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Salmonella enterica Serovar Typhimurium RamA, Intracellular Oxidative Stress Response, and Bacterial Virulence

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***Escherichia coli* and *Salmonella enterica* serovar Typhimurium have evolved genetic systems, such as the *soxR*/*S* and *marA* regulons, to detoxify reactive oxygen species, like superoxide, which are formed as by-products of metabolism. Superoxide also serves as a microbicidal effector mechanism of the host's phagocytes. Here, we investigate whether regulatory genes other than *soxR*/*S* and *marA* are active in response to oxidative stress in *Salmonella* and may function as virulence determinants. We identified a bacterial gene, which was designated *ramA* (342 bp) and mapped at 13.1 min on the *Salmonella* chromosome, that, when overexpressed on a plasmid in *E. coli* or *Salmonella*, confers a pleiotropic phenotype characterized by increased resistance to the redox-cycling agent menadione and to multiple unrelated antibiotics. The *ramA* gene is present in *Salmonella* serovars but is absent in *E. coli*. The gene product displays 37 to 52% homology to the transcriptional activators *soxR*/*S* and *marA* and 80 to 100% identity to a multidrug resistance gene in *Klebsiella pneumoniae* and *Salmonella enterica* serovar Paratyphi A. Although a *ramA soxR*/*S* double null mutant is highly susceptible to intracellular superoxide generated by menadione and displays decreased Mn-superoxide dismutase activity, intracellular survival of this mutant within macrophage-like RAW 264.7 cells and in vivo replication in the spleens in Ity^r mice are not affected. We concluded that despite its role in the protective response of the bacteria to oxidative stress in vitro, the newly identified *ramA* gene, together with *soxR*/*S*, does not play a role in initial replication of *Salmonella* in the organs of mice.**

Oxidative stress occurs when organisms encounter elevated levels of reactive oxygen species, such as superoxide anion, hydrogen peroxide, and hydroxyl radical. Reactive oxygen intermediates are produced at low rates during aerobic respiration in most cells, including prokaryotes. To cope with oxidative stress, bacteria have evolved protective responses that enable them to counter the damage and survive. These responses encompass active enzymatic detoxification of reactive oxygen intermediates, as well as repair of oxidative damage to bacterial DNA (20). Thus, superoxide dismutase (SOD) (encoded by *sodA*/*B*) converts the highly reactive superoxide to hydrogen peroxide, which in turn is converted by catalase (encoded by *katG*/*F*) into water and oxygen (8). When bacteria interact with a eukaryotic host, large quantities of reactive oxygen intermediates are produced by phagocytes during uptake of microorganisms, and this is a major microbicidal effector mechanism against pathogenic bacteria. Thus, the following question arises: Does the bacterial protective response to oxidative stress play a significant role in the intracellular fate of the microorganisms upon engulfment by phagocytes?

Bacterial genes that are induced upon exposure to oxidative stress have been studied extensively in *Escherichia coli*. These genes are organized in several stress regulons (8), and the *soxR*/*S* regulon appears to play a central role (1). This regulon includes at least a dozen genes involved in the protective response to oxidative stress, and all of the genes are transcriptionally induced or repressed by the SoxS protein. The synthesis of SoxS is controlled by the redox-sensing molecule SoxR, a homodimeric protein composed of two 17-kDa subunits, each of which contains a [2Fe-2S] cluster. In the absence of oxidative stress, the clusters are in a reduced state, and SoxR is inactive (11). When superoxide and/or nitric oxide radicals are encountered, the clusters are oxidized, and a conformational change triggers the transcription of SoxS. This protein interacts with the σ^{70} subunit of RNA polymerase to promote transcription of a variety of genes (7).

Given the homology between the *soxR*/*S* genes of *E. coli* and *Salmonella* in terms of both structure and function, it has been suggested that the *soxR*/*S* regulon plays an important role in *Salmonell*a virulence by mediating bacterial resistance to superoxide and nitrogen intermediates produced by phagocytes (7). Indeed, Fang et al. showed that a *soxS* null mutant of *Salmonella enterica* serovar Typhimurium was highly susceptible to paraquat (a redox-cycling, superoxide-generating agent) in vitro, but the mutation had no significant effect on the survival of bacteria in either the monocytic cell line J774.1 or peritoneal macrophages. Furthermore, upon intraperitoneal injection of the *soxS* null mutant, the survival of mice was identical to that observed after injection of the parental wild-type bacteria (7). These results suggested that the *soxR*/*S* regulon is not likely to be the only regulon involved in the protective response of *Salmonella* to macrophage-derived oxidative stress.

In the present study we identified another genetic determinant which mediates resistance to superoxide in *S. enterica* serovar Typhimurium. The gene product, designated RamA,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Origin or reference[a]
Strains		
14028s	Wild-type *S. enterica* serovar Typhimurium	ATCC
TS1	*ramA* knockout	This study
TS2	*soxR*/*S* knockout	This study
TS3	*ramA soxR*/*S* knockout	This study
EM1	*soxR*/*S* knockout	19
MC1061	*E. coli*	ATCC
DH5α	*E. coli*	ATCC
XII-blue	*E. coli*	ATCC
Plasmids		
pHisTrc-A	IPTG-inducible expression vector	Invitrogen
pTS008	pHisTrc-A containing *ramA*	This study
pGP704	Suicide vector	
pTS318	pGP704 containing disrupted *ramA*	This study
pWSK29	Low-copy-number plasmid	29
pTS206	pWSK29 containing *ramA*	This study
pBluescript SK(−)		Stratagene
pTS018	pBluescript containing 1,919 bp of the *Salmonella* genome	This study

[a] ATCC, American Type Culture Collection.

appears to be species restricted and belongs to the AraC transcriptional activator family. Our analysis of an *S. enterica* serovar Typhimurium *ramA* mutant showed that RamA is intimately involved in bacterial resistance to superoxide and hence may add to the resistance controlled by SoxR/S. We investigated the role of this gene as a virulence determinant for intracellular replication of *S. enterica* serovar Typhimurium in murine macrophages and in the spleens of intraperitoneally infected mice.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The bacterial strains and plasmids used are listed in Table 1. Microorganisms were grown in Luria-Bertani liquid culture medium at 37°C for 18 h with rigorous shaking. For plates, agar was added to a final concentration of 1.5%, and if required, the medium was supplemented with kanamycin (50 μg/ml) or ampicillin (50 μg/ml). For disk diffusion assays, M9 minimal medium plates with a standardized volume were used.

Mice. Female Swiss mice that were 10 to 12 weeks old were purchased from Charles River, Maastricht, The Netherlands.

Disk diffusion assay. To measure bacterial resistance to the redox-cycling agent menadione or to antibiotics, a disk diffusion assay was performed as described by Bauer et al. (2). Briefly, overnight or end-log-phase *Salmonella* cultures in Luria-Bertani medium were diluted 1:10 in phosphate-buffered saline (PBS) and spread on standardized M9 medium plates. A cotton disk (diameter, 6 mm; Schleicher & Schuell) containing the redox-cycling agent menadione (10 μl of a 3 M solution/disk; Sigma, St. Louis, Mo.), chloramphenicol (30 μg/disk), norfloxacin (30 μg/disk), gentamicin (100 μg/disk), or H_2O_2 (10 μl of a 3% solution/disk) was placed in the center of each plate. After overnight incubation at 37°C the diameter (in millimeters) of the bacterium-free zone was determined as a measure of resistance.

Construction of an *S. enterica* serovar Typhimurium DNA library and selection of superoxide-resistant clones. A random chemically mutagenized *S. enterica* serovar Typhimurium mutant that was resistant to menadione in vitro was used to select for genes involved in resistance to superoxide (26). The in vivo cloning technique developed by Groisman and Casadaban (13) was used. From menadione-resistant strains obtained in this way, plasmid DNA (cloning vector Mud5005; Cold Spring Harbor Laboratory collection) was isolated, and the DNA fragment contained about 23,000 bp of the *Salmonella* genome. Next, the plasmid was digested incompletely with *Sau*3A (Gibco BRL), and fragments were ligated into the *Bam*HI (Gibco BRL) site of pBluescript (Amp^r; Stratagene, La Jolla, Calif.). *E. coli* strain DH5α was used as a recipient of this library and was grown on plates containing ampicillin and a low concentration of menadione (0.5 mg/ml). Experiments typically resulted in about 300 colonies per plate, which were assayed for resistance to menadione, paraquat, and various antibiotics (e.g., norfloxacin and chloramphenicol). One of the resistant colonies was used for further analysis; this organism carried pBluescript with a 1,919-bp insert.

DNA manipulations. Standard DNA manipulations were performed as described by Sambrook et al. (21). Restriction enzymes and other modifying enzymes were purchased from Gibco BRL or Promega. The *ramA* reading frame was identified by sequencing a 1,919-bp *Sau*3A chromosomal DNA fragment ligated into pBluescript (Stratagene) by using an Amersham T7 sequencing kit. The reading frame was isolated from wild-type *Salmonella* by PCR by using primers 5′-ATTTGGATCCATGACCATTTCCGCTCAGG-3′ and 5′-TTTTAA GCTTCAATGCGTACGGCCATGC-3′ and was ligated into the low-copy-number plasmid pWSK29 (29).

Mapping the chromosomal location of *ramA*. Several methods were used to map the chromosomal location of the gene of interest, *ramA*. By using an *S. enterica* serovar Typhimurium Mud-P22 phage library developed by Youderian et al. (33) and Benson and Goldman (3), a multiple-dot blot hybridization assay was performed by isolating DNA from the Mud-P22 lysates, which covered the complete genome of *S. enterica* serovar Typhimurium. Hybridization of a ^{32}P-labeled *ramA* PCR product to the dot blot and exposure to X-ray film revealed the location of *ramA* with an accuracy of about 2 to 3 min on the *Salmonella* chromosome. Next, following placement of MudJ close to the *ramA* gene in a constitutive mutant, the genetic distances to known genes were determined in more detail by measuring cotransduction frequencies for P22 generalized transduction by using strains TA4190 (*ahpC*::Tn*10*), TN966 (*apeE2*::Tn*10*), TN1744 (*zbc-873*::Tn*10*), and TE3682 (*zbb*::Tn*10dCam*). All *Salmonella* LT2 mapping strains were provided by K. E. Sanderson, *Salmonella* Genetic Stock Center, Calgary, Alberta, Canada.

The presence of *ramA* in a set of gram-negative bacteria was determined by hybridization of the labeled *ramA* DNA fragment on a zoo blot containing *Eco*RI-digested chromosomal DNA fragments of 26 gram-negative bacteria (provided by J. G. Kusters, Rotterdam, The Netherlands).

Construction of a *ramA* knockout. A 2,486-bp DNA fragment spanning the *ramA* gene was isolated from the *Salmonella* wild-type chromosome by PCR by using primers 5′-AGCGCAAAGCTTTTGCCC-3′ and 5′-TCTGCCGCG ATATTGAAC-3′. This PCR product was ligated into pBluescript SK− (Stratagene) in which the *Pst*I restriction site was disrupted so that a 656-bp *Pst*I fragment containing 230 nucleotides of *ramA* and 570 nucleotides downstream of the *ramA* stop site could be replaced by a 1,500-bp kanamycin cassette. The 3,330-bp fragment was subsequently ligated into the suicide vector pGP704, yielding pTS318. Transformation of *Salmonella* by pTS318 and selection for kanamycin resistance and ampicillin sensitivity resulted in an inactive *ramA* gene (TS1). A *soxR*/*S ramA* double mutant (TS3) was obtained by P22 generalized transduction of a P22 lysate of *S. enterica* serovar Typhimurium (*ramA*::Km) into a *soxR*/*S* knockout strain (EM1, kindly provided by P. J. Pomposiello) (18). A *soxR*/*S* mutant with the parental wild-type background (TS2) was obtained by generalized transduction of 14028s by using a P22 lysate of EM1. All transduced strains were cleared of phage P22 by repeated isolation on EBU plates.

SOD activity of bacterial lysates. Lysates of end-log-phase *Salmonella* cultures (optical density at 600 nm, 0.8), grown in the presence or absence of 1 mM menadione for 1 h, were prepared by 10 cycles of freezing and thawing. Equal amounts of total proteins (15 μg) were separated on a native 11% polyacrylamide gel. The gel was rinsed with water and incubated in the presence of 1 mg of nitroblue tetrazolium (Roche Diagnostics GmbH) per ml for 20 min. After the gel was washed with water, it was incubated for 20 min in a solution consisting of 28 μM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 32 μM riboflavin, and 43 mM K_3PO_4.

In vivo replication of *S. enterica* serovar Typhimurium in spleens. Swiss mice were injected intraperitoneally with 10^2, 10^3, or 10^4 viable *Salmonella* cells in 500 μl of PBS. Briefly, overnight cultures of bacteria were diluted 1:10 in Luria-Bertani medium and grown to the mid-log phase. The bacteria were washed three times in PBS, and appropriate suspensions for intraperitoneal injection were prepared. The number of viable bacteria was confirmed by plating. On days 1, 2, and 4 after injection, mice were sacrificed, and the spleens were removed to determine the number of surviving bacteria. Single-cell suspensions were prepared by using sterile 70-μm cell strainers (Falcon); bacteria were recovered by lysis in water, and numbers of CFU were determined.

Nucleotide sequence accession number. The 342-bp open reading frame that was designated *ramA* has been deposited in the National Center for Biotechnology Information database under accession number AF288225.

TABLE 2. Susceptibility of *E. coli* MC1061 and *Salmonella* to redox-cycling agents and various antibiotics

Strain	Diam of inhibition zone (mm) (mean ± SD)[a]						
	Menadione	Paraquat	Sin-1	Chloramphenicol	Gentamicin	Nalidixic acid	H_2O_2
MC1061(pWSK29)	22 ± 1	ND[c]	ND	ND	ND	ND	ND
MC1061(TS206)	16 ± 1[b]	ND	ND	ND	ND	ND	ND
MC1061(pBluescript)	17 ± 1	ND	ND	30 ± 1	ND	ND	ND
MC1061(TS018)	14 ± 1[b]	ND	ND	24 ± 1	ND	ND	ND
14028s (wild type)	28 ± 1	46 ± 1	43 ± 1	30 ± 1	26 ± 1	28 ± 2	35 ± 1
TS1 (Δ*ramA*)	30 ± 2	45 ± 1	43 ± 1	32 ± 1	27 ± 1	31 ± 2	36 ± 1
TS2 (Δ*soxR*/*S*)	37 ± 1[b]	60 ± 1[b]	42 ± 1	24 ± 1	31 ± 1	21 ± 1	36 ± 1
TS3 (Δ*ramA* Δ*soxR*/*S*)	45 ± 1[b]	65 ± 1[b]	43 ± 1	30 ± 1	32 ± 1	27 ± 2	41 ± 1[b]

[a] In disk diffusion assays the diameters of the zones of inhibition of growth around cotton disks on M9 minimal medium agar plates were determined. The following amounts of compounds were used (per disk): menadione, 10 μl of a 3 M stock solution; paraquat, 10 μl of a 2% stock solution; Sin-1, 10 μl of a 500 mM stock solution; chloramphenicol, 30 μg; gentamicin, 100 μg; nalidixic acid, 10 μl of a 0.3% stock solution; H_2O_2, 10 μl of a 3% solution. Statistical analyses were performed by using the Student *t* test.

[b] The difference between the mutant and the parental strain (or vector control) is significant ($P < 0.05$).

[c] ND, not determined.

RESULTS

Screening an *S. enterica* serovar Typhimurium library in *E. coli* for the superoxide-resistant phenotype. To identify genetic determinants for superoxide resistance of *S. enterica* serovar Typhimurium, we constructed a genomic library of the 23,000-bp fragment in the Mud5005 plasmid that conferred menadione resistance in *Salmonella*, and this library was used to transform *E. coli.* One of the colonies that grew on plates containing menadione and consistently displayed enhanced resistance to menadione in disk diffusion assays was used for further analysis. Sequencing of the inserted *Salmonella Sau*3A DNA fragment revealed a 1,919-bp insert with one open reading frame, which was 342 bp long. Next, this open reading frame was PCR amplified from genomic DNA of parental *S. enterica* serovar Typhimurium and ligated into *Bam*HI-*Hin*dIII-restricted low-copy-number plasmid pWSK29 (29). The resulting plasmid was designated pTS206. Electroporation of *E. coli* MC1061 with plasmid pTS206 gave the transformed bacteria a phenotype identical to the phenotype of the bacteria transformed with the 1,919-bp fragment; i.e., the bacteria displayed increased resistance to menadione (diameter of bacterium-free zone, 15 to 16 mm; $n = 6$) when they were compared with the parental *E. coli* strain containing an empty vector (pWSK29) (diameter of bacterium-free zone, 21 to 22 mm; $n = 6$; $P < 0.01$ for comparison of pTS206 and empty vector pWSK29) (Table 2). Taken together, these results showed that an *S. enterica* serovar Typhimurium DNA fragment corresponding to a 342-bp open reading frame was sufficient to confer a menadione-resistant phenotype to *E. coli*. Overexpression of this gene in wild-type and *ramA* knockout *S. enterica* serovar Typhimurium also resulted in enhanced resistance to menadione (Table 3).

Sequence of the open reading frame, mapping of the gene, and homology analysis. The 342-bp open reading frame that was identified was initially designated *roxA* (for resistance to oxidative stress). Since *roxA* was 90% identical to the multidrug resistance gene *ramA* of *Klebsiella pneumoniae* described by George et al. (12), *roxA* was later renamed *ramA* after this gene. In a dot blot assay based on hybridization of the *ramA* probe to DNA isolated from Mud-P22 lysates (4), the *ramA* gene was mapped to a position between 12 and 17 min on the *S. enterica* serovar Typhimurium chromosome, showing the strongest hybridization with DNA isolated from TT15232 (*purE2154*::MudQ) and much less hybridization with DNA of TT15629 (*nadA219*::MudQ). Next, by determining the cotransduction frequencies of resistance markers (*ahpC*::Tn*10*, 0%; *apeE2*::Tn*10*, 20%; *zbb*::Tn*10dCam*, 0%), *ramA* was located in the *apeE-purE* intergenic region (i.e., between 12.6 and 13.5 min on the chromosome), just upstream of *apeE*.

To investigate the presence of *ramA* in gram-negative bacterial species, Southern blot hybridization was performed with *Eco*RI-digested chromosomal DNA of 26 different microorganisms. The *ramA* gene appeared to be restricted to *Salmonella* spp., with the notable exception of *S. enterica* serovar Arizona. *ramA* was not present in any of the *E. coli* strains tested (Fig. 1A). All the strains tested hybridized with an IS*1* transposon sequence used as a positive control (Fig. 1B).

To predict a possible function of RamA, a database search was performed, which revealed levels of amino acid homology of 37 to 52% with SoxS and MarA and 90% with RamA of *K. pneumoniae*, as well as 100% identity to Rma of *S. enterica* serovar Paratyphi A (Fig. 2) (1, 4, 5, 30, 31). These proteins belong to the AraC family of prokaryotic transcriptional regulators (9). In accordance with this, a DNA binding motif

TABLE 3. Menadione susceptibility of *Salmonella* strains expressing IPTG-induced *ramA*

Strain	Diam of inhibition zone (mm) (mean ± SD)[a]	
	Without IPTG	With IPTG
14028s (wild type)	30 ± 1	29 ± 1
TS1 (Δ*ramA*)	31 ± 2	30 ± 2
TS3 (Δ*ramA* Δ*soxR*/*S*)	45 ± 1[b]	45 ± 1[b]
14028s(pTS008)	38 ± 1	28 ± 1
TS1(pTS008)	37 ± 1	28 ± 1
TS3(pTS008)	46 ± 1[b]	37 ± 1[b]

[a] In disk diffusion assays the diameters of the zones of inhibition of growth around cotton disks on M9 minimal medium agar plates were determined in the presence or absence of 0.1 mM IPTG. Each disk contained 10 μl of a 3 M menadione stock solution. Statistical analyses were performed by using the Student *t* test (SPSS software).

[b] The difference between the mutant and the parental strain (or vector control) is significant ($P < 0.05$).

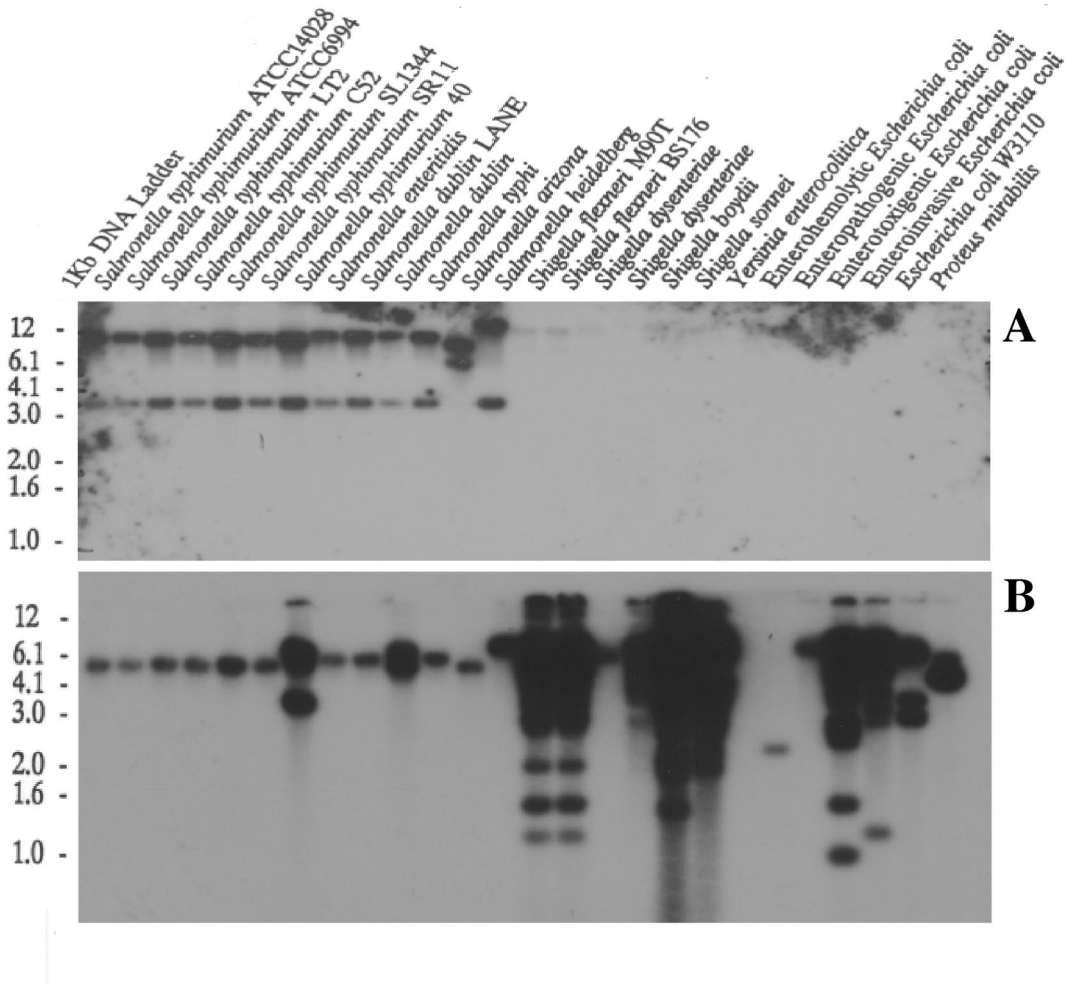

FIG. 1. Blot containing *Eco*RI-digested chromosomal DNA from a panel of 26 gram-negative bacteria. Each DNA was hybridized with a radioactively labeled DNA fragment containing the *ramA* reading frame. (A) Double hybridization bands are due to the fact that *Eco*RI cut the *Salmonella* DNA close to *ramA* in a region that overlapped the radioactive probe, resulting in bands at 2.5 and 6 kb. (B) Same blot hybridized with part of the IS*1* sequence as a control for hybridization.

(helix turn helix) was present in the RamA sequence, which suggested that the protein is a transcriptional activator that interacts with specific DNA sequences.

RamA is involved in bacterial resistance to intracellular superoxide. In order to assess the role of RamA in resistance to intracellular superoxide, we created a *ramA* null mutant of wild-type *Salmonella* (designated TS1) by replacing a kanamycin cassette with the 3′ end of *ramA* and downstream sequences. By using a P22 lysate from TS1, the disrupted *ramA* gene was also transduced in a *soxR*/*S* null mutant (designated TS3).

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RamA (S.t) MTISAQVIDTIVEWIDDNLNQPLRIDDIARHAGYSKWHLQRLFMQYKGESLGRY
SoxS         MSHQQIIQTLIEWIDEHIDQPLNIDVVAKKSGYSKWYLQRMFRTVTHQTLGEY
RamA (K.p) MTISAQVIDTIVEWIDDNLHQPLRIDDIARHAGYSKWHLQRLFLQYKGESLGRY
MarA  MTMSRRNTDAITIHSILDWIEDNLESPLSLEKVSERSGYSKWHLQRMFKKETGHSLGQY

RamA VRERKLKLAARDLLDTDQKVYDICLKYGFDSQQTFTRIFTRTFNLPPGAYRKEKHGRTHZ
SoxS IRQRRLLLAAVELRTTERPIFDIAMDLGYVSQQTFSRVFRREFDRTPSDYRHRLZ
RamA IRERKLLLAARDLRDTDQRVYDICLKYGFDSQQTFTRVFTRTFNQPPGAYRKENHSRAHZ
MarA IRSRKMTEIAQK
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FIG. 2. Comparison of the derived amino acid sequences of the *S. enterica* serovar Typhimurium (*S.t*) RamA protein, RamA of *K. pneumoniae* (*K.p*), and SoxS and MarA of *S. enterica* serovar Typhimurium. The DNA binding region (helix turn helix) is underlined. Identical amino acids are indicated by boldface type; RamA exhibits 37 to 52% homology with SoxS and MarA and 90% homology with RamA of *K. pneumoniae*. Rma of *S. enterica* serovar Paratyphi A was 100% identical to RamA of *S. enterica* serovar Typhimurium.

Expression of *ramA* on a plasmid (as part of a larger chromosomal DNA fragment in TS018 and as a single open reading frame in TS206) in the naturally *ramA*-negative organism *E. coli* resulted in increased resistance to intracellular superoxide, as determined by disk diffusion assays with the redox-cycling agent menadione; the bacterium-free zone diameters decreased from 17 to 14 mm and from 22 to 16 mm, respectively (Table 2). Remarkably, the *S. enterica* serovar Typhimurium *ramA* knockout was only slightly more susceptible to menadione than the parental wild-type bacteria (Table 2). However, when *ramA* was deleted in a *soxR*/*S* null mutant, the resulting double mutant was extremely susceptible to menadione, indicating that in the absence of SoxR and SoxS RamA plays an important role in defense against intracellular superoxide (Ta-

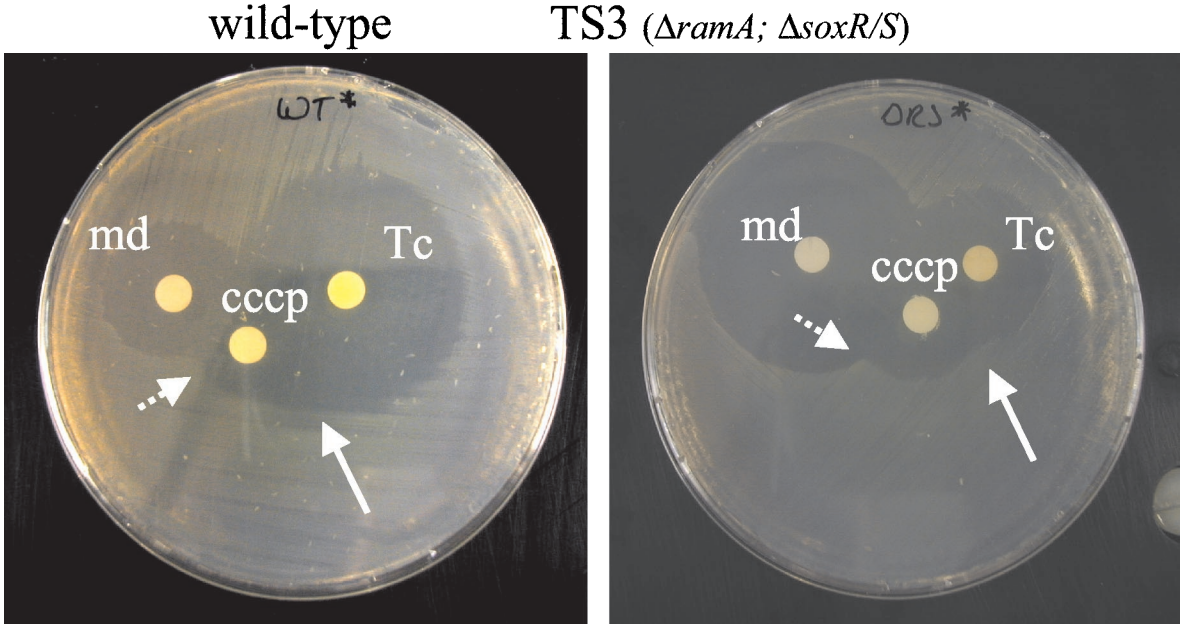

FIG. 3. Synergism between CCCP and tetracycline (Tc) but not menadione (md), as determined by the disk diffusion assay. The disks were arranged so that the predicted bacterium-free zones would overlap by about 1 mm. Bulging of the zone due to a synergistic effect between CCCP and tetracycline is indicated by an arrow; note that bulging of the bacterium-free zone was absent between CCCP and menadione (dashed arrows). The disks contained 10 μl of a 3 M menadione solution, 10 μl of a 50 mM CCCP solution, and 10 μl of a 50-mg/ml tetracycline solution.

ble 2). The menadione-susceptible phenotype could be restored fully by complementation with *ramA* on an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid; in the presence of IPTG the phenotype of the mutants reverted to that of parental wild-type *Salmonella* (Table 3). In addition, the susceptibility to intracellular superoxide was confirmed by using other redox-cycling agents, including paraquat. The mutants did not exhibit increased susceptibility to Sin-1 (which forms HOONO after generation of O_2^- and NO) or various unrelated antibiotics, including chloramphenicol, gentamicin, and nalidixic acid (Table 2).

To eliminate the possibility that the increased resistance to menadione in *Salmonella* overexpressing *ramA* on a plasmid is caused by increased efflux of the drug through induction of the multidrug pump AcrAB (17), we tested the effect of carbonyl cyanide *m*-chlorophenylhydrozone (CCCP), which blocks AcrAB activity, on resistance to menadione and tetracycline. CCCP markedly enhanced tetracycline susceptibility, which is known to depend on the AcrAB system (15, 32), but it had no effect on resistance to menadione (Fig. 3).

To confirm the role of RamA in resistance to intracellular superoxide but not to hydrogen peroxide, disk diffusion assays were performed with hydrogen peroxide (Table 2); no increase in sensitivity was observed for TS1 (Δ*ramA*) or TS2 (Δ*soxR*/*S*) compared to the sensitivity of the wild type. The double mutant (Δ*soxR*/*S* Δ*ramA*) was more sensitive, although the hydrogen peroxide sensitivity was not as markedly increased as the menadione sensitivity. This result indicates that RamA protects against elevated intracellular superoxide concentrations but not against hydrogen peroxide, which is the main degradation product of superoxide.

Mn-SOD activity is regulated by RamA. To establish whether RamA is involved in regulating the transcription of SODs, parental wild-type *Salmonella* and mutant strains were grown in the presence or absence of 1 mM menadione. Analysis of the SOD activity in whole-bacterial-cell lysates showed that the basal activities of Mn-SOD (SodA) were comparable in wild-type *Salmonella*, TS1 (Δ*ramA*), TS2 (Δ*soxR*/*S*), and TS3 (Δ*ramA* Δ*soxR*/*S*) (Fig. 4A). SodA was upregulated in the presence of menadione in parental wild-type *Salmonella* and TS1; however, the menadione-induced SodA activity in TS2 was 35% ± 6% of the activity found in the wild type (as determined densitometrically). The menadione-induced SodA activity in TS3 was even more reduced, to 25% ± 4% of the activity found in the parental wild-type strain (as determined densitometrically) (Fig. 4B). These findings indicate that expression of SodA is primarily under control of SoxS but is at least partially regulated by RamA. This conclusion was confirmed by the observation that in the various mutant strains SodA activity could be restored by overexpressing RamA on a plasmid under control of IPTG. Upon IPTG addition the SodA activity was induced in all four strains (Fig. 4C). Taken together, these data strongly suggest that SodA is (co-)regulated by RamA.

Replication of *Salmonella ramA* null mutants in mice. To determine the relevance of the *ramA* gene for replication of the bacteria in vivo, the course of infection after intraperitoneal injection of TS1 (Δ*ramA*), TS2(Δ*soxR*/*S*), TS3 (Δ*ramA* Δ*soxR*/*S*), and wild-type *Salmonella* was determined in the spleens of Swiss (*Ity*^{r}) mice for 4 days after injection. The results showed that the *ramA*, *soxR*/*S*, and *ramA soxR*/*S* null mutants were not attenuated in vivo but replicated with rates

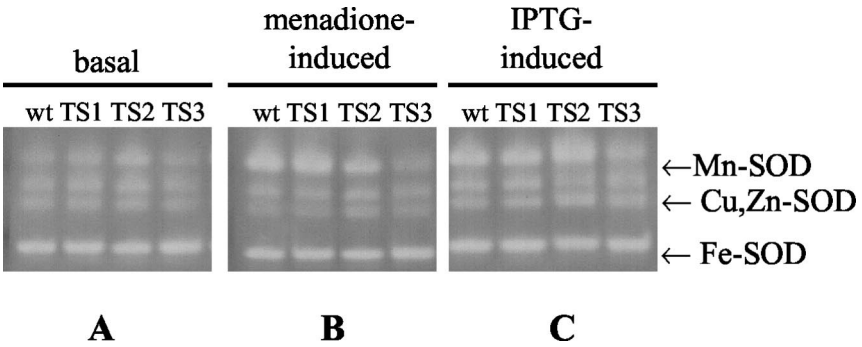

FIG. 4. SOD activities of the *Salmonella* strains. Lysates of the IPTG-inducible plasmid pHis-*ramA* (TS008) containing wild-type *Salmonella* (lanes 1, 5, and 9), TS1 (Δ*ramA*) (lanes 2, 6, and 10), TS2 (Δ*soxR*/*S*) (lanes 3, 7, and 11), and TS3 (Δ*ramA* Δ*soxR*/*S*) (lanes 4, 8, and 12) were assayed for basal SodA activity (A) and activity after menadione (B) or IPTG (C) addition. SOD activity is indicated by negative staining. The results of a representative experiment are shown. wt, wild type.

identical to the rate of the wild type (Fig. 5A). To eliminate the possibility that the effects of the mutated genes were overruled by the amount of bacteria injected, 10- and 100-fold-lower amounts (i.e., 10^3 and 10^2 CFU) of the wild type and TS3 were injected intraperitoneally into mice. Even at a low concentration the *Salmonella* strains were able to replicate at the same rate in the spleens of Swiss mice (Fig. 5B). The replication rates for the wild type and TS3 in the livers and spleens were determined by using the following formula (27): rate = (ln N4 − ln N1)/72, where N4 and N1 are the absolute amounts of the recovered bacteria on days 4 and 1, respectively. The replication rates in the spleens and livers were 0.078 ± 0.006 and 0.073 ± 0.004 h^{-1} (means ± standard deviations), respectively, for *Salmonella* wild-type strain 14028s and 0.092 ± 0.007 and 0.076 ± 0.010 h^{-1}, respectively, for TS3 (Δ*ramA* Δ*soxR*/*S*); these values are not significantly different.

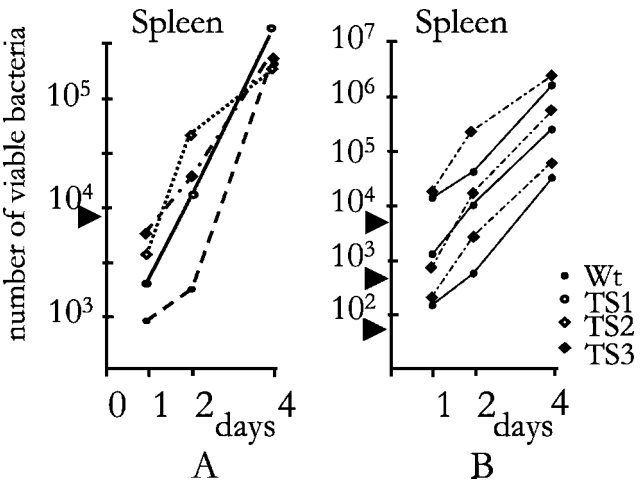

FIG. 5. In vivo survival of *Salmonella* strains in mice. Three multiplicities of infection were used, as indicated by arrows at zero time. (A) Recovery of *Salmonella* from spleens of infected mice. The changes in the numbers of intracellular wild-type *S. enterica* serovar Typhimurium 14028s (Wt), TS1 (Δ*ramA*), TS2 (Δ*soxR*/*S*), and TS3 (Δ*ramA* Δ*soxR*/*S*) were determined on days 1, 2, and 4 after intraperitoneal injection. The data are the means for four mice. No significant differences in outgrowth between the various *Salmonella* strains were detected ($P > 0.15$). (B) Recovery of *Salmonella* from spleens of infected mice. Three different multiplicities of infection (indicated by arrows) of wild-type and TS3 were used for infection. The changes in the numbers of intracellular bacteria were determined on days 1, 2, and 4 after intraperitoneal injection. The data are the means for four mice. No significant differences in outgrowth between the various *Salmonella* strains were detected ($P > 0.15$).

DISCUSSION

The main finding of the present study is that *S. enterica* serovar Typhimurium possesses, in addition to the *soxR*/*S* and *marA* regulons, a third transcriptional activator, *ramA*, which is located at about 13.1 min on the *Salmonella* chromosome and which responds to oxidative stress by activation of, for instance, cytoplasmic SodA, which is involved in a protective response against superoxide. The *ramA* gene appears to be specific for *Salmonella* serovars and is absent in many other gram-negative microorganisms; a notable exception is *K. pneumoniae*. When expressed on a plasmid in *E. coli* or *Salmonella*, *ramA* confers a pleiotropic phenotype consisting of combined menadione resistance and multiple antibiotic resistance (T. van der Straaten, R. Janssen, D. J. Mevius, and J. T. van Dissel, submitted for publication). Furthermore, like previous findings concerning the role of SoxR/S, RamA does not appear to play an important role as a virulence determinant of *Salmonella* in mice. This conclusion was based on the finding that after intraperitoneal injection the in vivo outgrowth of the *soxR*/*S ramA* null mutant strain in spleens was not attenuated. Subtle differences in bacterial outgrowth could not be established by injecting different numbers of bacteria.

What findings support our conclusion that *ramA* is a transcriptional activator in an oxidative stress regulon in *Salmonella*? First, the screening and selection procedure used to identify this gene was identical to that with which the *soxR*/*S* regulon was discovered in *E. coli*. Thus, *E. coli* and *Salmonella* carrying a plasmid expressing *ramA* displayed a pleiotropic phenotype consisting of combined multiple antibiotic and menadione resistance, the latter of which is related, at least partially, to enhanced activity of SodA. Second, an analysis of sequence homology showed that RamA, like SoxR/S and MarA, exhibits homology with the C-terminal region of a family of regulatory proteins that include AraC, the positive regulator of the arabinose operon in *E. coli* and other gram-negative bacteria (14, 22). The AraC family of gene regulators includes DNA binding proteins that contain putative helix-

turn-helix DNA recognition sequences and control expression of a variety of genes by binding to specific promoter sites. Because RamA has about 37 to 52% predicted amino acid homology with SoxS and MarA (known transcriptional activators involved in resistance of *E. coli* to oxidative stress, containing identical DNA binding domains), it is likely that overexpression of RamA in *E. coli* results in activation of at least some of the same genes that are activated by SoxS or MarA. Consistent with this, Yassien et al. showed that RamA binds to the *mar* box and activates *mar*-regulated genes in *E. coli* (32). Third, a major effect of inactivation of *ramA* on the susceptibility of *Salmonella* to menadione was observed in *soxR*/*S* null mutants. This suggests that genes induced by RamA may be under control of SoxS as well, providing an overlapping mode of genetic regulation. Since superoxide is a natural by-product of normal aerobic metabolism, most bacteria have evolved genetic protective systems to cope with oxidative stress. For *E. coli* and *Salmonella* the oxidative stress regulon *soxR*/*S* has been well described (1, 8). For instance, the expression of Mn-SOD (SodA) is known to be under control of SoxS, and oxidative stress induces Mn-SOD activity in wild-type bacteria but much less activity in *soxS* null mutants (18). This was found in the present study as well. Of the various bacterial SODs, cytoplasmatic SodA protects the bacteria against intracellular superoxide, whereas it has been suggested that periplasmic Cu,Zn-SOD plays a role in the defense against extracellular, phagocyte-derived reactive oxygen intermediates. Virulent *S. enterica* serovar Typhimurium contains two periplasmic Cu,Zn-SODs, and the virulence of *Salmonella* mutants lacking both of these enzymes is attenuated (6). Furthermore, *Salmonella* mutants that are less able to increase the expression of Mn-SOD (e.g., *soxS* null mutants) are not attenuated, suggesting that the expression of the periplasmic SODs is sufficient for virulence in mice (7, 23). Our findings extended these reports by showing that a *ramA soxR*/*S* double mutant, although extremely susceptible to the intracellular superoxide-generating agent menadione in vitro, is not attenuated in virulence for mice compared with the wild type. Furthermore, the mutants are just as able as wild-type *Salmonella* to survive within the macrophage-like RAW cells and to replicate within both normal and gamma interferon-activated cells (data not shown), although it may be argued that these findings do not definitely show that the mutants escape oxygen-dependent killing by these cells. *soxS* null mutants of *E. coli* are killed at somewhat higher rates than wild-type *E. coli* is killed upon ingestion by mouse peritoneal macrophages (16), but unlike *Salmonella*, *E. coli* is unable to replicate within these cells. Thus, an *E. coli* model has limitations when the role of oxidative stress regulons during phagocytosis and intracellular survival of the intracellular pathogen *Salmonella* in macrophages is studied.

Our findings suggest that during interaction with phagocytes, *S. enterica* serovar Typhimurium may rely on alternative protective mechanisms other than *soxR*/*S* and *ramA* regulons to cope with oxidative stress. One possible explanation may be related to the observation that unlike *E. coli*, *Salmonella* is equipped with a mechanism to inhibit the production of superoxide by phagocytes through exclusion of the NADPH oxidase from the phagosome and therefore does not encounter high levels of reactive oxygen intermediates inside macrophages (10, 28). Recently, we described an *S. enterica* serovar Typhimurium mutant with MudJ insertion inactivation of a newly described gene designated *sspJ*, which is as susceptible to menadione as the double *ramA soxR*/*S* null mutant described in this paper. However, in contrast to the latter strain, the *sspJ::*MudJ strain was unable to replicate within macrophages and was severely attenuated in vivo. This mutant regained virulence in mice incapable of producing superoxide (24, 25) due to a mutation in the p47 subunit of the NADPH oxidase. These results indicate that screening of mutagenized *S. enterica* serovar Typhimurium for susceptibility to redox-cycling agents like menadione may yield at least two classes of susceptible mutants. In the first class susceptibility to menadione in vitro correlates with attenuation of virulence for mice in vivo and replication in macrophages in vitro. The *sspJ* mutant is an example of this class of mutants (6, 26). In the second class in vitro susceptibility to redox-cycling agents is not associated with a change in mouse virulence or replication within macrophages in vitro, at least for early infection. The *ramA* null mutant described here is an example of the second class, which also includes *soxR*/*S* and *sodA* null mutants. Elucidation of the mechanisms underlying the intriguing differences between these two classes of mutants should help define the critical mechanisms of intracellular survival of *Salmonella*.

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