INFECTION AND IMMUNITY, May 1995, p. 1739–1744
0019-9567/95/$04.00+0
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Vol. 63, No. 5

Role of *Salmonella typhimurium* Mn-Superoxide Dismutase (SodA) in Protection against Early Killing by J774 Macrophages

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Received 18 October 1994/Returned for modification 9 December 1994/Accepted 10 February 1995

The *Salmonella typhimurium* gene for Mn-cofactored superoxide dismutase (*sodA*) was cloned by complementation of an *Escherichia coli sodA sodB* mutant for growth on minimal medium. Sequence analysis revealed an open reading frame of 618 bp encoding a polypeptide with 97% identity to *E. coli* SodA. A *S. typhimurium sodA* mutant was created by allelic exchange and tested for the ability to survive in the murine macrophage-like cell line J774. Growth of bacteria under iron-limiting conditions, inactivation of the Fur repressor, or expression of *sodA* from a plasmid resulted in increased resistance to early killing by J774 cells, which was abolished in the *sodA* mutant. These results suggest that resistance to the early oxygen-dependent microbicidal mechanisms of phagocytes involves the SodA gene product. The *S. typhimurium sodA* mutant was not significantly attenuated in mice, however, which suggests that resistance to early oxygen-dependent microbicidal mechanisms in vivo may play only a minor role in *Salmonella* pathogenesis.

The killing of most extracellular organisms by mononuclear phagocytes depends upon the capacity of these cells to convert oxygen to microbicidal metabolites, including reactive oxygen intermediates such as superoxide anions and hydrogen peroxide. Aerobic bacteria contain several protective enzymes which detoxify active oxygen species: superoxide dismutases (SODs; specifically, SodA and SodB), catalases (KatG and KatE), glutathione synthetase (GshAB), and glutathione reductase (Gor) (10). Several findings point to the importance of these enzymes in protection of bacteria from the oxidative killing mechanisms of host phagocytes. Exogenously added SOD or catalase was shown to protect *Escherichia coli* from phagocytic killing (1). In addition, studies on initial survival within phagocytes of *katFG* and *sodB* mutants of *Shigella flexneri*, a close relative of *E. coli*, indicated that the most efficient protective mechanism against oxygen toxicity in this species is formed by SOD, with catalase activity participating to a lesser extent (11). Conflicting results were reported by Papp-Szabò and coworkers, who showed that a *sodB* mutation in *E. coli* had no effect on killing by human polymorphonuclear leukocytes (26). Similarly, *Salmonella typhimurium* mutants in *katG* and *oxyR*, which are involved in defense against reactive oxygen intermediates, were found to resist killing by human polymorphonuclear leukocytes as well as the wild type, and a *katE katG* double mutant was found to have equal sensitivity to murine macrophages as the wild type (5, 25). Thus, the contribution of a particular reactive oxygen intermediate-detoxifying enzyme in protection against phagocytic killing mechanisms may vary with the organism and the model system studied.

During studies on survival of *Nocardia* spp. in polymorphonuclear leukocytes, it was demonstrated that bacteriocidal activity at early time points was due primarily to oxidative metabolism, whereas killing after 3 h was by both oxidative and nonoxidative mechanisms (1). Resistance to early killing in phagocytes is therefore most likely mediated by proteins which provide protection against oxygen-dependent microbicidal mechanisms. Since *S. typhimurium* is able to persist within macrophages in the liver and spleen of the mouse, it must also be able to circumvent these oxidative killing mechanisms. Since the manganese-cofactored SOD (MnSOD), SodA, is induced under conditions shown to exist intracellularly (i.e., low iron [12]), it might play an important role in defense against macrophage-induced oxidative damage in *S. typhimurium*. In this study, we investigated the role of MnSOD in the survival of *S. typhimurium* within the macrophage-like cell line J774 and in mouse virulence.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The strains used are listed in Table 1. All bacteria were cultured aerobically at 37°C. Antibiotics, when required, were included in the culture medium or plates at the following concentrations: kanamycin, 60 mg/liter; carbenicillin, 100 mg/liter; chloramphenicol, 20 mg/liter; and tetracycline, 20 mg/liter. Complementation of the growth defect of QC774 (*sodA sodB*) was performed on M9 agar plates (21). A spontaneous nalidixic acid-resistant derivative of *S. typhimurium* ATCC 14028 was selected by plating 10^9 bacteria on Luria-Bertani (LB) agar plates containing 50 mg of nalidixic acid per liter (31).

Conjugation. Conjugation between bacterial strains was performed overnight on LB agar plates. For selection on minimal plates, conjugation mixtures were resuspended and washed with 1× M9 salts (to remove residual nutrients from the LB agar) before plating on M9 agar. For allelic exchange using the suicide vector pEP185.2 (15), conjugation mixtures were plated on LB agar-selective plates, and individual colonies were screened for loss of the vector resistance marker on LB agar containing 20 mg of chloramphenicol per liter.

Recombinant DNA techniques. Plasmid DNA was isolated by use of ion-exchange columns from Qiagen (Hilden, Germany). Standard methods were used for restriction endonuclease analyses, ligation, and transformation of plasmid DNA and isolation of chromosomal DNA from bacteria. The construction of the gene bank from *S. typhimurium* ATCC 14028 in the cosmid vector pLAF RII has been described elsewhere (20).

Sequencing was performed by the dideoxy chain termination method described in a protocol of Kraft et al. with α-^{35}S-dATP (Amersham, Arlington Heights, Ill.) used for labelling (17).

The coding sequence of *sodA* was cloned without its upstream regulatory sequence by PCR amplification with *Taq* polymerase and primers 1, i.e., 5′-GCTCGACAACCATGGAGATGATTATGAG-3′, and 2, i.e., 5′-ACTCGCTTCTAGAGACGTGCAATGC-3′. The 695-bp PCR product was cloned behind the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible Trc promoter in the vector pTrc99A (Pharmacia, Alameda, Calif.) with the enzymes *Xba*I and *Nco*I.

Southern hybridization. Southern transfer of DNA onto a nylon membrane was performed as described previously. Labelling of DNA probes, hybridization, and immunological detection were performed with the DNA labelling and de-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
S. typhimurium		
ATCC 14028	Wild-type strain	ATCC
IR715	Nalidixic acid resistant derivative of ATCC 14028	I. Stojiljkovic (31)
SA1	IR715 *sodA*::Km	This study
E. coli		
LE392	F^- e14-(*mcrA*) *hsdR514* (r_K^- m_K^+)*supE44 supF58 lacY1* or Δ(*lacIZY*)6 *galK2 galT22 metB1 trpR55*	Lab collection
S17-1 λ*pir*	*prp thi recA hsdR*: chromosomal RP4-2 (Tn*1*::ISR*1 tet*::Mu Km::Tn*7*); λ*pir*	Lab collection (30)
QC772	F^- Δ*lac4169 rpsL* Φ(*sodA-lacZ*)49 Cm^r	D. Touati (7)
QC773	F^- Δ*lac4169 rpsL* Φ(*sodB-kan*)1-Δ2 Km^r	D. Touati (7)
QC774	F^- Δ*lac4169 rpsL* Φ(*sodA-lacZ*)49 Cm^r Φ(*sodB-kan*)1-Δ2 Km^r	D. Touati (7)
DH5α	*endA1 hsdR17*($r_K^-m_K^-$) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169 deoR* [φ80 *dlac* Δ(*lacZ*)*M15*]	Lab collection
Plasmids		
pBluescript KS	ColE1, *bla*	Stratagene
pTrc99A	P_{trc}, *lacI*^{q}, *bla*	Pharmacia
pMH152	pACYC184, Fur^-	K. Hantke (4)
pEP185.2	pGP704, pBluescript MCS, *cat*	J. Pepe (15)

tection kit (nonradioactive) from Boehringer GmbH (Mannheim, Germany). The DNA was labelled by random-primed incorporation of digoxigenin-labelled dUTP. Hybridization was performed at 65°C in solutions without formamide. For Southern hybridization with cosmids of a gene bank or with chromosomal DNA, a nonstringent wash (10 min at room temperature in 2× SSC–0.1% sodium dodecyl sulfate [SDS] and a stringent wash (30 min at 65°C in 0.2× SSC–0.1% SDS) were performed (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybrids were detected by an enzyme-linked immunoassay using an antibody conjugate (anti-digoxigenin alkaline phosphatase conjugate) and the substrate AMPPD [3-(2′-spiroademantane)-4-methoxy-4-(3″-phosphoryloxy-)phenyl-1,2-dioxethane; Boehringer). The light emitted by the dephosphorylated AMPPD was detected by X-ray film.

Computer analysis. The nucleotide sequences were compared with nonredundant updates of SWISS-PROT, PIR(R), and GenPept by use of the program blastX and with nonredundant updates of GenBank and EMBL by use of the program blastN. Nucleotide sequences were further analyzed with the PC/GENE software package.

SOD activity assay. Bacterial lysates were prepared by a modification of the procedure described by Touati (32). A volume of an overnight culture in LB agar equivalent to 5×10^9 bacteria (calculated by optical density measurements at 578 nm) was centrifuged briefly at 14,000 rpm to pellet cells. Cells were washed by resuspending them in phosphate-buffered saline (PBS) and pelleting again. Bacteria were then resuspended in 1/20 volume of 10 mM potassium phosphate–0.1 mM EDTA buffer containing 0.3 mg of lysozyme per ml and subjected to 10 freeze-thaw cycles by dipping tubes alternately for 1 min in an ethanol-dry ice bath and a 42°C water bath. Lysates were cleared by centrifugation at 14,000 rpm for 10 min. The total protein content of the lysates was determined by the Bradford assay (3). Equivalent amounts of total protein were loaded onto a polyacrylamide gel run under nondenaturing conditions. SOD bands were visualized in gels by the activity staining method of Beauchamp and Fridovich (2).

Cell culture techniques and macrophage survival assay. The macrophage cell line J774 was cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, Md.) supplemented with 10% heat-inactivated equine serum (Gibco BRL), 1% nonessential amino acids, and 1 mM glutamine (DMEMsup). J774 cells were tested periodically for production of oxygen radicals via the hexose monophosphate shunt (oxidative burst) after induction with phorbol myristate acetate (Consolidated Midland Corp., Brewster, N.Y.) as described elsewhere (9).

For macrophage survival assays, the bacteria were grown overnight, washed in PBS, and opsonized in fresh mouse serum for 20 min. Twenty-four-well microtiter plates were seeded with macrophages at a concentration of 5×10^5 cells per well in 0.5 ml of DMEMsup and incubated overnight at 37°C in 5% CO_2. The bacterial cultures were then diluted, and about 5×10^6 bacteria in 0.25 ml of DMEMsup were added to each well of macrophages. To create iron limitation, the overnight cultures were grown in LB broth plus 0.2 mM 2,2′-dipyridyl, harvested, and grown for 2 h in DMEMsup plus 2 mg of apotransferrin per ml (Boehringer) and 10 mM sodium bicarbonate buffer. After opsonization, the bacteria were diluted in DMEMsup plus 2 mg of apotransferrin per ml and 10 mM sodium bicarbonate buffer.

Microtiter plates were centrifuged at 250 × *g* for 5 min at room temperature to synchronize infection. Cells were incubated for 15 min at 37°C in 5% CO_2, free bacteria were removed by three washes with PBS, and the zero time point was taken as described below. The washing solutions were collected, and extracellular bacteria were quantified by dilution in sterile PBS and plating on LB agar. DMEMsup plus 6 μg of gentamicin per ml was added to the wells, and the cells were incubated at 37°C in 5% CO_2. Wells were sampled at appropriate time points after infection by aspirating the medium, lysing the macrophages with 0.5 ml of 1% deoxycholate, and rinsing each well with 0.5 ml of PBS. Viable bacteria were quantified by dilution in sterile PBS and plating on LB agar. All experiments were performed independently at least three times, and the standard error for each time point was calculated.

Infection of mice. Virulence of the *sodA* mutant was tested by infecting 6- to 8-week-old female BALB/c ByJ mice obtained from Jackson Laboratories (Bar Harbor, Maine). Serial 10-fold dilutions ranging from 9.2×10^7 to 9.2×10^4 were made in LB agar, and 0.2 ml of these dilutions was administered intragastrically to groups of four mice. Mortality was recorded at 28 days postinfection, and the 50% lethal dose (LD_{50}) values were calculated by the method of Reed and Muench (28). Stability of the *sodA* mutation in vivo was assessed by plating the liver and spleen of a moribund infected mouse. The liver and spleen were each homogenized in 1 ml of PBS with a stomacher (Tekmar, Cincinnati, Ohio), and 0.1 ml of diluted homogenate was plated on both LB agar plus nalidixic acid and LB agar plus kanamycin. Viable counts were compared on each plate to determine the stability of the *sodA*::*kan* mutation. From plates with LB agar plus nalidixic acid, 10 colonies were picked and grown for SOD activity assays as described above.

RESULTS

Cloning and sequencing of the *S. typhimurium sodA* gene. To clone the *S. typhimurium sodA* gene, we used a strategy which has been described previously (for examples, see references 6 and 13). The *E. coli* strain QC774 (*sodA sodB*) exhibits a growth defect when grown aerobically on minimal medium. Growth can be restored by introduction of either *sodA* or *sodB* on a plasmid. We therefore attempted to clone *S. typhimurium sodA* by complementation of QC774 for growth on minimal medium. A cosmid library of *S. typhimurium* ATCC 14028 constructed in pLAFRII was introduced by conjugation into QC774 on LB agar. Twelve exconjugants were picked and assayed for SOD activity (2). One of these clones, designated pSA8.0, showed SOD activity corresponding to *E. coli sodA* on an SOD activity gel. This cosmid was digested with *Eco*RV, and the fragments were cloned into pBluescript KS. A pool of subclones was used to transform QC774, and the transformants were tested for complementation of the growth defect on minimal medium. A clone able to grow on M9 plates was found to contain a plasmid (pSA8.1) carrying a 2-kb *Eco*RV fragment. Plasmid pSA8.1 was shown to encode *sodA* by activity staining (data not shown). For sequencing, further subclones of pSA8.1 were constructed with the enzymes *Sal*I, *Pst*I, and *Eco*RI.

```
   1 CGCCGCC CGT TTT CTC TTC CAC TTG CTG CCC CAG GAA GTG CAA CGC ATC
  50 TTT TAC CGG CTG GCT ATT TTC AGC GTA GGC CAG GCG CAA AAT GGT TTC
  98 GCC GTG GGA GAA GAA AGA GAG TGT GGA GAG TAA GGC CAA CGT AGC CAG
 146 ACG TAT AAA ACC AGG TTG CTT CAT GAT TCC CTC GCA ATT GTA ATA ATT
 194 TAC CGG GAA TGT AAT TGC AGT GTG AAA TTA TAA CCT TCT TGA TTT TGC
 242 CAC CGC TGA CAA AAT TTG GCA CCA AAA GAA AGA TTT TGA AAC CCT GTT
                             ___EcoRI___
 290 TCA CAT TTC ATG ATT TCC AGG AAT TCA TCA ACA GGC GGT TTC GGT TGG
 338 AGG CGT AAA AAA CGG TTT TTT CAG CGG ATG CCG TAA CGT TTA TAA CCC
                                         _-35_
                             ______Fur box_______________
 386 TGG AAA AAG TAC GGC ATT GAT AAT CAT TTT CAA TAT CAT TTA ATT AAC
     __-10______                                         >__________
 434 TAT AAT GAA CCA ACT GCT TAC GCG GCG TTA ACA CTG TGC CGC TCG ACA
     ______Primer1_____________>
 482 ATA ATG GAG ATG ATT ATG AGT TAT ACA CTG CCA TCC CTG CCG TAC GCT
                         Met Ser Tyr Thr Leu Pro Ser Leu Pro Tyr Ala

 530 TAT GAT GCA CTG GAA CCG CAC TTC GAT AAG CAG ACG ATG GAG ATT CAC
     Tyr Asp Ala Leu Glu Pro His Phe Asp Lys Gln Thr Met Glu Ile His

 578 CAC ACC AAA CAC CAT CAA ACC TAT GTC AAC AAC GCT AAC GCG GCG CTG
     His Thr Lys His His Gln Thr Tyr Val Asn Asn Ala Asn Ala Ala Leu

 626 GAA AAC CTG CCT GAG TTT GCC AGC CTG CCG GTT GAA GAA CTG ATT ACT
     Glu Asn Leu Pro Glu Phe Ala Ser Leu Pro Val Glu Glu Leu Ile Thr

 674 AAA CTG GAC CAG GTG CCA GCG GAC AAA AAA ACT GTG CTG CGT AAC AAC
     Lys Leu Asp Gln Val Pro Ala Asp Lys Lys Thr Val Leu Arg Asn Asn

 722 GCG GGC GGC CAT GCT AAC CAC AGC CTG TTC TGG AAA GGG CTG AAA ACA
     Ala Gly Gly His Ala Asn His Ser Leu Phe Trp Lys Gly Leu Lys Thr

 770 GGC ACC ACT CTG CAG GGT GAT CTG AAA GCG GCT ATC GAG CGT GAC TTC
     Gly Thr Thr Leu Gln Gly Asp Leu Lys Ala Ala Ile Glu Arg Asp Phe
                                     _EcoRI_
 818 GGT TCC GTT GAC AAC TTC AAA GCT GAA TTC GAA AAA GCA GCA GCA ACC
     Gly Ser Val Asp Asn Phe Lys Ala Glu Phe Glu Lys Ala Ala Ala Thr

 866 CGT TTC GGC TCC GGC TGG GCG TGG CTG GTG CTG AAA GGC GAC AAA CTG
     Arg Phe Gly Ser Gly Trp Ala Trp Leu Val Leu Lys Gly Asp Lys Leu

 914 GCT GTG GTT TCT ACC GCA AAC CAG GAT TCC CCG CTG ATG GGT GAA GCC
     Ala Val Val Ser Thr Ala Asn Gln Asp Ser Pro Leu Met Gly Glu Ala

 962 ATT TCC GGC GCT TCC GGC TTC CCG ATC CTG GGC CTG GAC GTG TGG GAA
     Ile Ser Gly Ala Ser Gly Phe Pro Ile Leu Gly Leu Asp Val Trp Glu

1010 CAC GCT TAC TAC CTG AAA TTC CAG AAC CGC CGC CCG GAC TAC ATC AAA
     His Ala Tyr Tyr Leu Lys Phe Gln Asn Arg Arg Pro Asp Tyr Ile Lys

1058 GAG TTC TGG AAC GTG GTG AAC TGG GAC GAA GCA GCA GCG CGT TTC GCG
     Glu Phe Trp Asn Val Val Asn Trp Asp Glu Ala Ala Ala Arg Phe Ala
                 <__________Primer 2_______________<
1106 CTA AAA TAA TTT GCA TTG CAC GTC TGT AGA AGC GAG TCT GAT GAC TCG
     Leu Lys
1154 CTT TTT TTG TAT CCG CGT AAG GAG CAG CAG ATG CAT TAT CCG GTT GAC
1202 GTG TTT ATT GG
```

FIG. 1. DNA sequence and deduced amino acid sequence of *S. typhimurium sodA*. Potential −10 and −35 sequences and a potential Fur binding site (identified by homology to the *E. coli* sequence) are shown. *Eco*RI sites used to generate the *sodA* mutation are indicated. Annealing sites of primers 1 and 2 used to amplify the *sodA* coding sequence by PCR are shown with arrows. This sequence has been assigned GenBank accession number U20645.

Sequence analysis revealed an open reading frame of 618 bp (Fig. 1). A potential Fur box located upstream of the open reading frame was identical to the Fur box located in the promoter region of *E. coli sodA* (23) (Fig. 1). The deduced amino acid sequence of the open reading frame shared 97% identity with *E. coli* SodA (Fig. 2). These data show that the cloned DNA fragment encoded *sodA* and therefore confirmed the data obtained by complementation of QC774 and SOD activity staining.

Construction of an *S. typhimurium sodA* mutant. A chromosomal *sodA* mutant of *S. typhimurium* IR715 was created by marker exchange. A pBluescript KS derivative lacking the *Eco*RI polylinker site was created by digestion with *Eco*RV and *Sma*I and subsequent religation. The 2-kb *Eco*RV insert in pSA8.1 was cloned into this pBluescript KS derivative to create plasmid pSA8.5. The kanamycin resistance cassette KIXX (Pharmacia) was introduced into the *Eco*RI sites indicated at nucleotides 310 and 842 of Fig. 1. The resulting insert was cloned into the suicide vector pEP185.2 with enzymes *Xba*I and *Kpn*I and the host strain S17-1 λ*pir* (30) for propagation of the suicide vector. This construct (pSA8.8) was mated into

```
E. c.     MSYTLPSLPYAYDALEPHFDKQTMEIHHTKHHQTYVNNANAALESLPEFA
S. t.     --------------------------------------------N-----

E. c.     NLPVEELITKLDQLPADKKTVLRNNAGGHANHSLFWKGLKKGTTLQGDLK
S. t.     S------------V--------------------------T---------

E. c.     AAIERDFGSVDNFKAEFEKAAASRFGSGWAWLVLKGDKLAVVSTANQDSP
S. t.     ----------------------T---------------------------

E. c.     LMGEAISGASGFPIMGLDVWEHAYYLKFQNRRPDYIKEFWNVVNWDEAAA
S. t.     --------------L-----------------------------------

E. c.     RFAAKK
S. t.     ---L-
```

FIG. 2. Comparison of the deduced amino acid sequences of *E. coli* (E.c.) and *S. typhimurium* (S.t.) SodA. Identical amino acids are indicated by dashes. The sequence alignment was prepared with the CLUSTALV program.

IR715, and exconjugants were selected on plates containing kanamycin and nalidixic acid. Exconjugants were restreaked on plates containing chloramphenicol to test for loss of the suicide vector. Exconjugants sensitive to chloramphenicol but resistant to kanamycin originate from allelic exchange between the chromosomal *sodA* and the mutated copy on pSA8.8.

To confirm the marker exchange, one of these exconjugants, designated SA1, was characterized further by Southern hybridization (Fig. 3). Using the insert of plasmid pSA8.1 as a probe, a 2-kb fragment and a 3.2-kb fragment were detected in *Eco*RV-digested chromosomal DNA of IR715 and SA1, respectively. The change in size of 1.2 kb is that which would be expected as a result of the allelic exchange. In addition, SA1 was found to show no detectable SodA activity by SOD activity staining (Fig. 4A). Introduction of pSA8.1 into SA1 resulted in SodA activity, as detected by activity staining (Fig. 4A). These results confirmed the inactivation of *sodA* by marker exchange in SA1.

Survival of SA1 in J774 macrophages. To determine whether MnSOD is involved in bacterial defense against oxygen-dependent microbicidal mechanisms of macrophages, strains IR715 and SA1 were tested for their ability to survive in the cell line J774. Since the oxidative burst is thought to occur immediately upon contact of macrophages with microbes (1), we investigated the ability of *S. typhimurium* to survive the initial contact with J774 cells (Fig. 5). The *sodA* mutant strain survived in macrophages at a rate similar to that of the wild

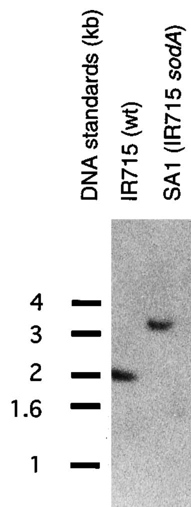

FIG. 3. Southern hybridization of *Eco*RV-digested chromosomal DNA prepared from IR715 and SA1 (IR715 *sodA*) with a probe containing *sodA*. Sizes and positions of DNA standards are given on the left.

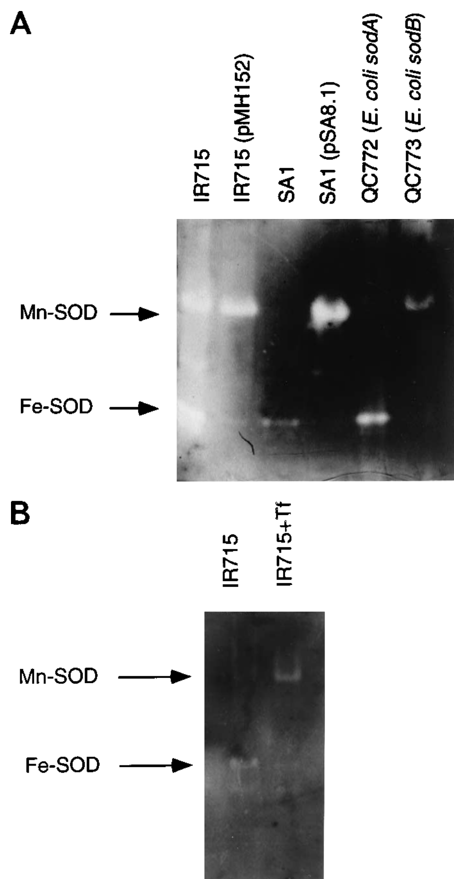

FIG. 4. (A) SOD activity gel of *S. typhimurium* IR715 and derivatives. Arrows indicate bands of SOD activity corresponding to *E. coli* SodA (MnSOD) and SodB (FeSOD). About 30 μg of total protein (20 μl of lysate) was loaded per lane. (B) SOD activity of IR715 grown under iron-replete (LB agar) and iron-limiting (DMEM plus 2 mg of apotransferrin per ml [Tf]) conditions. About 10 μg of total protein was loaded per lane. Lysates were prepared and activity staining was performed as described in Materials and Methods.

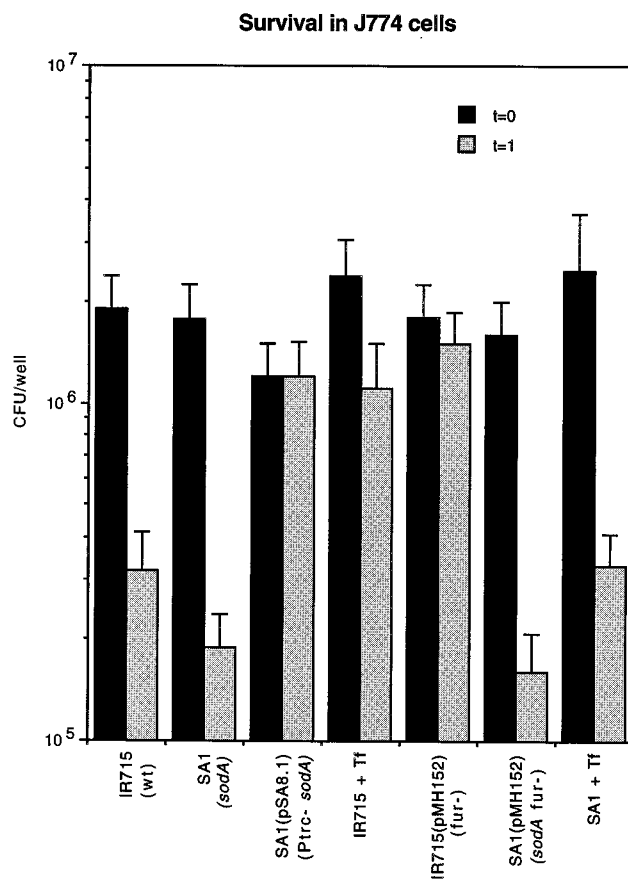

FIG. 5. Survival of *S. typhimurium* strains in J774 cells. Strains were assayed for survival at 0 and 1 h after infection of cells as described in Materials and Methods. Bars indicate averages of at least three experiments ± standard errors of the mean. Tf indicates growth of bacterial inoculum with 2 mg of apotransferrin per ml to create iron starvation.

type (Fig. 5). These data thus indicated that under the assay conditions used, MnSOD activity does not contribute significantly to macrophage survival of *S. typhimurium.*

In *E. coli*, *sodA* expression is subject to regulation by six regulatory proteins (8). Therefore, to ensure expression of *sodA* under the assay conditions used, we cloned by PCR a promoterless *S. typhimurium sodA* gene into plasmid pTrc99A (Pharmacia), yielding pSA8.9. The annealing sites for the primers used to amplify *sodA* are indicated in Fig. 1. In pSA8.9, *sodA* expression is under the control of the *trc* promoter. In this construct, we found the *trc* promoter to be leaky, allowing high levels of expression of MnSOD even without IPTG induction (data not shown). Addition of IPTG increased MnSOD expression even further. Plasmid pSA8.9 was introduced into SA1, and the resulting strain (pregrown without IPTG) was tested for survival within J774 cells. The number of bacteria recovered from macrophages 1 h after infection of J774 cells increased three- to ninefold compared with the numbers recovered from strains SA1 or IR715 (Fig. 5). SOD activity gels showed high levels of MnSOD activity in SA1(pSA8.9) (data not shown). Thus, the elevated level of MnSOD present in SA1(pSA8.9) seemed to confer protection against early killing in J774 cells. Introduction of the empty vector, pTrc99A, into SA1 had no effect on survival within J774 cells (data not shown).

To determine whether elevated amounts of MnSOD are also expressed under conditions which more closely resemble the environment in the host, we performed macrophage survival assays with bacterial inocula pregrown under iron-limiting conditions. In *E. coli*, *sodA* is repressed by the iron response regulator Fur. Fur has been shown to strongly repress *sodA* expression if *E. coli* is grown in iron-rich medium (8, 23). If the iron concentration decreases, Fur dissociates from the *sodA* promoter, thereby allowing elevated expression of *sodA*. The availability of iron for microbes has been shown to be low in serum as well as in an intracellular habitat (12), and thus iron limitation may more closely resemble in vivo growth conditions. IR715 did indeed survive better in macrophages if the bacteria were iron starved prior to infection (Fig. 5). We next investigated whether this increased bacterial survival could also be observed in the absence of Fur. Negative complementation was used to create a Fur^- phenotype in IR715. By introducing a mutated *fur* gene carrying a point mutation in the DNA binding domain on a low-copy-number plasmid (pMH152), inactive heterodimers which are unable to bind DNA are formed. As a result, the merodiploid strain behaves like a *fur* mutant with respect to expression of *fur*-regulated genes (4). Like expression of *sodA* in *S. typhimurium* from a plasmid or iron starvation of IR715, negative complementation resulted in increased resistance to early killing in J774 cells (Fig. 5). An increased amount of MnSOD activity was detected

in IR715(pMH152) by SOD activity staining (Fig. 4A). This was accompanied by a decrease in FeSOD (SodB) activity. This finding is in agreement with the results of Niederhoffer et al., who found that *sodB* expression was strongly reduced in a *fur* mutant (23). The relative decrease in FeSOD was also observed when IR715 was grown under iron-limiting conditions (Fig. 4B). To ensure that the increased resistance to macrophage killing was due to derepression of *sodA*, plasmid pMH152 was introduced into the *sodA* mutant SA1, and the resulting strain was tested for survival in J774 cells. The *sodA fur* merodiploid strain survived at rates similar to that of the parent SA1. In addition, no increase in survival was observed when SA1 was iron starved prior to infection of macrophages (Fig. 5). Thus, the increase in macrophage survival caused by inactivation of Fur or pregrowth of bacteria under iron-limiting conditions can be abolished by a mutation in *sodA*. These data show that under conditions of iron starvation, elevated levels of MnSOD contribute to survival of *S. typhimurium* in J774 cells.

Virulence of the *S. typhimurium sodA* mutant in mice. To determine whether the increased resistance to macrophage killing mediated by elevated levels of MnSOD is important in vivo, the virulence of SA1 was compared with that of its parent, IR715, in the murine typhoid model of infection. The *sodA* mutant was found to be only slightly attenuated in mice. After intragastric infection, the LD_{50} of SA1 was 1.6×10^6 bacteria, while IR715 had a LD_{50} of 6×10^5 bacteria. The *sodA* mutation did not revert in vivo, since equal numbers of bacteria were enumerated on plates containing nalidixic acid (resistance of the parent strain) and on those containing kanamycin from the liver and spleen of an infected mouse. In addition, 10 colonies isolated from the spleen and picked at random showed no SodA activity when examined by SOD activity staining of extracts. Thus, under the conditions used for infection, MnSOD does not appear to play a crucial role for *S. typhimurium* virulence in mice.

DISCUSSION

In this report, we demonstrated that high levels of MnSOD can protect *S. typhimurium* against early killing by J774 cells. These expression levels can be achieved by growth of bacteria under iron-limiting conditions, expression of *sodA* from a strong promoter, or by inactivation of the Fur repressor. Growth under iron deficiency, which induces expression of *sodA*, is likely to more closely resemble the in vivo situation since *S. typhimurium* faces a low-iron (transferrin-containing) environment (body fluids) prior to phagocytosis. Genes repressed by Fur have also been shown to be induced during growth in epithelial cells, which precedes contact with the lymphatic fluid during the course of a *Salmonella* infection (12).

Although elevated MnSOD levels were protective in the intracellular survival assay using J774 cells, the *sodA* mutant was only weakly attenuated in the mouse model of infection. One possible explanation for this apparent discrepancy is that bacteria are equipped with multiple enzymes to protect them against oxidative damage. A defect in only one enzyme may thus be insufficient to render the bacterium incompetent to withstand oxidative stress in vivo. In *E. coli*, single mutations in *sodA* or *sodB* resulted in a slightly increased (up to 10-fold in rich medium) sensitivity to paraquat in vitro, whereas the double mutant *sodA sodB* was approximately 1,000-fold more sensitive to paraquat than the wild type (7). The *sodA sodB* mutant also exhibits an aerobic growth defect on minimal medium, which has been attributed to the sensitivity of enzymes necessary for synthesis of branched-chain amino acids to oxygen radicals (7). This growth defect of the double mutant, which we presume would also occur in a *sodA sodB* mutant of *S. typhimurium*, would render it difficult to assess the role of SOD in virulence by determining the LD_{50} of the double mutant.

A second possible reason for the only moderate attenuation of the *S. typhimurium sodA* mutant in mice is that the defense against oxidative killing mechanisms of phagocytes is not crucial for a successful infection. This latter hypothesis is supported by the finding that *Salmonella typhi* and *S. typhimurium* have been reported to elicit little or no oxidative burst upon entry into phagocytic cells (16, 22, 33). Other macrophage pathogens, such as *Legionella* and *Mycobacterium* spp., have been shown to enter the macrophage via complement receptors (27, 29). Uptake by this route has been shown not to trigger the release of oxygen intermediates by macrophages and would thus allow these pathogens to avoid the toxic consequences of the oxidative burst. Similarly, complement receptors have been implicated in the uptake of *S. typhimurium* by macrophages (14). Complement receptors are expressed at elevated levels in resident macrophages, as compared with those in activated macrophages (18). *S. typhimurium* may persist in vivo preferentially in resident macrophages of the liver and spleen, which are in a lower state of activation for oxidative killing (19, 24). Thus, like other intracellular pathogens, *S. typhimurium* may evade the oxidative burst of phagocytes, perhaps by selecting the appropriate set of phagocytic receptors for entry into its intracellular niche. *S. typhimurium* may therefore elicit only a weak oxidative burst upon contact with its target phagocytes, making MnSOD function dispensable. The J774 cells used for the in vitro assays are different from these resident macrophages, which might explain the contribution of oxygen-dependent killing mechanisms in this in vitro model. The contribution of oxygen-dependent microbicidal activity against *S. typhimurium* in macrophages studied outside the native context of host organs may also differ from that occurring in vivo, which might explain the differences between the results achieved in our in vivo and in vitro models.

ACKNOWLEDGMENTS

We would like to thank D. Touati, K. Hantke, J. Pepe, and I. Stojiljkovic for providing bacterial strains, I. Stojiljkovic for suggestions on the manuscript, and S. Anic and J. Lipps for technical assistance.

A.J.B. was supported by a stipend from the Deutsche Forschungsgemeinschaft (Ba 1337/1-2).

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