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

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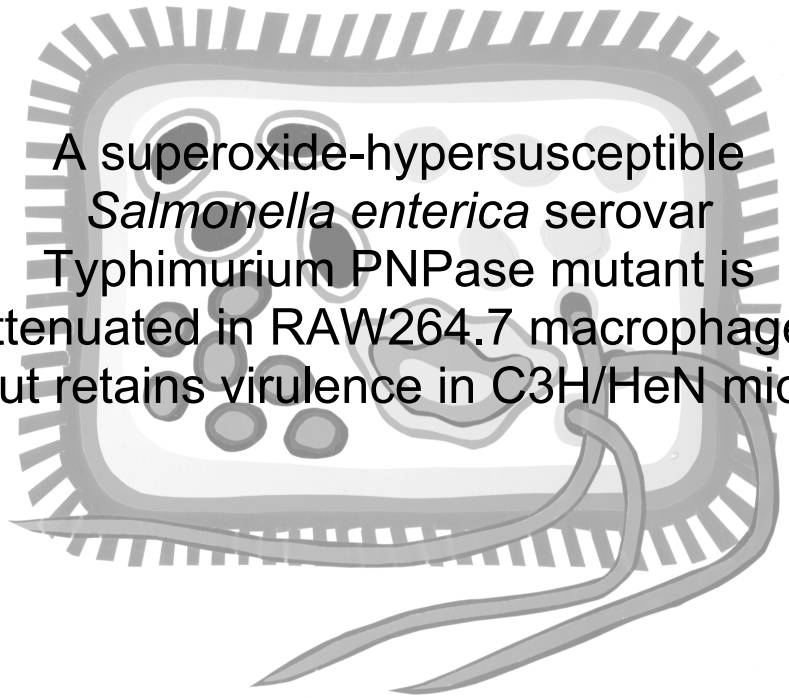
chapter five



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

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Abstract

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

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

Introduction

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

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

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

Materials and Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

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

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

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

[a] nd, not determined

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

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

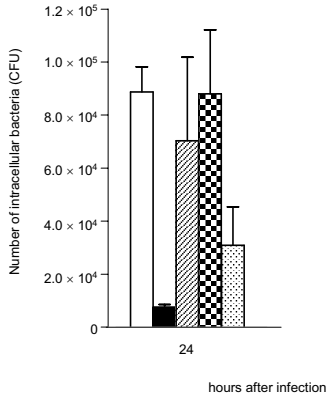

Figure 1. Number of intracellular bacteria in RAW264.7 murine macrophage-like cells infected with wild-type *S. enterica* serovar Typhimurium 14028s (white bar) and the superoxide hypersusceptible mutants AVD101 (black bar), AVD102 (dashed bar), AVD103 (checkered bar), and AVD104 (dotted bar). At 24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

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

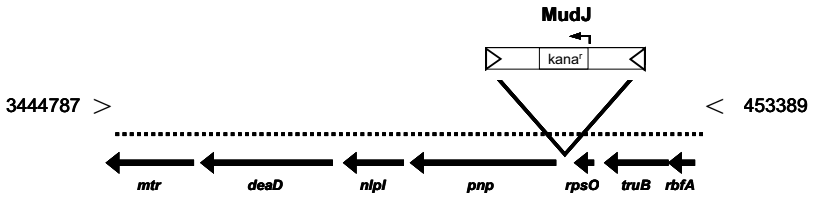

Figure 2. Position of the MudJ transposon in the genome of *S. enterica* serovar Typhimurium. The MudJ transposon had inversely inserted in the promotor region of *pnp*, the gene encoding PNPase.

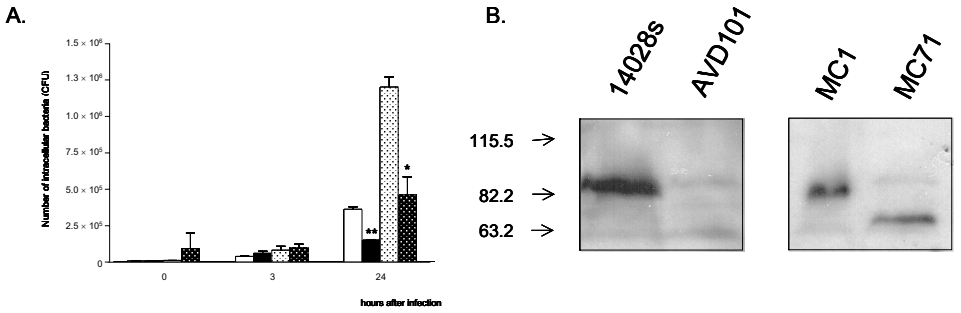

Figure 3. Intracellular *S. enterica* serovar Typhimurium in RAW264.7 mouse macrophage-like cells at 0, 3, and 24 h after infection (A) and expression of PNPase in a total extract (B) of *S. enterica* serovar Typhimurium 14028s (white bars, wild-type), AVD101 (black bars, 14028s *pnp*::MudJ), MC1 (white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

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

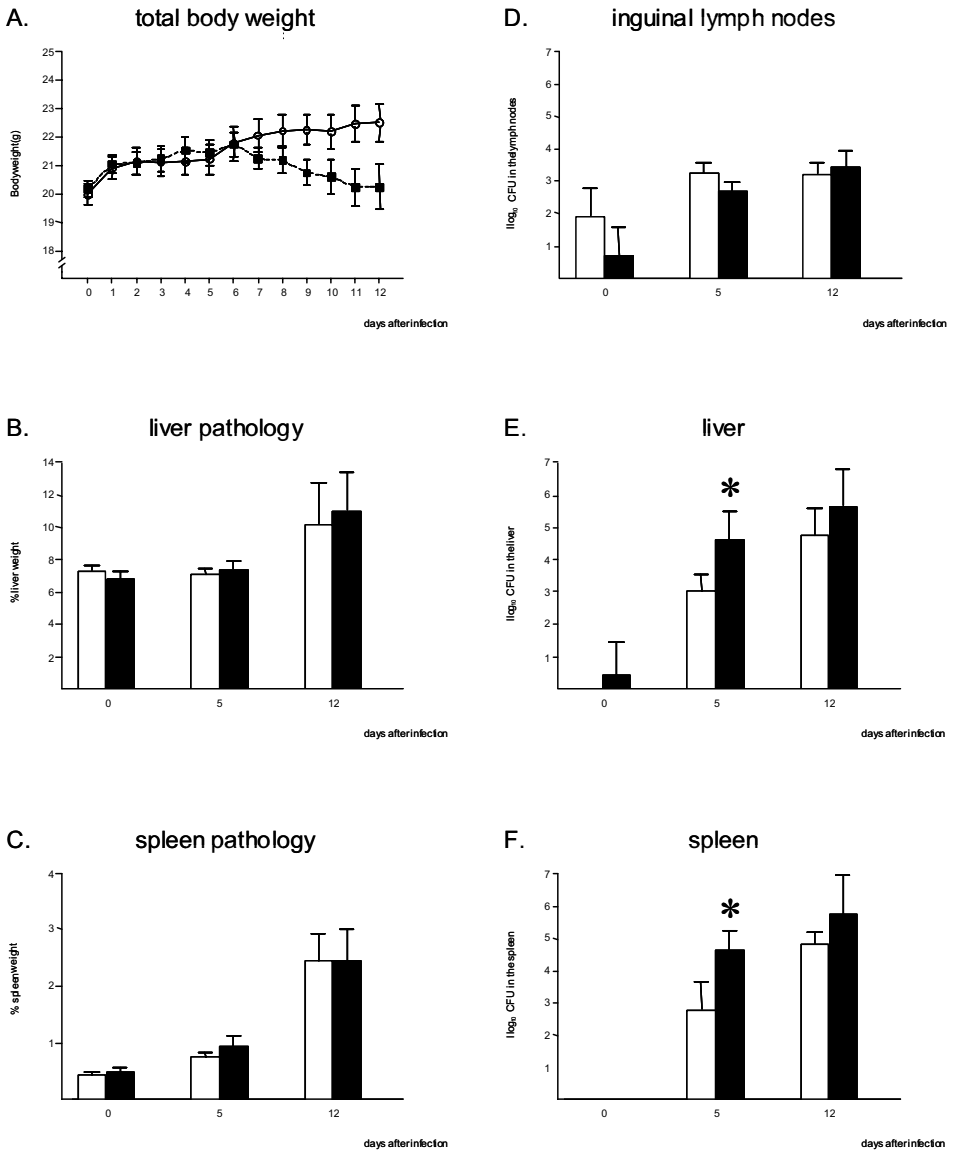

Figure 4. Total body weight (A), induced pathology in the spleen (B) and liver (C), number of bacteria in the inguinal lymph nodes (D), liver (E), and spleen (F), and intracellular number of bacteria in the spleen (G). Mice were injected subcutaneously in the flanks with 3×10^4 CFU of 14028s (white dots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

Discussion

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

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

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

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

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

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