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

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chapter ten

Summarizing discussion

Partly adapted from:

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

Summarizing discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Menadione

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

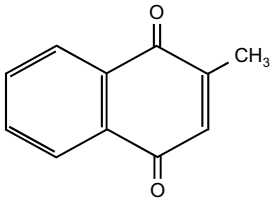

Figure 1. Chemical structure of menadione (2-methyl-1,4-naphtoquinone).

Quinones

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

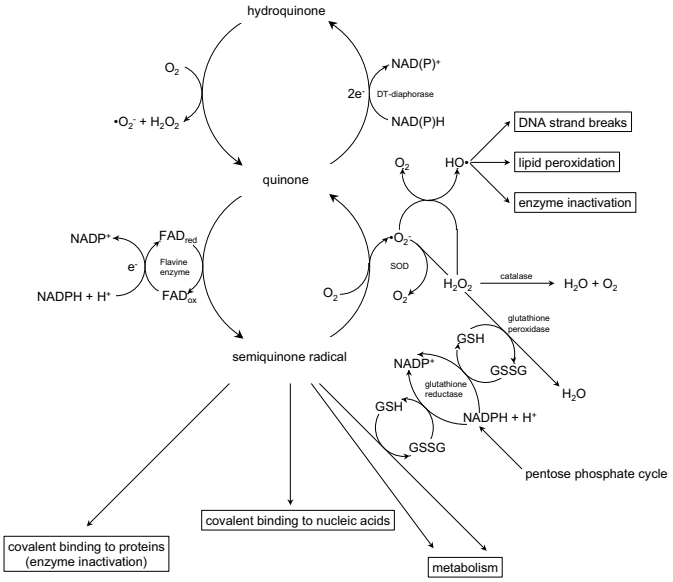

Figure 2. schematic overview of the redox cycle of quinones, the damage that can be induced by the radicals that are formed, and defense mechanisms of the host (reviewed in (19)).

Menadione-susceptible mutants

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

Salmonella genes involved in oxidative stress

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

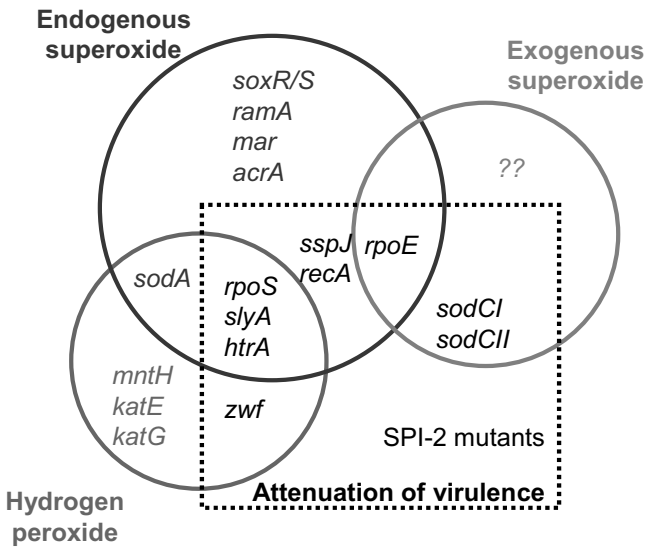

Figure 3. Classification of *Salmonella* mutants by in vitro and in vivo phenotype. Mutants that are sensitive to exogenous superoxide generated by the host NADPH-oxidase, endogenously produced superoxide and hydrogen peroxide are shown, and the consequences for virulence in mice are indicated. *soxR/S*,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

Mutants sensitive to endogenously produced superoxide

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

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

Table 1. Phenotype of *Salmonella* null mutants

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

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

Mutants sensitive to endogenous and exogenous superoxide

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

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

Mutants sensitive to exogenous, NADPH-oxidase-generated superoxide

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

Mutants disturbed in prevention of NADPH-oxidase-generated superoxide

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

Concluding remarks

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

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