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

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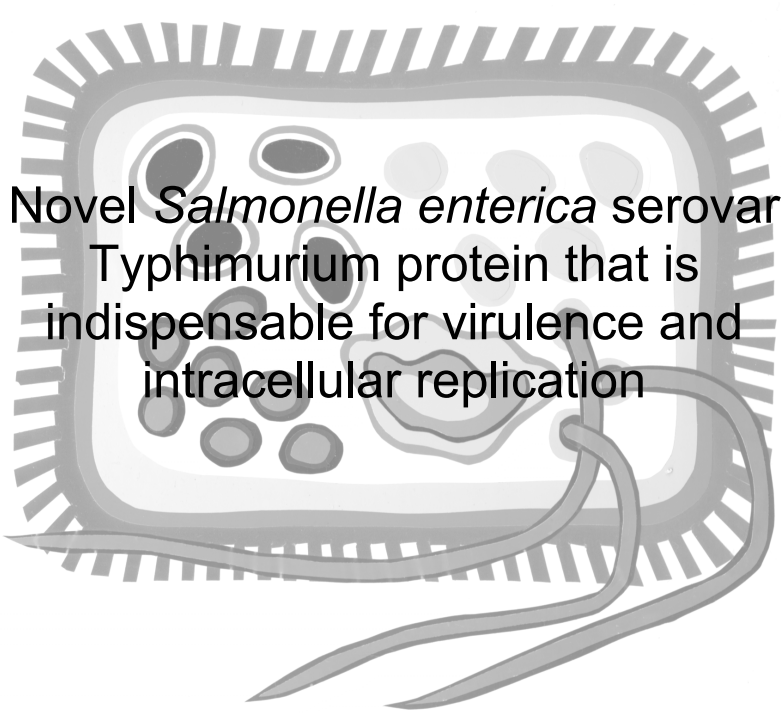
chapter six



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

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Abstract

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

Introduction

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

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

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

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

Materials and Methods

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

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

Table 1. Salmonella strains and plasmids used in this study

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

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

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

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

[a] nd, not determined

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

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

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

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

[a] nd, not done

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

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

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

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

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

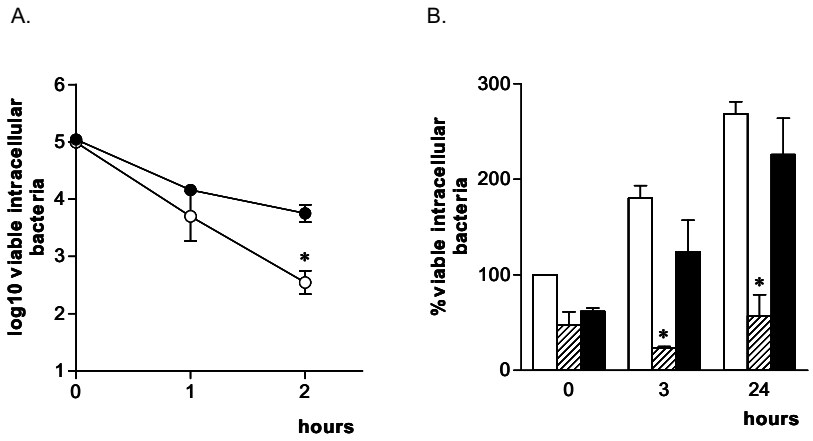

Figure 1. In vitro intracellular killing of DLG294 *(ssp*::MudJ; open circles) and wild-type *S. enterica* serovar Typhimurium 14028s (solid circles) by J774 macrophage-like cells (A). After uptake of the bacteria and removal of remaining extracellular microorganisms, at various time points the number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

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

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

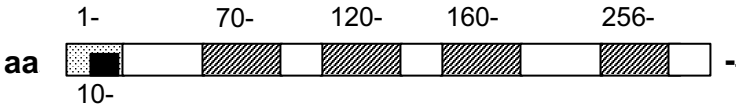

Figure 2. Schematic drawing of homologous domains within SspJ protein, 392 aminoacids. Depicted are a leader sequence from amino acids 1 to 22, lipid membrane attachment site from amino acids 10 to 21, and four PQQ domains from amino acids 70 to 107, 120 to 157, 160 to 197 and 256 to 293.

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

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

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

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

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

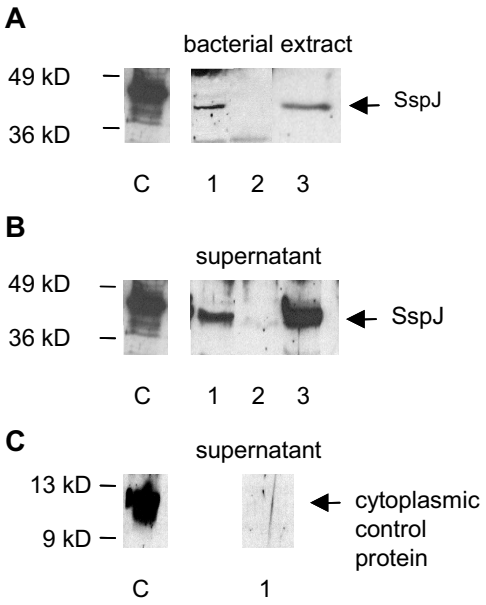

Figure 3. Expression of SspJ by *Salmonella* strains. Panel A shows expression of SspJ in a total extract of *S. enterica* serovar Typhimurium (wild type) and in DLG294(*sspJ*::MudJ) complemented by plasmid pBluescript carrying *sspJ* (DLG294-pTS125)) (lanes 1 and 3, respectively), but not in DLG294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

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

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

Discussion

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

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

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

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

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

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

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

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