Salmonella typhimurium and its host : host-pathogen cross-talk, immune evasion, and persistence

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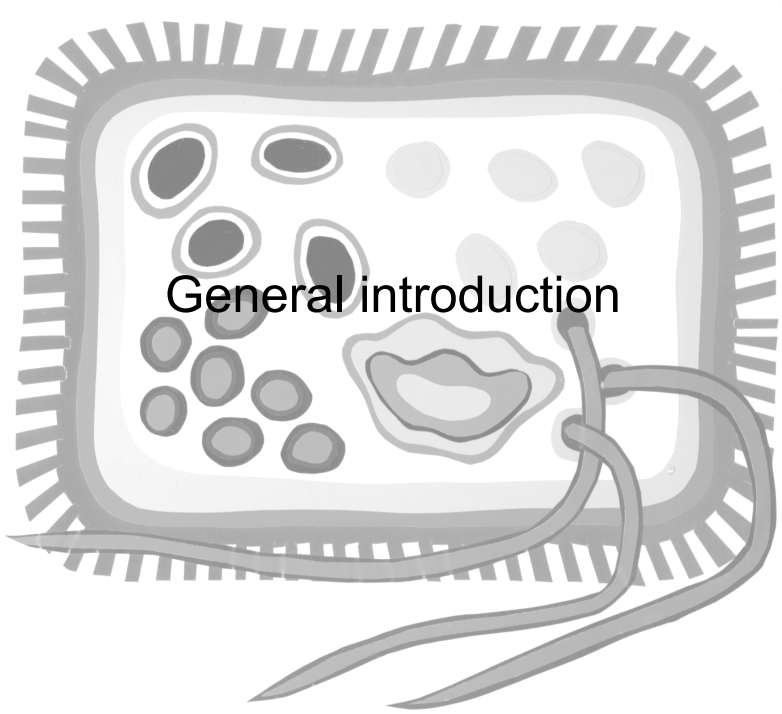
chapter one



General introduction

Partly adapted from:

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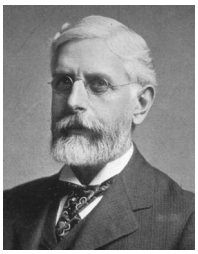
History

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.

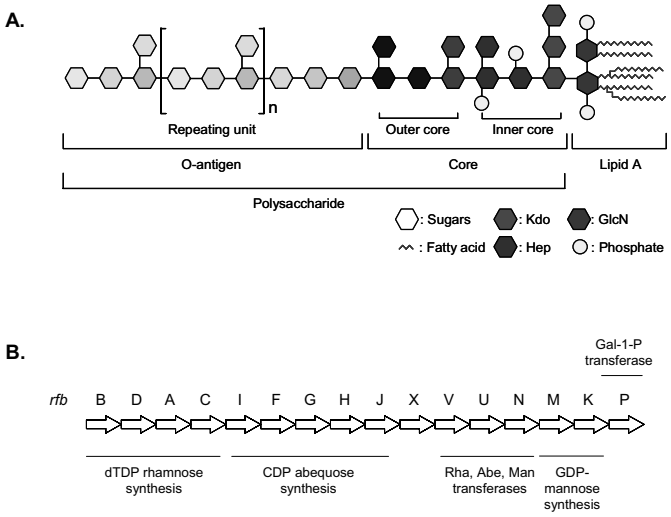


Figure 1. LPS structure of *S. enterica* serovar Typhimurium (A) and organization of the *rfb* operon encoding genes involved in the formation of the O-antigen (B).

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 β1 (IL12Rβ1) deficiency (2, 3, 33, 164) or Interferon γ receptor 1 (IFNγR1) 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.

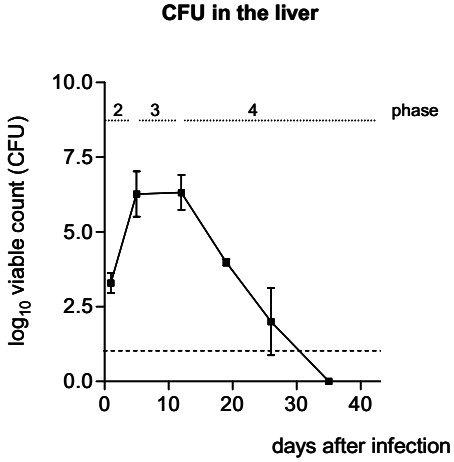


Figure 2. Three of the four phases of primary *S. enterica* serovar Typhimurium infection in the livers of C3H/HeN mice. The first phase is characterized by rapid killing of the bacteria by resident macrophages and complement. During the second phase, when the bacteria have spread to the liver and spleen 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-1β converting enzyme capable of cleaving the pro-forms of the inflammatory cytokines IL-1β and IL-18 (65). Caspase-1 activation then leads to the secretion of IL-1β 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 IFNγ and TNFα 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.

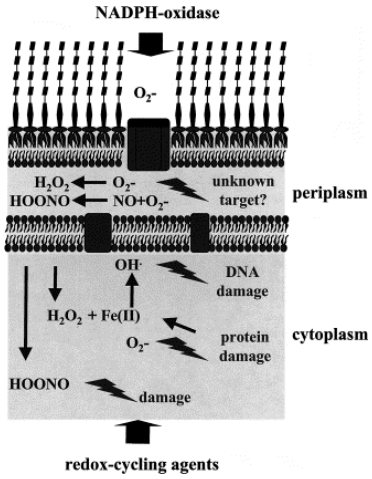


Figure 3. Schematic overview of superoxide-mediated damage. Superoxide produced by the NADPH-oxidase passes the outer membrane and may cause damage to periplasmic targets or can be converted into hydrogen peroxide, which will pass the cytoplasmic membrane. In conjunction with Fe(II), hydroxyl radicals 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 $NADP^+$.

$$GSH + \cdot HOO^- \longrightarrow GS\cdot + H_2O_2$$
$$GS\cdot + GS\cdot \longrightarrow GSSG$$
$$GSSG + 2NADPH \longrightarrow 2GSH + 2NADP^+$$

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 IFNγ, as shown by others, resulted in reactivation of the *S. enterica* serovar typhimurium infection we investigated in Chapter 3 whether neutralization of TNFα, 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.

References

1. **Abshire, K. Z. and F. C. Neidhardt.** 1993. Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. J. Bacteriol. **175:**3734-3743.
2. **Altare, F., A. Durandy, D. Lammas, J. F. Emile, S. Lamhamedi, D. F. Le, P. Drysdale, E. Jouanguy, R. Doffinger, F. Bernaudin, O. Jeppsson, J. A. Gollob, E. Meinl, A. W. Segal, A. Fischer, D. Kumararatne, and J. L. Casanova.** 1998. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science **280:**1432-1435.
3. **Altare, F., D. Lammas, P. Revy, E. Jouanguy, R. Doffinger, S. Lamhamedi, P. Drysdale, D. Scheel-Toellner, J. Girdlestone, P. Darbyshire, M. Wadhwa, H. Dockrell, M. Salmon, A. Fischer, A. Durandy, J. L. Casanova, and D. S. Kumararatne.** 1998. Inherited interleukin 12 deficiency in a child with bacille Calmette-Guerin and *Salmonella enteritidis* disseminated infection. J. Clin. Invest **102:**2035-2040.
4. **Babior, B. M.** 1999. NADPH oxidase: an update. Blood **93:**1464-1476.
5. **Baumler, A. J. and F. Heffron.** 1995. Identification and sequence analysis of lpfABCDE, a putative fimbrial operon of *Salmonella typhimurium*. J. Bacteriol. **177:**2087-2097.

6. **Baumler, A. J., R. M. Tsolis, and F. Heffron.** 1996. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. Infect. Immun. **64:**1862-1865.
7. **Beuzon, C. R., S. Meresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot, and D. W. Holden.** 2000. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. EMBO J. **19:**3235-3249.
8. **Boise, L. H. and C. M. Collins.** 2001. *Salmonella*-induced cell death: apoptosis, necrosis or programmed cell death? Trends Microbiol. **9:**64-67.
9. **Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan.** 2000. *Salmonella* nomenclature. J. Clin. Microbiol. **38:**2465-2467.
10. **Brumell, J. H., D. L. Goosney, and B. B. Finlay.** 2002. SifA, a type III secreted effector of *Salmonella typhimurium*, directs *Salmonella*-induced filament (Sif) formation along microtubules. Traffic. **3:**407-415.
11. **Brumell, J. H., C. M. Rosenberger, G. T. Gotto, S. L. Marcus, and B. B. Finlay.** 2001. SifA permits survival and replication of *Salmonella typhimurium* in murine macrophages. Cell Microbiol. **3:**75-84.
12. **Brumell, J. H., P. Tang, S. D. Mills, and B. B. Finlay.** 2001. Characterization of *Salmonella*-induced filaments (Sifs) reveals a delayed interaction between *Salmonella*-containing vacuoles and late endocytic compartments. Traffic. **2:**643-653.
13. **Buchmeier, N., S. Bossie, C. Y. Chen, F. C. Fang, D. G. Guiney, and S. J. Libby.** 1997. SlyA, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. Infect. Immun. **65:**3725-3730.
14. **Buchmeier, N. A. and F. Heffron.** 1991. Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. Infect. Immun. **59:**2232-2238.
15. **Buchmeier, N. A., S. J. Libby, Y. Xu, P. C. Loewen, J. Switala, D. G. Guiney, and F. C. Fang.** 1995. DNA repair is more important than catalase for *Salmonella* virulence in mice. J. Clin. Invest **95:**1047-1053.
16. **Buchmeier, N. A., C. J. Lipps, M. Y. So, and F. Heffron.** 1993. Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. Mol. Microbiol. **7:**933-936.
17. **Burckhardt, B., P. Sendi, D. Pfluger, W. Zimmerli, R. Nuesch, H. C. Bucher, J. Drewe, N. Gyr, and M. Battegay.** 1999. Rare AIDS-defining diseases in the Swiss HIV Cohort Study. Eur. J. Clin. Microbiol. Infect. Dis. **18:**399-402.
18. **Burns-Keliher, L., C. A. Nickerson, B. J. Morrow, and R. Curtiss, III.** 1998. Cell-specific proteins synthesized by *Salmonella typhimurium*. Infect. Immun. **66:**856-861.
19. **Casimir, C. M., H. N. Bu-Ghanim, A. R. Rodaway, D. L. Bentley, P. Rowe, and A. W. Segal.** 1991. Autosomal recessive chronic granulomatous disease caused by deletion at a dinucleotide repeat. Proc. Natl. Acad. Sci. U. S. A. **88:**2753-2757.
20. **Cheminay, C., D. Chakravortty, and M. Hensel.** 2004. Role of neutrophils in murine salmonellosis. Infect. Immun. **72:**468-477.
21. **Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow.** 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. **30:**175-188.
22. **Clark, R. A., H. L. Malech, J. I. Gallin, H. Nunoi, B. D. Volpp, D. W. Pearson, W. M. Nauseef, and J. T. Curnutte.** 1989. Genetic variants of chronic granulomatous disease: prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. N. Engl. J. Med. **321:**647-652.
23. **Clegg, S., S. Hull, R. Hull, and J. Pruckler.** 1985. Construction and comparison of recombinant plasmids encoding type 1 fimbriae of members of the family Enterobacteriaceae. Infect. Immun. **48:**275-279.
24. **Clegg, S., B. K. Purcell, and J. Pruckler.** 1987. Characterization of genes encoding type 1 fimbriae of *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Serratia marcescens*. Infect. Immun. **55:**281-287.
25. **Collins, F. M.** 1969. Effect of specific immune mouse serum on the growth of *Salmonella enteritidis* in nonvaccinated mice challenged by various routes. J. Bacteriol. **97:**667-675.
26. **Conlan, J. W.** 1996. Neutrophils prevent extracellular colonization of the liver microvasculature by Salmonella typhimurium. Infect. Immun. **64:**1043-1047.
27. **Conlan, J. W. and R. J. North.** 1992. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. Infect. Immun. **60:**5164-5171.
28. **Crosa, J. H., D. J. Brenner, W. H. Ewing, and S. Falkow.** 1973. Molecular relationships among the *Salmonelleae*. J. Bacteriol. **115:**307-315.
29. **Crump, J. A., S. P. Luby, and E. D. Mintz.** 2004. The global burden of typhoid fever. Bull. World Health Organ **82:**346-353.
30. **Curnutte, J. T.** 1992. Molecular basis of the autosomal recessive forms of chronic granulomatous disease. Immunodefic. Rev. **3:**149-172.
31. **Curnutte, J. T.** 1993. Chronic granulomatous disease: the solving of a clinical riddle at the molecular level. Clin. Immunol. Immunopathol. **67:**S2-15.
32. **De Groote M.A., U. A. Ochsner, M. U. Shiloh, C. Nathan, J. M. McCord, M. C. Dinauer, S. J. Libby, A. Vazquez-Torres, Y. Xu, and F. C. Fang.** 1997. Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. Proc. Natl. Acad. Sci. U. S. A. **94:**13997-14001.
33. **De Jong, R., F. Altare, I. A. Haagen, D. G. Elferink, T. Boer, P. J. Breda Vriesman, P. J. Kabel, J. M. Draaisma, J. T. van Dissel, F. P. Kroon, J. L. Casanova, and T. H. Ottenhoff.** 1998. Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. Science **280:**1435-1438.

34. **Dinauer, M. C., E. A. Pierce, G. A. Bruns, J. T. Curnutte, and S. H. Orkin.** 1990. Human neutrophil cytochrome b light chain (p22-phox). Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. J. Clin. Invest. **86:**1729-1737.
35. **Dubrac, S. and D. Touati.** 2000. Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the sodB promoter. J. Bacteriol. **182:**3802-3808.
36. **Dunlap, N. E., W. H. Benjamin, Jr., A. K. Berry, J. H. Eldridge, and D. E. Briles.** 1992. A 'safe-site' for *Salmonella typhimurium* is within splenic polymorphonuclear cells. Microb. Pathog. **13:**181-190.
37. **Dunlap, N. E., W. H. Benjamin, Jr., R. D. McCall, Jr., A. B. Tilden, and D. E. Briles.** 1991. A 'safe-site' for *Salmonella typhimurium* is within splenic cells during the early phase of infection in mice. Microb. Pathog. **10:**297-310.
38. **Edelman, R. and M. M. Levine.** 1986. Summary of an international workshop on typhoid fever. Rev. Infect. Dis. **8:**329-349.
39. **Fang, F. C., M. A. DeGroote, J. W. Foster, A. J. Baumler, U. Ochsner, T. Testerman, S. Bearson, J. C. Giard, Y. Xu, G. Campbell, and T. Laessig.** 1999. Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. Proc. Natl. Acad. Sci. U. S. A. **96:**7502-7507.
40. **Fang, F. C. and A. Vazquez-Torres.** 2002. Nitric oxide production by human macrophages: there's NO doubt about it. Am. J. Physiol Lung Cell Mol. Physiol **282:**L941-L943.
41. **Farr, S. B. and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561-585.
42. **Farrant, J. L., A. Sansone, J. R. Canvin, M. J. Pallen, P. R. Langford, T. S. Wallis, G. Dougan, and J. S. Kroll.** 1997. Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. Mol. Microbiol. **25:**785-796.
43. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. U. S. A. **83:**5189-5193.
44. **Figueroa-Bossi, N., S. Uzzau, D. Maloriol, and L. Bossi.** 2001. Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in *Salmonella*. Mol. Microbiol. **39:**260-271.
45. **Friedrich, M. J., N. E. Kinsey, J. Vila, and R. J. Kadner.** 1993. Nucleotide sequence of a 13.9 kb segment of the 90 kb virulence plasmid of *Salmonella typhimurium*: the presence of fimbrial biosynthetic genes. Mol. Microbiol. **8:**543-558.
46. **Galan, J. E. and A. Collmer.** 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284:**1322-1328.
47. **Galan, J. E. and R. Curtiss, III.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. U. S. A **86:**6383-6387.
48. **Gallois, A., J. R. Klein, L. A. Allen, B. D. Jones, and W. M. Nauseef.** 2001. *Salmonella* pathogenicity island 2-encoded type iii secretion system mediates exclusion of nadph oxidase assembly from the phagosomal membrane. J. Immunol. **166:**5741-5748.
49. **Garcia-Del Portillo, F.** 2001. *Salmonella* intracellular proliferation: where, when and how? Microbes. Infect. **3:**1305-1311.
50. **Garvis, S. G., C. R. Beuzon, and D. W. Holden.** 2001. A role for the PhoP/Q regulon in inhibition of fusion between lysosomes and *Salmonella*-containing vacuoles in macrophages. Cell Microbiol. **3:**731-744.
51. **Ginocchio, C., J. Pace, and J. E. Galan.** 1992. Identification and molecular characterization of a *Salmonella typhimurium* gene involved in triggering the internalization of salmonellae into cultured epithelial cells. Proc. Natl. Acad. Sci. U. S. A **89:**5976-5980.
52. **Gordon, M. A., H. T. Banda, M. Gondwe, S. B. Gordon, M. J. Boeree, A. L. Walsh, J. E. Corkill, C. A. Hart, C. F. Gilks, and M. E. Molyneux.** 2002. Non-typhoidal *Salmonella* bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. AIDS **16:**1633-1641.
53. **Gordon, S.** 2002. Pattern recognition receptors: doubling up for the innate immune response. Cell **111:**927-930.
54. **Gorvel, J. P. and S. Meresse.** 2001. Maturation steps of the *Salmonella*-containing vacuole. Microbes. Infect. **3:**1299-1303.
55. **Govoni, G., S. Vidal, M. Cellier, P. Lepage, D. Malo, and P. Gros.** 1995. Genomic structure, promoter sequence, and induction of expression of the mouse *Nramp1* gene in macrophages. Genomics **27:**9-19.
56. **Graham, S. M.** 2002. Salmonellosis in children in developing and developed countries and populations. Curr. Opin. Infect. Dis. **15:**507-512.
57. **Gralnick, J. and D. Downs.** 2001. Protection from superoxide damage associated with an increased level of the YggX protein in *Salmonella enterica*. Proc. Natl. Acad. Sci. U. S. A **98:**8030-8035.
58. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A **87:**6181-6185.
59. **Groisman, E. A. and H. Ochman.** 1993. Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. EMBO J. **12:**3779-3787.
60. **Guignot, J., E. Caron, C. Beuzon, C. Bucci, J. Kagan, C. Roy, and D. W. Holden.** 2004. Microtubule motors control membrane dynamics of *Salmonella*-containing vacuoles. J. Cell Sci. **117:**1033-1045.
61. **Hashim, S., K. Mukherjee, M. Raje, S. K. Basu, and A. Mukhopadhyay.** 2000. Live *Salmonella* modulate expression of Rab proteins to persist in a specialized compartment and escape transport to lysosomes. J. Biol. Chem. **275:**16281-16288.
62. **Henderson, B., S. Poole, and M. Wilson.** 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60:**316-341.

63. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden.** 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. **30:**163-174.
64. **Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky.** 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc. Natl. Acad. Sci. U. S. A **96:**2396-2401.
65. **Hersh, L. B. and M. S. Jorns.** 1975. Use of 5-deazaFAD to study hydrogen transfer in the D-amino acid oxidase reaction. J. Biol. Chem. **250:**8728-8734.
66. **Hess, J., C. Ladel, D. Miko, and S. H. Kaufmann.** 1996. *Salmonella typhimurium* aroA- infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. J. Immunol. **156:**3321-3326.
67. **Holden, D. W.** 2002. Trafficking of the *Salmonella* vacuole in macrophages. Traffic. **3:**161-169.
68. **Hormaeche, C. E.** 1979. Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. Immunology **37:**311-318.
69. **Hung, C. C., S. M. Hsieh, C. F. Hsiao, M. Y. Chen, and W. H. Sheng.** 2001. Risk of recurrent non-typhoid *Salmonella* bacteraemia after early discontinuation of ciprofloxacin as secondary prophylaxis in AIDS patients in the era of highly active antiretroviral therapy. AIDS **15:**645-647.
70. **Imlay, J. A. and S. Linn.** 1988. DNA damage and oxygen radical toxicity. Science **240:** 1302-1309.
71. **Jenkins, C. R. and D.Rowley**. 1959. Opsonins as determinants of survival in intraperitoneal infections in mice. Nature (London) **184:**474-475.
72. **Jepson, M. A. and M. A. Clark.** 2001. The role of M cells in *Salmonella* infection. Microbes. Infect. **3:**1183-1190.
73. **Jiang, X. M., B. Neal, F. Santiago, S. J. Lee, L. K. Romana, and P. R. Reeves.** 1991. Structure and sequence of the rfb (O antigen) gene cluster of *Salmonella* serovar typhimurium (strain LT2). Mol. Microbiol. **5:**695-713.
74. **Jones, B. D. and S. Falkow.** 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect. Immun. **62:**3745-3752.
75. **Jones, B. D., C. A. Lee, and S. Falkow.** 1992. Invasion by *Salmonella typhimurium* is affected by the direction of flagellar rotation. Infect. Immun. **60:**2475-2480.
76. **Jouanguy, E., S. Lamhamedi-Cherradi, F. Altare, M. C. Fondaneche, D. Tuerlinckx, S. Blanche, J. F. Emile, J. L. Gaillard, R. Schreiber, M. Levin, A. Fischer, C. Hivroz, and J. L. Casanova.** 1997. Partial interferon-gamma receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis. J. Clin. Invest **100:**2658-2664.
77. **Katscher, F.** 1997. *Salmonella* or *Smithella*? Nature **388:**320.
78. **Kauffmann, F.** 1966. The Bacteriology of Enterobacteriacaea. Munksgaard, Copenhagen, Denmark.
79. **Kehres, D. G., M. L. Zaharik, B. B. Finlay, and M. E. Maguire.** 2000. The NRAMP proteins of *Salmonella typhimurium* and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. Mol. Microbiol. **36:**1085-1100.
80. **Kelly, K. J. and D.A.Owen.** 2004. Bacterial diarrheas and dysenteries., pp. 421-424. *In* D. H. Conner and F.W.Chandler (ed.), Pathology of infectious diseases.
81. **Knodler, L. A., J. Celli, W. D. Hardt, B. A. Vallance, C. Yip, and B. B. Finlay.** 2002. *Salmonella* effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems. Mol. Microbiol. **43:**1089-1103.
82. **Kuo, C. F., T. Mashino, and I. Fridovich.** 1987. α,β-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme. J. Biol. Chem. **262:**4724-4727.
83. **Lee, A. K., C. S. Detweiler, and S. Falkow.** 2000. OmpR regulates the two-component system SsrA-ssrB in *Salmonella* pathogenicity island 2. J. Bacteriol. **182:**771-781.
84. **Lencer, W. I. and M. R. Neutra.** 2000. *Salmonella* pathogenesis: the trojan horse or the New York shuttle? Gastroenterology **118:**803-805.
85. **Li, Q. and B. J. Cherayil.** 2003. Role of Toll-like receptor 4 in macrophage activation and tolerance during *Salmonella enterica* serovar Typhimurium infection. Infect. Immun. **71:**4873-4882.
86. **Liang-Takasaki, C. J., N. Grossman, and L. Leive.** 1983. *Salmonellae* activate complement differentially via the alternative pathway depending on the structure of their lipopolysaccharide O-antigen. J. Immunol. **130:**1867-1870.
87. **Liang-Takasaki, C. J., P. H. Makela, and L. Leive.** 1982. Phagocytosis of bacteria by macrophages: changing the carbohydrate of lipopolysaccharide alters interaction with complement and macrophages. J. Immunol. **128:**1229-1235.
88. **Liang-Takasaki, C. J., H. Saxen, P. H. Makela, and L. Leive.** 1983. Complement activation by polysaccharide of lipopolysaccharide: an important virulence determinant of *salmonellae*. Infect. Immun. **41:**563-569.
89. **Licht, T. R., K. A. Krogfelt, P. S. Cohen, L. K. Poulsen, J. Urbance, and S. Molin.** 1996. Role of lipopolysaccharide in colonization of the mouse intestine by *Salmonella typhimurium* studied by in situ hybridization. Infect. Immun. **64:**3811-3817.
90. **Liese, J., S. Kloos, V. Jendrossek, T. Petropoulou, U. Wintergerst, G. Notheis, M. Gahr, and B. H. Belohradsky.** 2000. Long-term follow-up and outcome of 39 patients with chronic granulomatous disease. J. Pediatr. **137:**687-693.

91. **Lo, W. F., H. Ong, E. S. Metcalf, and M. J. Soloski.** 1999. T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to *Salmonella* infection and the involvement of MHC class Ib molecules. J. Immunol. **162:**5398-5406.
92. **Lu, S., P. B. Killoran, F. C. Fang, and L. W. Riley.** 2002. The global regulator ArcA controls resistance to reactive nitrogen and oxygen intermediates in *Salmonella enterica* serovar Enteritidis. Infect. Immun. **70:**451-461.
93. **Marlovits, T. C., T. Kubori, A. Sukhan, D. R. Thomas, J. E. Galan, and V. M. Unger.** 2004. Structural insights into the assembly of the type III secretion needle complex. Science **306:**1040-1042.
94. **Masse, E. and S. Gottesman.** 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A **99:**4620-4625.
95. **Mastroeni, P., J. A. Chabalgoity, S. J. Dunstan, D. J. Maskell, and G. Dougan.** 2001. *Salmonella*: immune responses and vaccines. Vet. J. **161:**132-164.
96. **Mastroeni, P. and N. Menager.** 2003. Development of acquired immunity to *Salmonella*. J. Med. Microbiol. **52:**453-459.
97. **Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche, and G. Dougan.** 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. J. Exp. Med. **192:**237-248.
98. **Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche.** 1992. Role of T cells, α and IFNγ in recall of immunity to oral challenge with virulent salmonellae in mice vaccinated with live attenuated aro$^-$ *Salmonella* vaccines. Microb. Pathog. **13:**477-491.
99. **Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche.** 1993. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. Infect. Immun. **61:**3981-3984.
100. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852-856.
101. **McCormick, M. L., G. R. Buettner, and B. E. Britigan.** 1998. Endogenous superoxide dismutase levels regulate iron-dependent hydroxyl radical formation in *Escherichia coli* exposed to hydrogen peroxide. J. Bacteriol. **180:**622-625.
102. **McSorley, S. J. and M. K. Jenkins.** 2000. Antibody is required for protection against virulent but not attenuated *Salmonella enterica* serovar Typhimurium. Infect. Immun. **68:**3344-3348.
103. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607-625.
104. **Meresse, S., K. E. Unsworth, A. Habermann, G. Griffiths, F. Fang, M. J. Martinez-Lorenzo, S. R. Waterman, J. P. Gorvel, and D. W. Holden.** 2001. Remodelling of the actin cytoskeleton is essential for replication of intravacuolar *Salmonella*. Cell Microbiol. **3:**567-577.
105. **Mermin, J. H., J. M. Townes, M. Gerber, N. Dolan, E. D. Mintz, and R. V. Tauxe.** 1998. Typhoid fever in the United States, 1985-1994: changing risks of international travel and increasing antimicrobial resistance. Arch. Intern. Med. **158:**633-638.
106. **Miao, E. A., C. A. Scherer, R. M. Tsolis, R. A. Kingsley, L. G. Adams, A. J. Baumler, and S. I. Miller.** 1999. *Salmonella typhimurium* leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. Mol. Microbiol. **34:** 850-864.
107. **Miller, S. I. and D.A.Pegues.** 2004. *Salmonella* species, including *Salmonella typhi*., pp. 2344-2359. *In* G. L. Mandell, J.E.Bennet, and R.Dolin (ed.), Principles and practice of infectious diseases, 3rd ed.
108. **Mills, S. D. and B. B. Finlay.** 1998. Isolation and characterization of *Salmonella typhimurium* and *Yersinia pseudotuberculosis*-containing phagosomes from infected mouse macrophages: *Y. pseudotuberculosis* traffics to terminal lysosomes where they are degraded. Eur. J. Cell Biol. **77:**35-47.
109. **Mittrucker, H. W., B. Raupach, A. Kohler, and S. H. Kaufmann.** 2000. Cutting edge: role of B lymphocytes in protective immunity against *Salmonella typhimurium* infection. J. Immunol. **164:**1648-1652.
110. **Monack, D. M., D. M. Bouley, and S. Falkow.** 2004. *Salmonella typhimurium* Persists within Macrophages in the Mesenteric Lymph Nodes of Chronically Infected Nramp1$^{+/+}$ Mice and Can Be Reactivated by IFNγ Neutralization. J. Exp. Med. **199:**231-241.
111. **Monack, D. M., D. Hersh, N. Ghori, D. Bouley, A. Zychlinsky, and S. Falkow.** 2000. *Salmonella* exploits caspase-1 to colonize Peyer's patches in a murine typhoid model. J. Exp. Med. **192:**249-258.
112. **Mukherjee, K., S. Parashuraman, M. Raje, and A. Mukhopadhyay.** 2001. SopE acts as an Rab5-specific nucleotide exchange factor and recruits non-prenylated Rab5 on *Salmonella*-containing phagosomes to promote fusion with early endosomes. J. Biol. Chem. **276:**23607-23615.
113. **Nauciel, C.** 1990. Role of CD4+ T cells and T-independent mechanisms in acquired resistance to *Salmonella typhimurium* infection. J. Immunol. **145:**1265-1269.
114. **Netea, M. G., B. J. Kullberg, L. A. Joosten, T. Sprong, I. Verschueren, O. C. Boerman, F. Amiot, W. B. van den Berg, and J. W. Van der Meer.** 2001. Lethal *Escherichia coli* and *Salmonella typhimurium* endotoxemia is mediated through different pathways. Eur. J. Immunol. **31:**2529-2538.
115. **Neutra, M. R.** 1998. Current concepts in mucosal immunity. V Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system. Am. J. Physiol **274:**G785-G791.
116. **Nevola, J. J., B. A. Stocker, D. C. Laux, and P. S. Cohen.** 1985. Colonization of the mouse intestine by an avirulent *Salmonella typhimurium* strain and its lipopolysaccharide-defective mutants. Infect. Immun. **50:**152-159.

117. **Newport, M. J., C. M. Huxley, S. Huston, C. M. Hawrylowicz, B. A. Oostra, R. Williamson, and M. Levin.** 1996. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N. Engl. J. Med. **335:**1941-1949.
118. **O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. MacDermott, and S. B. Formal.** 1980. Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. J. Immunol. **124:**20-24.
119. **O'Brien, A. D., D. A. Weinstein, M. Y. Soliman, and D. L. Rosenstreich.** 1985. Additional evidence that the Lps gene locus regulates natural resistance to *S. typhimurium* in mice. J. Immunol. **134:**2820-2823.
120. **Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman.** 1996. Identification of a pathogenicity island required for Salmonella **survival in host cells. Proc. Natl. Acad. Sci. U. S. A 93:**7800-7804.
121. **Ochs, R. Dinner with Typhoid Mary. Http://newsday.com/extras/lihistory/7/hs702a.htm**
122. **Oliver, D. M.** 2004. Periplasm, pp. 88-103. *In* F. C. Neidhardt, R.Curtiss III, J.L.Ingraham, E.C.C.Lin, K.Brooks Low, B.Magasanik, W.S.Reznikoff, M.Riley, M.Schaechter, and H.E.Umbarger (ed.), *Escherichia coli and Salmonella* cellular and molecular biology, second ed. ASm Press, Washington D.C.
123. **Ouellette, A. J.** 2004. Defensin-mediated innate immunity in the small intestine. Best. Pract. Res. Clin. Gastroenterol. **18:**405-419.
124. **Pang, T., Z. A. Bhutta, B. B. Finlay, and M. Altwegg.** 1995. Typhoid fever and other salmonellosis: a continuing challenge. Trends Microbiol. **3:**253-255.
125. **Penner, J. L.** 1988. International Committee on Systematic Bacteriology Taxonomic Subcommittee on Enterobacteriacaea. Int. J. Syst. Bacteriol. **38:**223-224.
126. **Pfeifer, C. G., S. L. Marcus, O. Steele-Mortimer, L. A. Knodler, and B. B. Finlay.** 1999. *Salmonella typhimurium* virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. Infect. Immun. **67:**5690-5698.
127. **Plant, J. and A. A. Glynn.** 1974. Natural resistance to *Salmonella* infection, delayed hypersensitivity and Ir genes in different strains of mice. Nature **248:**345-347.
128. **Plant, J. E., J. M. Blackwell, A. D. O'Brien, D. J. Bradley, and A. A. Glynn.** 1982. Are the Lsh and Ity disease resistance genes at one locus on mouse chromosome 1? Nature **297:**510-511.
129. **Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler.** 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science **282:**2085-2088.
130. **Pomposiello, P. J., M. H. Bennik, and B. Demple.** 2001. Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. J. Bacteriol. **183:**3890-3902.
131. **Pomposiello, P. J. and B. Demple.** 2000. Identification of SoxS-regulated genes in *Salmonella enterica* serovar typhimurium. J. Bacteriol. **182:**23-29.
132. **Popoff, M. Y., J. Bockemuhl, and F. W. Brenner.** 2000. Supplement 1998 (no. 42) to the Kauffmann-White scheme. Res. Microbiol. **151:**63-65.
133. **Purcell, B. K., J. Pruckler, and S. Clegg.** 1987. Nucleotide sequences of the genes encoding type 1 fimbrial subunits of *Klebsiella pneumoniae* and *Salmonella typhimurium*. J. Bacteriol. **169:**5831-5834.
134. **Raetz, C. R. H.** 2004. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles, pp. 1035-1063. *In* F. C. Neidhardt, R.Curtiss III, J.L.Ingraham, E.C.C.Lin, K.Brooks Low, B.Magasanik, W.S.Reznikoff, M.Riley, M.Schaechter, and H.E.Umbarger (ed.), *Escherichia coli* and *Salmonella* cellular and molecular biology, second ed. ASm Press, Washington D.C.
135. **Rajagopalan, P., R. Kumar, and A. N. Malaviya.** 1981. Immunological studies in typhoid fever. I. Immunoglobulins, C3, antibodies, rheumatoid factor and circulating immune complexes in patients with typhoid fever. Clin. Exp. Immunol. **44:**68-73.
136. **Reeves, M. W., G. M. Evins, A. A. Heiba, B. D. Plikaytis, and J. J. Farmer, III.** 1989. Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. nov. J. Clin. Microbiol. **27:**313-320.
137. **Richter-Dahlfors, A., A. M. Buchan, and B. B. Finlay.** 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. J. Exp. Med. **186:**569-580.
138. **Roos, D.** 1994. The genetic basis of chronic granulomatous disease. Immunol. Rev. **138:**121-157.
139. **Roos, D., B. M. de, F. Kuribayashi, C. Meischl, R. S. Weening, A. W. Segal, A. Ahlin, K. Nemet, J. P. Hossle, E. Bernatowska-Matuszkiewicz, and H. Middleton-Price.** 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood **87:**1663-1681.
140. **Ruiz-Albert, J., X. J. Yu, C. R. Beuzon, A. N. Blakey, E. E. Galyov, and D. W. Holden.** 2002. Complementary activities of SseJ and SifA regulate dynamics of the *Salmonella typhimurium* vacuolar membrane. Mol. Microbiol. **44:**645-661.
141. **Salmon, D. E. and T. Smith.** 1886. The bacterium of swine-plague. American Monthly Microscopical Journal **7:**204-205.
142. **Saxen, H., I. Reima, and P. H. Makela.** 1987. Alternative complement pathway activation by *Salmonella* O polysaccharide as a virulence determinant in the mouse. Microb. Pathog. **2:**15-28.
143. **Scherer, C. A. and S.I.Miller.** 2004. Molecular pathogenesis of *Salmonellae*, pp. 266-316. *In* Principles of bacterial pathogenesis.
144. **Schnaitman, C. A. and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**655-682.

145. **Seymour, R. L., P. V. Mishra, M. A. Khan, and M. P. Spector.** 1996. Essential roles of core starvation-stress response loci in carbon-starvation-inducible cross-resistance and hydrogen peroxide-inducible adaptive resistance to oxidative challenge in *Salmonella typhimurium*. Mol. Microbiol. **20:**497-505.
146. **Shaio, M. F. and H. Rowland.** 1985. Bactericidal and opsonizing effects of normal serum on mutant strains of *Salmonella typhimurium*. Infect. Immun. **49:**647-653.
147. **Shiloh, M. U., J. D. MacMicking, S. Nicholson, J. E. Brause, S. Potter, M. Marino, F. Fang, M. Dinauer, and C. Nathan.** 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity. **10:**29-38.
148. **Shiloh, M. U., J. Ruan, and C. Nathan.** 1997. Evaluation of bacterial survival and phagocyte function with a fluorescence-based microplate assay. Infect. Immun. **65:** 3193-3198.
149. **Sinha, K., P. Mastroeni, J. Harrison, R. D. de Hormaeche, and C. E. Hormaeche.** 1997. *Salmonella typhimurium aroA*, *htrA*, and *aroD htrA* mutants cause progressive infections in athymic (nu/nu) BALB/c mice. Infect. Immun. **65:**1566-1569.
150. **Skamene, E., P. Gros, A. Forget, P. A. Kongshavn, C. St Charles, and B. A. Taylor.** 1982. Genetic regulation of resistance to intracellular pathogens. Nature **297:**506-509.
151. **Smith, R. M. and J. T. Curnutte.** 1991. Molecular basis of chronic granulomatous disease. Blood **77:**673-686.
152. **Staretz-Haham, O., R. Melamed, M. Lifshitz, N. Porat, C. Fieschi, J. L. Casanova, and J. Levy.** 2003. Interleukin-12 receptor beta1 deficiency presenting as recurrent *Salmonella* infection. Clin. Infect. Dis. **37:**137-140.
153. **Stein, M. A., K. Y. Leung, M. Zwick, F. Garcia-Del Portillo, and B. B. Finlay.** 1996. Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. Mol. Microbiol. **20:**151-164.
154. **Stemmer, F. and M. Loos.** 1985. Evidence for direct binding of the first component of complement, C1, to outer membrane proteins from *Salmonella minnesota*. Curr. Top. Microbiol. Immunol. **121:73-84.:**73-84.
155. **Stock, J. B., B. Rauch, and S. Roseman.** 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **252:**7850-7861.
156. **Storz, G. and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188-194.
157. **Suffredini, A. F., G. Fantuzzi, R. Badolato, J. J. Oppenheim, and N. P. O'Grady.** 1999. New insights into the biology of the acute phase response. J. Clin. Immunol. **19:**203-214.
158. **Sundquist, M., A. Rydstrom, and M. J. Wick.** 2004. Immunity to *Salmonella* from a dendritic point of view. Cell Microbiol. **6:**1-11.
159. **Suvarnapunya, A. E., H. A. Lagasse, and M. A. Stein.** 2003. The role of DNA base excision repair in the pathogenesis of *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. **48:**549-559.
160. **Swanson, R. N. and A. D. O'Brien.** 1983. Genetic control of the innate resistance of mice to *Salmonella typhimurium*: Ity gene is expressed in vivo by 24 hours after infection. J. Immunol. **131:**3014-3020.
161. **Swenson, D. L. and S. Clegg.** 1992. Identification of ancillary fim genes affecting fimA expression in *Salmonella typhimurium*. J. Bacteriol. **174:**7697-7704.
162. **Thrasher, A. J., N. H. Keep, F. Wientjes, and A. W. Segal.** 1994. Chronic granulomatous disease. Biochim. Biophys. Acta **1227:**1-24.
163. **Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman.** 1999. A *Salmonella* virulence protein that inhibits cellular trafficking. EMBO J. **18:**3924-3933.
164. **Van Dissel, J. T., S. M. Arend, and T. H. Ottenhoff.** 2001. Infections with non-tuberculous mycobacteria and salmonellae in patients with genetic defects in the interleukin-12/interferon-gamma-mediated pathway of macrophage activation. Neth. J. Med. **59:**90-94.
165. **Vazquez-Torres, A., J. Jones-Carson, A. J. Baumler, S. Falkow, R. Valdivia, W. Brown, M. Le, R. Berggren, W. T. Parks, and F. C. Fang.** 1999. Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. Nature **401:**804-808.
166. **Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos, and F. C. Fang.** 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. J. Exp. Med. **192:**227-236.
167. **Vazquez-Torres, A., B. A. Vallance, M. A. Bergman, B. B. Finlay, B. T. Cookson, J. Jones-Carson, and F. C. Fang.** 2004. Toll-like receptor 4 dependence of innate and adaptive immunity to *Salmonella*: importance of the Kupffer cell network. J. Immunol. **172:**6202-6208.
168. **Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni, and F. C. Fang.** 2000. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science **287:**1655-1658.
169. **Vidal, S., M. L. Tremblay, G. Govoni, S. Gauthier, G. Sebastiani, D. Malo, E. Skamene, M. Olivier, S. Jothy, and P. Gros.** 1995. The *Ity*/*Lsh*/*Bcg* locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. J. Exp. Med. **182:**655-666.
170. **Vidal, S. M., D. Malo, K. Vogan, E. Skamene, and P. Gros.** 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. Cell **73:**469-485.
171. **Vidal, S. M., E. Pinner, P. Lepage, S. Gauthier, and P. Gros.** 1996. Natural resistance to intracellular infections: *Nramp1* encodes a membrane phosphoglycoprotein absent in macrophages from susceptible ($Nramp1^{D169}$) mouse strains. J. Immunol. **157:**3559-3568.
172. **Warren, J., P. Mastroeni, G. Dougan, M. Noursadeghi, J. Cohen, M. J. Walport, and M. Botto.** 2002. Increased susceptibility of C1q-deficient mice to *Salmonella enterica* serovar Typhimurium infection. Infect. Immun. **70:**551-557.

173. **Waterman, S. R. and D. W. Holden.** 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cell Microbiol. **5:**501-511.
174. **Wick, M. J.** 2004. Living in the danger zone: innate immunity to *Salmonella*. Curr. Opin. Microbiol. **7:**51-57.
175. **Yamamoto, Y., T. W. Klein, and H. Friedman.** 1996. Induction of cytokine granulocyte-macrophage colony-stimulating factor and chemokine macrophage inflammatory protein 2 mRNAs in macrophages by Legionella pneumophila or *Salmonella typhimurium* attachment requires different ligand-receptor systems. Infect. Immun. **64:**3062-3068.
176. **Yethon, J. A., J. S. Gunn, R. K. Ernst, S. I. Miller, L. Laroche, D. Malo, and C. Whitfield.** 2000. *Salmonella enterica* serovar typhimurium waaP mutants show increased susceptibility to polymyxin and loss of virulence In vivo. Infect. Immun. **68:**4485-4491.
177. **Zheng, M., B. Doan, T. D. Schneider, and G. Storz.** 1999. OxyR and SoxRS regulation of *fur*. J. Bacteriol. **181:**4639-4643.
178. **Zheng, M., X. Wang, L. J. Templeton, D. R. Smulski, R. A. LaRossa, and G. Storz.** 2001. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. J. Bacteriol. **183:**4562-4570.
179. **Zhou, D. and J. Galan.** 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes. Infect. **3:**1293-1298.