic compounds and as a result, more damage to the bacterium.

## Menadione

Most *S. enterica* serovar Typhimurium mutants described in this thesis were generated by ramdom MudJ transposon insertion into the wild-type 14028s strain and those mutants were selected that displayed increased or decreased susceptibility to intracellular superoxide generated by menadione. Menadione is a synthetic Vitamin $K_3$ supplement that is fat-soluble and is used in medicine to help in the clotting of blood. Menadione is a quinone (2-methyl-1,4-naphtoquinone; Fig. 1) that is converted into menaquinone in the liver.

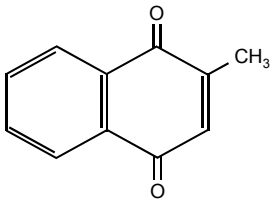


**Figure 1.** Chemical structure of menadione (2-methyl-1,4-naphtoquinone).

**Quinones**

Quinones are widely distributed in nature as they are abundant in burnt organic material and in food (reviewed in (1, 24)) and have antitumor activity (6, 20). Quinones are substrates for several flavoenzymes such as NADPH cytochrome P-450 reductase, DT-diaphorase, NADH-cytochrome $b_5$ reductase, and NADH-ubiquinone oxidoreductase, and can be converted into hydroquinone via a two-electron reduction or reaction with glutathione (GSH) and into semiquinone radical via a one-radical reduction (Fig. 2 and reviewed in (19)). In the two-electron reduction pathway that is catalyzed by the FAD-dependent DT-diaphorase, the quinones are converted into hydroquinone and is considered a cellular protector against oxidative stress. However, hydroquinones can be converted into quinones in the presence of oxygen and in this reaction superoxide radicals are formed. Xanthine oxidase and Xanthine dehydrogenase can also catalyze the two-electron reduction of quinones, but these enzymes are also capable of catalyzing the one-electron reduction of quinones that leads to the formation of semiquinone radicals causing damage to the cell by covalent binding to proteins or nucleic acids or by influencing the cells' metabolism. Semiquinones can react together to form hydroquinones and quinones, but in aerobic conditions, most of these semiquinones react with $O_2$ to regenerate the quinone and to form $O_2^{-}\bullet$. These radicals react with hydrogen peroxide, which is formed by dismutation of superoxide, and the more toxic radical HO• is formed which can damage the cell by causing DNA strand breaks, lipid peroxidation, or enzyme inactivation (Fig. 2). However, the cell is equipped with defense mechanisms such as superoxide dismutases, catalases, glutathione reductase, and glutathione peroxidase to convert these toxic compounds into $H_2O_2$ and $H_2O$. However, if either one of these mechanisms is not working properly, the cell might get damaged.

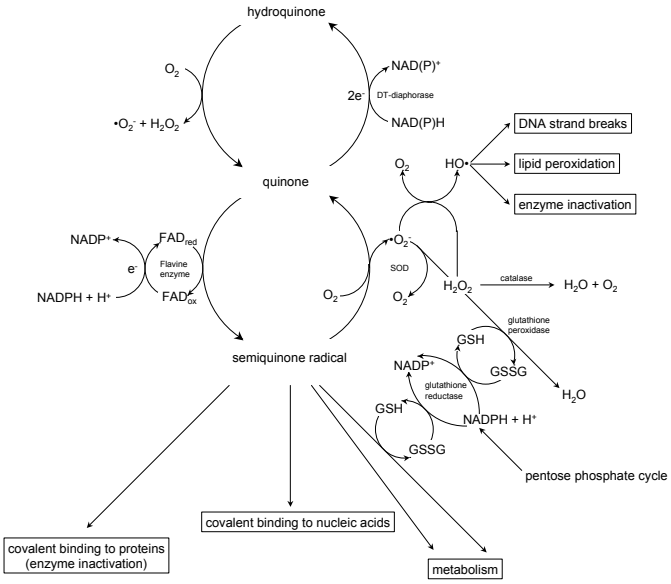


**Figure 2.** schematic overview of the redox cycle of quinones, the damage that can be induced by the radicals that are formed, and defense mechanisms of the host (reviewed in (19)).

**Menadione-susceptible mutants**

Mutants with increased susceptibility to the redox cycling agent menadione, can display several underlying defects causing this phenotype. The first option is the decreased detoxification of the toxic compounds that are formed inside the cell and might involve, for instance, mutants deficient in SOD, catalase, glutathione peroxidase, and glutathione dismutase. However, since the redox cycle of the quinones is rather complicated as many enzymes are involved and is strongly dependent upon the circumstances and the host's reaction to this compound, mutations in either one of the routes involved might cause the same menadione-susceptible phenotype. Other types of mutants might be those with reduced capacity to repair the damage that is caused by the radicals. Since semiquinone radicals can directly influence the proteins and nucleic acids by covalent binding and can influence metabolism, it is likely that mutants unable to deal with this are hypersusceptible. Menadione also induces the formation of the highly reactive hydroxyl radicals (OH•) that can cause DNA strand breaks, lipid peroxidation, and enzyme inactivation and can kill *Salmonella* through these mechanisms if it is unable to repair or prevent the damage. Another explanation for increased susceptibility to menadione might be decreased efflux or increased influx of this redox cycling agent resulting in concentrations of this toxic compound that are too high for *Salmonella* to deal with. All intracellular defense mechanisms will be turned on and will be able to prevent or clear part of the damaging radicals, but this will not be sufficient and the bacterium will die as a consequence. For the menadione-hypersusceptible mutant DLG294 described in this thesis, it is most likely that the in vitro and in vivo phenotypes can be explained by decreased membrane integrity and subsequent increased uptake of toxic compounds such as menadione. Since the known defense and virulence mechanisms of DLG294 were not differently expressed compared to the wild-type strain as was shown in Chapter 9, this mutant probably has a leaky membrane through which more menadione and other toxic compounds can enter the cell and can cause damage when the defense systems are completely occupied.

## *Salmonella* genes involved in oxidative stress

Research on many mutant strains of *E. coli* as well as *Salmonella* has led to the identification of a range of genes that are necessary for survival during oxidative stress generated in vitro. However, the exact role of many of these genes both in survival within macrophages and in virulence in mice is not understood. With the studies described in Chapter 5 and Appendix 1 we tried to identify more genes that are involved in resistance to superoxide stress and analyzing them for the ability to survive within macrophages. In literature, many mutants have been described that can be divided in different classes based on their phenotypes (Fig. 3, Table 1). These studies have shown that the mechanisms that *Salmonella* uses to cope with superoxide stress are very diverse and illustrate the complexity of the superoxide stress response of *Salmonella* (reviewed in(13)).



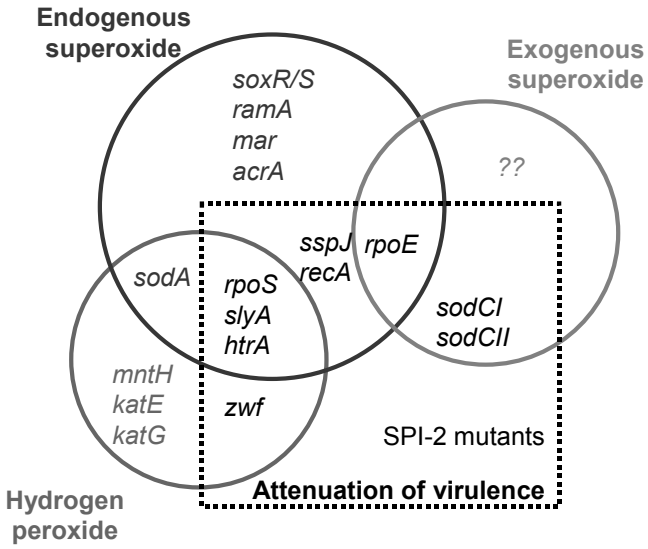


**Figure 3.** Classification of *Salmonella* mutants by in vitro and in vivo phenotype. Mutants that are sensitive to exogenous superoxide generated by the host NADPH-oxidase, endogenously produced superoxide and hydrogen peroxide are shown, and the consequences for virulence in mice are indicated. *soxR/S*, *ramA*, *mar, acrA* and *oxyR* encode transcriptional regulators; *sodCI* and *sodCII* encode periplasmic copper-zinc superoxide dismutases; *sodA* encodes the cytoplasmic manganese superoxide dismutase; *zwf* encodes the glucose 6-phosphate dehydrogenase; *htrA* encodes a periplasmic protease; *rpoE* encodes an "extracytoplasmic function" sigma factor; *rpoS* and *slyA* encode transcriptional regulators; *recA* encode a recombinase important for DNA repair; *sspJ* encodes a periplasmic protein that interferes with superoxide levels; Spi2 is the pathogenicity island of *Salmonella* that contains genes encoding the second type-III secretion system.

**Mutants sensitive to endogenously produced superoxide**

One of the methods to identify genes involved in survival under superoxide stress is the isolation of mutant strains that are either more sensitive or more resistant to redox-cycling agents that generate intracellular superoxide (Appendix 1). A range of mutants that are more sensitive to the redox-cycling agents menadione and paraquat has been isolated in this way. Whereas the in vitro phenotype, i.e., sensitivity to intracellular superoxide, is the same, there are huge differences between these mutants in the importance of the inactivated gene products in the survival of *Salmonella* in macrophages and in virulence in mice.

For instance, mutants in the *soxR/S* regulon, the cytoplasmic manganese dismutase SodA, the transcriptional regulator RamA, or the global regulator AcrA are all highly susceptible to superoxide in vitro, however, these mutants are not attenuated in vivo (9, 16, 18, 25, 28). It cannot be ruled out, however, that following interaction with the host in vivo, alternative mechanisms are activated and compensate for lack of expression of either one of these proteins.

**Table 1. Phenotype of *Salmonella* null mutants**

| Gene | Intracellular superoxide[a] | Extracellular superoxide | Hydrogen peroxide | Virulent/ attenuated[b] | References |
|---|---|---|---|---|---|
| Regulators of gene expression | | | | | |
| *acrA* | [c] | | S | V | (16) |
| *oxyR* | S | | S | | (5) |
| *ramA* | S | | R | V | (28) |
| *rpoE* | S | S | | A | (23) |
| *rpoS* | S | | S | A | (8, 22) |
| *slyA* | S | | S | A | (2) |
| *soxR/S* | S | R | | V | (9) |
| Neutralization of scavenging of ROI | | | | | |
| *katE* | | | S | V | (3) |
| *katG* | | | S | V | (3) |
| *mntH* | R | | S | V | (15) |
| *sodA* | S | R | S | V/A | (25) |
| *sodCI* | R | S | | V/A | (7, 21, 26) |
| *sodCII* | R | S | | V/A | (7, 21) |
| *sodCIII* | R | | | | (10) |
| *zwf* | S | S | | A | (17) |
| Protection against ROI damage | | | | | |
| *htrA* | S/R | R | S | V | (14, 23) |
| *recA* | S | | | A | (3) |
| Prevention of ROI production by macrophages | | | | | |
| Spi-2 encoded genes | | | | A | (11, 31) |
| Unknown function | | | | | |
| *sspJ* | S | R | | A | (27, 29) |

[a] R: resistant, S: sensitive.
[b] V: virulent, A: attenuated, V/A: conflicting data.
[c] Open space: no data available

**Mutants sensitive to endogenous and exogenous superoxide**

In contrast to the mutants described above, the same experimental approach has also led to identification of genes that are important for survival in the host. For instance, *sspJ* encoding a putative serine/threonine kinase-like protein, *htrA* encoding a periplasmic protease, *rpoE* encoding the sigma factor identified in *E. coli* as being important for survival under extreme heat-stress, *zwf* encoding the glucose-6-phosphate dehydrogenase, or the transcriptional regulator *rpoS* and *slyA* (2, 8, 14, 17, 23, 27). All these mutants show increased susceptibility to endogenously as well as exogenously produced superoxide and as a consequence display attenuated in vitro or in vivo phenotypes, although for some

mutants it is not completely known whether the attenuation is due to the superoxide susceptibility or that additional factors are responsible for the reduced virulence.

**Mutants sensitive to exogenous, NADPH-oxidase-generated superoxide**

The identification of periplasmic superoxide dismutases in *Salmonella* has led to the observation that mutants in the two *sodC* genes do not display an increased sensitivity to redox-cycling agents but are sensitive only to extracellular superoxide generated by the xanthine/xanthine oxidase system in vitro or superoxide produced by the macrophage. Although periplasmic SodC proteins are important for virulence, the exact role of each of the two SodC proteins is still unclear and rather confusing (7, 21, 26). To add to the confusion, the group of Imlay has proposed a model that *Salmonella* SodC mutants are killed because they are more sensitive to hydrogen peroxide (12). Interestingly, not all *Salmonella* strains contain two periplasmic superoxide dismutases. For instance, *S. typhi* and some *S. paratyphi* strains only contain the *sodCII* gene (7). *E. coli* also contains this *sodCII* gene. This must mean that periplasmic Sod's are not always essential determinants of virulence of *Salmonella*.

**Mutants disturbed in prevention of NADPH-oxidase-generated superoxide**

It is clear that *Salmonella* has evolved mechanisms to cope with both superoxide released endogenously as a by-product of its own respiration and superoxide produced by macrophages. When cells are infected with wild-type *S. typhimurium* there is hardly any superoxide production in the vicinity of internalized bacteria, indicating that *Salmonella* can prevent superoxide production by phagocytes (reviewed in (30)). The mechanism is dependent on the second type-III secretion system encoded by SPI2, since mutants in this system are unable to inhibit superoxide production by phagocytes (31). Recently, it became apparent that translocation of $gp91^{phox}$ and $gp47^{phox}$ of the NADPH-oxidase complex to the *Salmonella*-containing phagosome is inhibited in phagosomes containing wild-type *Salmonella* (11, 31). In addition, it appeared that relocalization of iNOS to *Salmonella* - containing phagosomes is also inhibited by a type-III secretion system-dependent mechanism (4), suggesting that this system is involved in interfering with vesicular transport in host cells. Clearly, prevention of superoxide production in the vicinity of intracellular *Salmonella* is an excellent survival strategy. If this survival mechanism is very efficient, and as a result, the levels of superoxide encountered by *Salmonella* in vivo are very low, this would also explain why so many mutants that are superoxide-sensitive in vitro are not attenuated in vivo. All the mechanisms described in the above paragraphs would then be a second barrier against ROI. When *Salmonella* is no longer able to prevent superoxide production, for instance, in activated phagocytic cells, these systems will become active and neutralize toxic oxygen intermediates or repair or prevent the damage induced by these compounds.



## Concluding remarks

*Salmonella* has evolved complex mechanisms to cope with ROI, and it is clear that different defense barriers exist that either prevent production of these compounds altogether or limit the damage that is done by the low amounts of toxic compounds that are still produced. In addition, like every aerobically growing organism, *Salmonella* and *E. coli* also have to protect themselves from endogenously produced ROI or environmental conditions that increase the endogenous superoxide concentration. Extensive research on gene-deleted *E. coli* and *Salmonella* strains has vastly increased our knowledge about the mechanisms that these bacteria employ to cope with ROI. As illustrated, it is at present not possible to adequately predict from the in vitro phenotype of a mutant, what the consequences of this mutation are for in vivo virulence. Although mutants that are overly sensitive to extracellular superoxide are likely to be attenuated in vivo, it is conceivable that mutants made using such a selection criterion could also lead to the identification of genes that are not essential for intracellular survival and virulence of *Salmonella*. It is clear that several "barriers of defense" against ROI exist. Some of these mechanisms may only be important for coping with ROI-inducing substances in the environment and may in this way determine *Salmonella* fitness, whereas other mechanisms are also crucial for virulence and determine survival of *Salmonella* in the host. Novel techniques, such as microarray analysis, have already contributed to our understanding of the various oxidative stress regulons. In the near future more extensive use of such techniques to analyze the response of *Salmonella* or *E. coli* to either intracellular or extracellular superoxide stress will give a more detailed view of the regulons induced, and from this point the contribution of candidate genes to virulence can be established.

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# Samenvatting in het Nederlands

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*Salmonella* is een staafvormige bacterie die overal in de natuur voorkomt. De meeste *Salmonella*'s kunnen geen ziekte veroorzaken doordat ze niet overleven in dieren of mensen. Deze komen alleen in de natuur of in het riool voor en zijn ongevaarlijk. Er zijn er echter een aantal die wel kunnen overleven en dit zijn de soorten die verantwoordelijk zijn voor ziekte in mens en dier. *Salmonella* infecties zijn een van de meest voorkomende voedsel-gerelateerde infecties in de wereld en vormen een groot probleem voor mensen in ontwikkelingslanden en voor mensen met een niet goed functionerend immuunsysteem. Ziektebeelden kunnen variëren van asymptomatisch dragerschap waarbij de bacteriën in de darm blijven en via de ontlasting uitgescheiden worden, tot levensbedreigende sepsis waarbij de bacteriën in het bloed terecht komen. Het ziektebeeld is afhankelijk van de *Salmonella* stam waarmee iemand besmet raakt en betreft meestal besmettingen met *S. enterica* serovar Enterica of serovar Typhimurium via met dierlijk afval besmet voedsel of water.

Besmetting met *Salmonella* gebeurt via het eten of drinken van besmet voedsel of water. Het maagzuur zorgt ervoor dat een groot deel van de bacteriën gedood wordt. Echter, wanneer de zuurgraad iets hoger is dan normaal, dan krijgt een deel van de bacteriën de kans om de maag te passeren en in de darm terecht te komen. Daar komen de bacteriën verscheidene andere afweermechanismen tegen zoals een dikke slijmlaag en de natuurlijke darmflora waarmee *Salmonella* een competitie om voedsel moet aangaan. Als *Salmonella* dit redt, zal het uiteindelijk in de dunne darm de Peyerse platen bereiken waarin zich de M cellen bevinden. *Salmonella* invadeert deze M cellen en afhankelijk van de *Salmonella* stam, kunnen twee ziektebeelden optreden. Bij infectie met de typhoïde stammen *S. enterica* serovar Typhi of serovar Paratyphi, stammen die alleen infectie in mensen veroorzaken, zullen de bacteriën zich verspreiden naar de onderliggende lymfoïde weefsels en zullen ze macrofagen binnen treden. De bacteriën komen in het bloed terecht, bereiken de lever en de milt en veroorzaken een ernstige ontstekingsreactie (inflammatoire respons) die typhoïde koorts of buiktyfus genoemd wordt en in 10% van de gevallen dodelijk is wanneer de patient niet behandeld wordt. Wanneer het echter een infectie met de niet-typhoïde stammen betreft zoals *S. enterica* serovar Typhimurium, dan blijven de bacteriën in de Peyerse platen en induceren daar een inflammatoire repons die gemediëerd wordt door cytokines, chemokines en neutrofiele granulocyten. Deze vorm van infectie wordt gastroenteritis genoemd en leidt tot diarrhee en gaat na een aantal dagen vanzelf over. In bepaalde patiënten, zoals mensen met een minder goed functionerend immuunsysteem, kan infectie met deze in principe ongevaarlijke stam wel leiden tot ernstige ziekte. Een ander probleem van *S. enterica* serovar Typhimurium zijn terugkerende infecties. Patiënten leiden dan aan steeds terugkerende infecties met hetzelfde isolaat. Dit is beschreven voor o.a. AIDS patienten en mensen die bestraling hebben ondergaan.

Bij de meeste wetenschappelijke studies naar *Salmonella* wordt gebruikt gemaakt van een in vivo muis model. Infectie met *S. enterica* serovar Typhimurium, wat bij mensen

gastroenteritis veroorzaakt, leidt in muizen tot een ziektebeeld dat overeenkomt met typhoïde koorts en is daarom een goed model voor humane infectie met *S. enterica* serovar Typhi of Paratyphi. De natuurlijke infectieroute is de orale (via de mond), maar experimentele infecties kunnen ook intraveneus (in een ader), intraperitoneaal (in de buikholte), of subcutaan (onder de huid) geïnitieerd worden. Zodra de bacteriën in het bloed terecht komen, verlopen al deze infecties op dezelfde manier en lijken op het verloop van typhoïde koorts in de mens. Binnen een paar dagen verspreiden de bacteriën zich naar de lever en de milt waar ze verblijven en zich vermenigvuldigen in macrofagen. Dit leidt, samen met de influx van immuuncellen (macrofagen en neutrofiele granulocyten), tot een vergroting van de lever en de milt (hepatosplenomegalie). Afhankelijk van het soort muis en de dosis, zullen de muizen als gevolg van de infectie doodgaan, of er wordt een immuunrespons geïnduceerd waardoor de muizen in leven blijven en beschermd zullen zijn tegen een tweede infectie. Natuurlijke bescherming tegen een *S. enterica* serovar Typhimurium infectie wordt o.a. bepaald door de genen *ity* en *lps*. *Ity* is het gen dat codeert voor Nramp1, een membraan fosfoglycoproteine dat alleen tot expressie wordt gebracht door macrofagen van de reticulo-endotheliale organen zoals lever en milt en dat betrokken is bij de weerstand tegen *Salmonella*. Muizen die het resistente allel van *Nramp1* (*Nramp1*$^{G169}$) tot expressie brengen kunnen de groeisnelheid van *S. enterica* serovar Typhimurium in vivo controleren zodat er een T cel-gemediëerde immuunrespons kan worden geïnitieerd die nodig is voor de uiteindelijke klaring van de bacteriën. Echter, muizen die het gevoelige allel *Nramp1*$^{D169}$ tot expressie brengen kunnen dit niet en zullen aan de infectie overlijden. Expressie van *Nramp1* wordt aanzienlijk vergroot na activatie van de macrofagen door IFN$\gamma$ and LPS. Het andere gen dat bescherming biedt is *lps*, het gen dat codeert voor Toll-like receptor 4 (TLR4). TLR4 is een receptor die betrokken is bij de herkenning van lipopolysaccharide (LPS) wat uitbundig tot expressie wordt gebracht door Gram-negatieve bacteriën zoals *Salmonella*. Deze receptor raakt dan geactiveerd en geeft signalen door die de celkern instrueren om bepaalde genen af te schrijven en bepaalde cytokines te maken, waaronder IFN$\gamma$, Il-18 en TNF$\alpha$. Deze cytokines spelen een belangrijke rol in het activeren van macrofagen en dus bij de afweer tegen *S. enterica* serovar Typhimurium. Muizen met een mutatie in het gen dat codeert voor TLR4, zoals in C3H/HeJ muizen, reageren niet op LPS als gevolg van een niet goed functionerend TLR4 en zijn daarom extreem gevoelig voor *S. enterica* serovar Typhimurium infecties.

Bij de afweer tegen *S. enterica* serovar Typhimurium spelen met name macrofagen een belangrijke rol. Macrofagen zijn fagocyterende cellen die uitgerust zijn met een heel scala aan mechanismen om micro-organismen te doden. Echter, *S. enterica* serovar Typhimurium heeft zich volledig aangepast en is in staat om te overleven en zelfs te delen in dit vijandig milieu. Een belangrijk afweermechanisme van de macrofaag tegen micro-organismen is de productie van zuurstofradicalen. Deze radicalen zijn zeer schadelijk en zouden *S. enterica* serovar Typhimurium doden als die zich niet aangepast zou hebben. In het genoom van *Salmonella* bevinden zich twee pathogeniciteitseilanden (SPI-1 en SPI-2) die coderen voor type III secretie systemen (TTSS). SPI-1 is een TTSS die betrokken is bij de opname door de cel. Het codeert voor een soort injectiespuit waarmee bepaalde

eiwitten in de cel geïnjecteerd worden waardoor er allerlei veranderingen in het membraan van de cel optreden en de bacterie opgenomen wordt, zelfs door niet-fagocyterende cellen. Dit proces wordt *Salmonella*-geïnduceerde opname genoemd. *S. enterica* serovar Typhimurium komt dan in een fagosoom terecht waar het kan overleven doordat het tweede TTSS gecodeerd door SPI-2 in werking treedt. Hierbij worden weer eiwitten in het cytoplasma geïnjecteerd waardoor allerlei antibacteriële processen verstoord worden en *S. enterica* serovar Typhimurium kan overleven. Zo wordt via SPI-2 gecodeerde eiwitten o.a. voorkomen dat er superoxide geproduceerd kan worden doordat deze eiwitten de translocatie en vorming van het actieve NADPH oxidase complex voorkomen. Naast SPI-1 en SPI-2 gecodeerde eiwitten spelen ook andere genen zoals *soxR/S*, *phoP/Q* een belangrijke rol in de afweer tegen superoxide en in overleving in de gastheer.

Onderzoek naar de rol van superoxide-resistentiegenen in overleving van *S. enterica* serovar Typhimurium in macrofagen en muizen en onderzoek naar reactiverende *Salmonella* infecties leidt uiteindelijk tot meer inzicht in de strategieën die *Salmonella* gebruikt om te overleven.

Hoofdstuk 1 geeft een samenvatting van wat er bekend is over *Salmonella*, de interactie met de gastheer en systemen die een rol spelen bij de afweer tegen superoxide en overleving in macrofagen.

In hoofdstuk 2 wordt het opzetten van een in vivo muizenmodel voor een reactiverende *S. enterica* serovar Typhimurium infectie door middel van bestraling en depletie van $CD4^+$ T cellen beschreven. Een belangrijke complicatie van *S. enterica* serovar Typhimurium infecties in sommige patiënten is het steeds terugkeren van de infectie. De infectie wordt opgeruimd, maar toch ziet *Salmonella* de kans om zich schuil te houden voor het immuunsysteem en te overleven en weer toe te slaan zodra het immuunsysteem verstoord is. Het is echter nog niet precies bekend waar *Salmonella* zich schuil houdt en welke processen een rol spelen bij de onderdrukking van groei tijdens de fase waarin *Salmonella* zich schuil houdt (persistentie/latentie fase). Reactiverende *S. enterica* serovar Typhimurium infecties betreffen met name patiënten die bestraling hebben ondergaan, patienten met AIDS of andere immuunstoornissen. We laten met ons reactivatiemodel zien dat $CD4^+$ T cellen een rol spelen bij de onderdrukking van replicatie van *S. enterica* serovar Typhimurium tijdens de fase van persistentie.

Sinds een aantal jaren wordt er voor de behandeling van rheumatoïde arthritis en de ziekte van Crohn een behandeling toegepast waarbij de patiënten behandeld worden met Infliximab of Ethanercept. Deze vorm van behandeling werkt voor deze patiënten erg goed, maar een groot nadeel van deze vorm van behandeling die in een kleine groep patiënten een rol speelt, is het opspelen van allerlei infecties met intracellulaire pathogenen, zoals *Mycobacterium tuberculosis*. Het kan dan gaan om nieuwe infecties, maar ook om reactiverende infecties. De behandeling is gebaseerd op het toedienen van antilichamen gericht tegen tumor necrosis factor $\alpha$ (TNF$\alpha$). TNF$\alpha$ is een cytokine dat een belangrijke rol speelt bij de activatie van macrofagen en bij de afweer tegen allerlei pathogenen, waaronder *Salmonella*. Voor *Salmonella* infecties is het wel bekend dat neutralisatie van TNF$\alpha$ leidt tot verhoogd risico op ernstige *Salmonella* infecties, maar of deze behandeling

ook tot reactivatie van een latente *Salmonella* infectie leidt, zoals bij latente *Mycobacterium tuberculosis* infectie, is nog onduidelijk. In hoofdstuk 3 is onderzocht of TNFα ook een rol speelt bij de onderdrukking van *S. enterica* serovar Typhimurium tijdens de fase van persistentie in muizen. In het model dat wij gebruikt hebben, zagen we geen reactivatie van de *S. enterica* serovar Typhimurium infectie na behandeling van de muizen met een antilichaam gericht tegen TNFα. Verder hebben we gezien dat toevoeging van anti-TNFα aan IFNγ-gestimuleerde muizemacrofagen (RAW264.7 cellen) geen effect had op het IFNγ effect van verminderde uitgroei van *S. enterica* serovar typhimurium. Dit zou erop kunnen duiden dat TNFα een ondergeschikte rol speelt aan IFNγ bij een infectie met *S. enterica* serovar Typhimurium. Zolang IFNγ geproduceerd door $CD4^+$ T cellen aanwezig blijft, zal de latent aanwezige *S. enterica* serovar Typhimurium onderdrukt blijven en zal geen reactivatie plaatsvinden.

Hoofdstuk 4 behandelt het onderzoek naar mutanten die in staat zijn om langer te overleven in macrofagen. We hebben daarbij *S. enterica* serovar Typhimurium mutanten gemaakt door middel van P22 MudJ transposon insertie. MudJ transposon is een groot stuk DNA dat met behulp van een enzym (een transposase) at random ingebouwd kan worden in het genoom en hierdoor een bepaald, van tevoren niet te voorspellen, gen kan inactiveren. We hebben op die manier verschillende mutanten verkregen die vervolgens geselecteerd zijn op de mogelijkheid om langer te overleven in muizemacrofagen. Uiteindelijk bleven er twee mutanten over die na inverse PCR en sequentie-analyse dezelfde bleken te zijn, omdat het MudJ transposon op precies dezelfde plaats in het genoom was ingebouwd. Bij deze twee mutanten bleek het MudJ transposon ingebouwd te zijn in *rmlC*, het gen dat codeert voor het enzym dTDP-4-deoxyrhamnose 3,5-epimerase dat betrokken is bij de vorming van het O-antigen van het lipopolyaccharide (LPS). Analyse van het LPS liet ook zien dat deze mutant een verkort LPS heeft en lijkt heel veel op een *S. enterica* serovar Typhimurium stam met het ruwe Ra chemotype. Dit is een mutant die het O-antigen mist en alleen het lipid A deel en het core van het LPS heeft. Ook deze Ra mutant bleek in staat om langer te overleven in RAW264.7 cellen en was deze zelfs na 48 uur nog in hoge aantallen aanwezig. Desondanks bleken deze mutanten toch niet in staat om in muizen een ernstige infectie te veroorzaken, wat niet verklaard kon worden door de verhoogde gevoeligheid voor complement in het bloed.

Door random MudJ transposon insertie in wildtype *S. enterica* serovar Typhimurium 14028s hebben we vele mutanten verkregen die vervolgens geselecteerd zijn op gevoeligheid voor het superoxide-genererende stofje menadione. Dit is gedaan door vele kolonies te strijken op agarplaten met en zonder menadione en die mutanten te selecteren die niet groeiden op de platen met menadione en wel op de controle plaat zonder menadione. We hebben op die manier een aantal superoxide-gevoelige mutanten verkregen die vervolgens getest zijn in een in vitro infectie-experiment in RAW264.7 macrofagen zoals weergegegven in Appendix 1. Vervolgens is er met behulp van inverse PCR en sequentie-analyse bepaald waar het MudJ transposon was ingebouwd en welk gen geïnactiveerd was door dit transposon. Eén van de mutanten die op die manier verkregen is staat beschreven in Hoofdstuk 5 en betreft een mutant waarbij het MudJ



transposon was ingebouwd in de promotor regio van het gen *pnp* dat codeert voor PNPase, een enzym dat betrokken is bij de afbraak van RNA en bij de adaptate van groei bij lage temperaturen en wordt gezien als een regulator van virulentie en persistenie van *S. enterica* serovar Typhimurium. Wij beschrijven een additionele rol voor PNPase in de resistentie tegen superoxide en in overleving in macrofagen.

Bij het onderzoek naar de rol van superoxide-resistentie genen beschrijven we in Hoofdstuk 6 de isolatie en karakterisatie van DLG294, een *S. enterica* serovar Typhimurium mutant waarbij door een MudJ transposon een tot dan toe onbekend gen geïnactiveerd is en dat *sspJ* genoemd is. Het eiwit SspJ wordt niet meer gevormd in de mutant en zorgt ervoor dat de mutant veel gevoeliger is geworden voor menadione, een redox-cycling agens dat superoxide radicalen genereert in de bacterie. DLG294 bleek minder virulent te zijn in vitro in macrofagen en in vivo in muizen. Door *sspJ* op een plasmide constitutief tot expressie te brengen in DLG294 is het fenotype van de mutant hersteld tot dat van de wildtype stam. Hieruit bleek heel duidelijk de rol van SspJ in de afweer tegen superoxide en in virulentie, maar de exacte rol en manier van functioneren is nog niet geheel duidelijk.

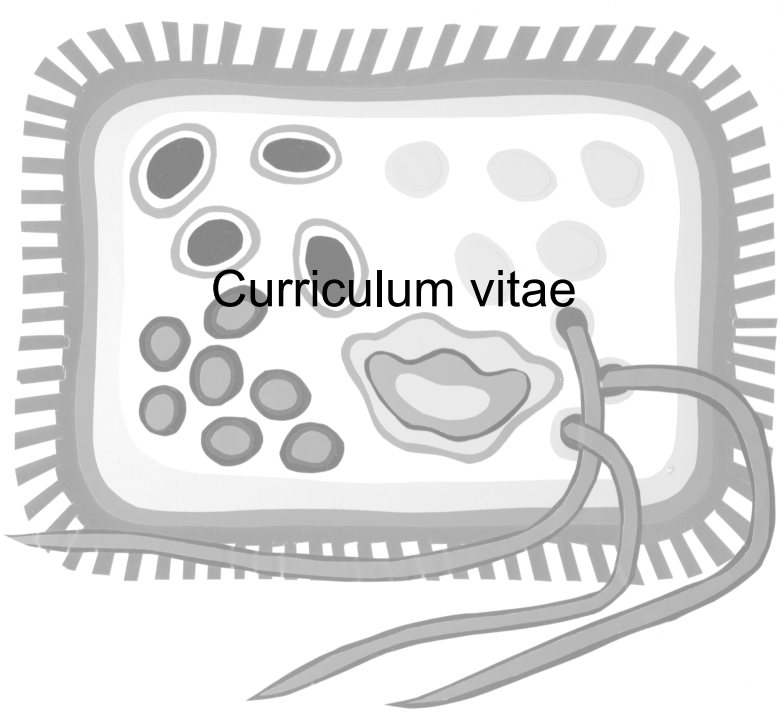
DLG294 is nader onderzocht in vivo in muizen, zoals beschreven staat in Hoofdstuk 7. DLG294 induceert bijna geen granulomateuze lesies in de lever na subcutane infectie van *Salmonella*-resistente ($Ity^r$) C3H/HeN muizen met $3 \times 10^4$ CFU en de aantallen bacteriën waren 3 log eenheden lager dan die van de wild-type stam op dag 5 na infectie. Daarentegen was DLG294 wel net zo virulent als wildtype en induceerde het een vergelijkbare leverpathologie in $p47^{phox-/-}$ muizen. Deze muizen missen een functioneel NADPH oxidase systeem door het ontbreken van de $p47^{phox}$ en kunnen geen superoxide produceren. Ook in beenmergmacrofagen van deze $p47^{phox-/-}$ muizen en in X-CGD PLB985 cellen waren de bacterie-aantallen van DLG294 net zo hoog als die van de wild-type stam op 24 uur na infectie. Deze resultaten suggereren dat SspJ een rol speelt bij de resistentie tegen oxidatieve stress en in overleving en replicatie van *S. enterica* serovar Typhimurium zowel in vitro als in vivo.

Macrofagen spelen een belangrijke rol bij *Salmonella* infecties. Ze spelen hierbij een dubbele rol, die van gastheer- en effectorcel. DLG294 is gevoeliger voor superoxide en is geattenueerd in macrofagen en we weten dat superoxide hierbij een belangrijke rol speelt, maar wellicht dat andere processen ook een rol spelen en ervoor zorgen dat DLG94 slechter in staat is om te overleven. Door de infectie met *S. enterica* serovar Typhimurium zal de macrofaag geactiveerd worden om de bacteriën te doden. Hierbij spelen diverse mechanismen een rol. Wij zijn in Hoofdstuk 8 nagegaan wat het effect is van wildtype en DLG249 infectie op de genexpressie in de macrofagen met behulp van een Affymetrix gene chip. Met deze chip kunnen 6.400 genen tegelijkertijd bestudeerd worden. Wildtype *S. enterica* serovar Typhimurium en DLG294 bleken beide de expressie van vele genen te beïnvloeden, maar er waren geen grote verschillen te zien. Hieruit konden we concluderen dat het feit dat DLG294 slechter in staat is om uit te groeien in macrofagen te wijten is aan de mutatie in *sspJ* en niet aan een veranderde activatiestatus van de macrofagen t.o.v. wildtype geïnfecteerde macrofagen.

Omdat het nog steeds onduidelijk was waarom DLG294 geattenueerd is en welke rol SspJ precies speelt, hebben we in Hoofdstuk 9 het fenotype van DLG294 nader bestudeerd met behulp van een fenotypische array waarbij tegelijkertijd vele processen bestudeerd kunnen worden. Het fenotype van DLG294 is vergeleken met de wildtype stam en liet zien dat DLG294 beter in staat was on stikstofbronnen te gebruiken voor groei, maar dat het daarnaast gevoeliger is voor een groot aantal antibiotica en voor hoge of lage pH. Daarnaast hebben we gekeken naar de genexpressie van intracellulaire DLG294 en hebben deze vergeleken met de genexpressie van intracellulaire wildtype *S. enterica* serovar Typhimurium. Daaruit bleek dat er slechts een paar kleine verschillen tussen DLG294 en de wildtype stam waren en dat er waarschijnlijk iets mis is met de membraanintegriteit van DLG294 waardoor er meer schadelijke stoffen worden opgenomen en deze stam hierdoor meer schade ondervindt.

**Concluderende opmerkingen**

*Salmonella* heeft allerlei complexe mechanismen ontwikkeld om om te kunnen gaan met reactive zuurstofradicalen. *Salmonella* bezit verschillende afweer barrières die de productie van zuurstofradicalen kunnen voorkomen verschillende of die de aangerichte schade door het beetje schadelijke stoffen dat toch nog gevormd wordt zoveel mogelijk beperken. Bovendien is het zo dat ieder organisme (o.a. *Salmonella* en *E. coli*) dat in de aanwezigheid van zuurstof groeit (aeroob) zichzelf moet kunnen beschermen tegen zuurstofradicalen die door het organisme zelf gemaakt worden of tegen invloeden vanuit de omgeving die de superoxide concentratie in de cel kunnen beïnvloeden. Uitgebreid onderzoek naar mutanten van *E. coli* en *Salmonella* heeft onze kennis over afweermechanismen tegen zuurstofradicalen al behoorlijk vergroot. Maar, zoals ook in dit proefschrift beschreven staat, is het op dit moment niet mogelijk om aan de hand van het in vitro fenotype van een mutant te voorspellen hoe deze zich in vivo zal gaan gedragen. Hoewel mutanten die extreem gevoelig zijn voor extracellulair superoxide waarschijnlijk wel geattenueerd zullen zijn in vivo, is het toch ook denkbaar dat met deze selectie criteria ook genen geïdentificeerd kunnen worden die niet essentieel zijn voor intracellulaire overleving en voor virulentie van *Salmonella*. Het is duidelijk dat er verschillende afweerbarrières tegen reactieve zuurstofradicalen zijn. Sommige van deze mechanismen zijn alleen van belang bij het omgaan met reactieve zuurstofradicalen in het milieu en bepalen de fitheid van *Salmonella* terwijl andere mechanismen ook van cruciaal belang zijn voor virulentie en bepalen daarmee de overlevingskansen van *Salmonella* in de gastheer. Nieuwe technieken, zoals microarray analyse, hebben al bijgedragen aan onze kennis over de verschillende genen en regulons die betrokken zijn bij afweer tegen oxidatieve stress. In de nabije toekomst zal uitgebreider gebruik van deze technieken een gedetailleerder beeld geven van de regulons die geïnduceerd worden. Zo kan de reactie van *Salmonella* op intra- of extracellulaire superoxide stress onderzocht worden en kan vervolgens de bijdrage van mogelijke virulentiegenen bevestigd worden.



# Curriculum vitae

curriculum vitae

## Curriculum Vitae

Op 11 november 1975 werd ik geboren te Hattem. Vanaf augustus 1988 tot en met mei 1995 volgde ik het Voorbereidend Wetenschappelijk Onderwijs (V.W.O.) aan de Christelijke Scholengemeenschap "De Heertganck" te Heerde. Na het behalen van mijn V.W.O. diploma ben ik richting Utrecht getrokken om daar Medische Biologie aan de faculteit Geneeskunde van de Universiteit Utrecht te gaan studeren.

Voor mijn eerste wetenschappelijke stage ben ik in Utrecht gebleven om daar mijn hoofdvakstage van 9 maanden te volgen bij de afdeling Immunohematologie in het Universitair Medisch Centrum Utrecht onder begeleiding van Dr. Ger J. J. C. Boonen en Dr. René H. Medema. Hier heb ik gewerkt aan het onderzoek naar de "Relatie tussen cycline D3 expressie en de negatieve groeiregulatie van Jurkat leukemische T cellen na T cel receptor stimulatie".

Voor mijn bijvakstage ben ik eens een kijkje gaan nemen in het wetenschappelijke bedrijfsleven. Gedurende 6 maanden heb ik bij de Bacteriologische Research & Development afdeling van Intervet International B.V. te Boxmeer gewerkt aan "Antilichaam-gemediëerde reductie van *Campylobacter jejuni* in kippen" onder directe begeleiding van Dr. Anton A. C. Jacobs en Dr. Piet J. M. Nuijten en onder toezicht van Dr. Harm Snippe van het Eijkman-Winkler Instituut voor Medische Microbiologie van de Universiteit Utrecht.

In december 1999 heb ik het doctoraal examen behaald en op 1 januari 2000 ben ik begonnen als Assistent In Opleiding (AIO) bij de afdeling Infectieziekten in het Leids Universitair Medisch Centrum, onder supervisie van Prof. Dr. Jaap T. van Dissel en Dr. Riny Janssen. Het onderzoek dat ik daar heb gedaan, staat beschreven in dit proefschrift. In het kader van de SMBWO opleiding tot Medisch Microbiologisch Onderzoeker heb ik tijdens mijn AIO-periode bij de afdeling Infectieziekten de cursussen Medische Parasitologie, Medische Mycologie en Virologie mogen volgen en ben ik nu nog in afwachting van de officiële erkenning.

Per 1 april 2005 ben ik in dienst getreden bij het Laboratorium Kindergeneeskunde Infectieziekten in het Universitair Medisch Centrum Nijmegen en werk daar nu als onderzoeker aan het proteomics deelonderwerp van het VIRGO project onder leiding van Dr. Peter W. M. Hermans en Prof. Dr. Ronald de Groot.

# List of publications

publications

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Ger J. J. C. Boonen, Brigitte van Oirschot, **Angela van Diepen**, Wendy J. Mackus, Leo F. Verdonck, Gert Rijksen, and René H. Medema. 1999. Cyclin D3 regulates proliferation and apoptosis of leukemic T cell lines. J. Biol. Chem. **274:**34676-34682.

Tahar van der Straaten, **Angela van Diepen**, Kitty Kwappenberg, Sjaak van Voorden, Kees Franken, Riny Janssen, Johannes G. Kusters, Donald L. Granger, and Jaap T. van Dissel. 2001. Novel *Salmonella enterica* serovar Typhimurium protein that is indispensable for virulence and intracellular replication. Infect Immun. **69:**7413-7418.

**Angela van Diepen**, Tahar van der Straaten, Steven M. Holland, Riny Janssen, and Jaap T. van Dissel. 2002. A superoxide-hypersusceptible *Salmonella enterica* serovar Typhimurium mutant is attenuated but regains virulence in p47(phox-/-) mice. Infect Immun. **70:**2614-2621.

Riny Janssen, Tahar van der Straaten, **Angela van Diepen**, Jaap T. van Dissel. 2003. Responses to reactive oxygen intermediates and virulence of *Salmonella typhimurium*. Microbes Infect. **5:**527-34. Review.

Tahar van der Straaten, Laurence Zulianello, **Angela van Diepen**, Donald L. Granger, Riny Janssen, and Jaap T. van Dissel. 2004. *Salmonella enterica* serovar Typhimurium RamA, intracellular oxidative stress response, and bacterial virulence. Infect Immun. **72:**996-1003.

**Angela van Diepen**, Joke S. van de Gevel, Margaretha M. Koudijs, Ferry Ossendorp, Henry Beekhuizen, Riny Janssen, and Jaap T. van Dissel. 2004. Gamma irradiation and CD4+ T cell depletion causes reactivation of latent *Salmonella typhimurium* infection in C3H/HeN Mice. Infect. Immun. **73:**2857-2862.

**Angela van Diepen**, Cerithsa A. E. Martina, Riny Janssen, and Jaap T. van Dissel. 2005. Treatment with anti-TNF$\alpha$ does not induce reactivation of latent *Salmonella enterica* serovar Typhimurium infection in C3H/HeN mice. Submitted for publication.

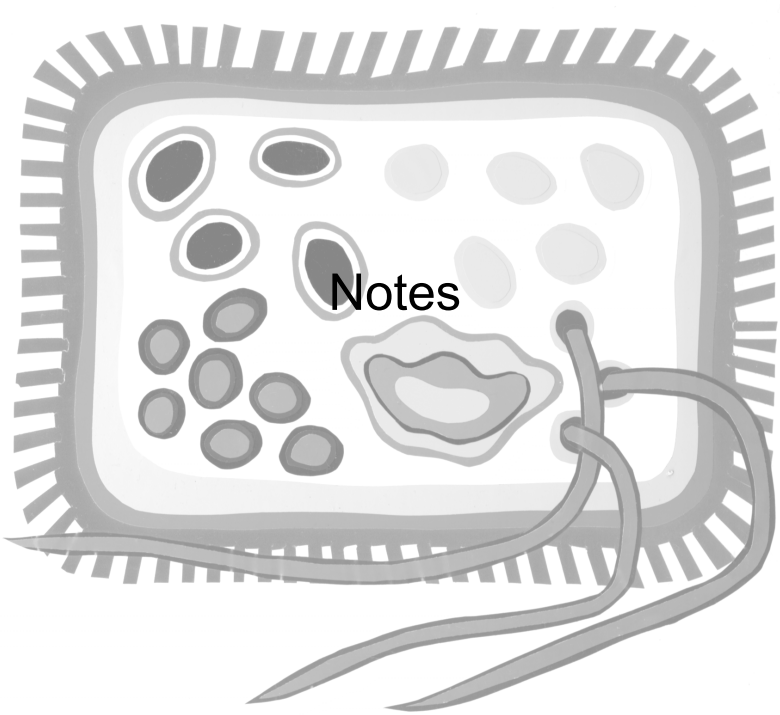
**Angela van Diepen**, Els Verhard, Jaap T. van Dissel, and Riny Janssen. An *rmlC Salmonella enterica* serovar Typhimurium mutant is attenuated in vivo but is able to persist in RAW264.7 macrophages. Submitted for publication.

**Angela van Diepen**, Natasha A. N. Deters, Martijn den Reijer, Davy van Doren, Linda van Diemen, Riny Janssen, and Jaap T. van Dissel. A superoxide-hypersusceptible *Salmonella enterica* serovar Typhimurium PNPase mutant is attenuated in RAW264.7 macrophages but is more virulent in C3H/HeN mice. Submitted for publication.



**Angela van Diepen**, Riny Janssen, and Jaap T. van Dissel. Comparison of mRNA levels from RAW264.7 macrophages infected with *Salmonella enterica* serovar Typhimurium 14028s and the superoxide-hypersusceptible mutant DLG294 by microarray analysis. Manuscript in preparation.

**Angela van Diepen**, Yvette Wormstone, Vittoria Danino, Riny Janssen, Jay C. D. Hinton, and Jaap T. van Dissel. Gene expression profiling of intracellular wild-type *Salmonella enterica* serovar Typhimurium and mutant DLG294 that is hypersusceptible to the redox cycling agent menadione. Manuscript in preparation.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