Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase

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ABSTRACT  Superoxide dismutase (SOD) catalyzes the conversion of superoxide radical to hydrogen peroxide. Periplasmic localization of bacterial Cu/Zn-SOD has suggested a role of this enzyme in defense against extracellular phagocyte-derived reactive oxygen species. Sequence analysis of regions flanking the *Salmonella typhimurium* *sodC* gene encoding Cu/Zn-SOD demonstrates significant homology to α-phage proteins, reflecting possible bacteriophage-mediated horizontal gene transfer of this determinant among pathogenic bacteria. *Salmonella* deficient in Cu/Zn-SOD has reduced survival in macrophages and attenuated virulence in mice, which can be restored by abrogation of either the phagocyte respiratory burst or inducible nitric oxide synthase. Moreover, a *sodC* mutant is extremely susceptible to the combination of superoxide and nitric oxide. These observations suggest that SOD protects periplasmic or inner membrane targets by diverting superoxide and limiting peroxynitrite formation, and they demonstrate the ability of the respiratory burst and nitric oxide synthase to synergistically kill microbial pathogens in *vivo*.

Cu/Zn-superoxide dismutase (SOD) was first identified in bovine erythrocytes (1) and recognized as a prokaryotic enzyme in *Photobacterium leiognathi* in 1974 (2). Although initially regarded as a biological curiosities, prokaryotic Cu/Zn-SOD was eventually found in a number of other bacterial species including *Salmonella* (3, 4). In part, the initial failure to recognize the presence of Cu/Zn-SOD in bacteria may have resulted from its low concentrations relative to Mn- and Fe-SODs (5). Periplasmic localization of prokaryotes (6, 7) led to the proposal that Cu/Zn-SOD may defend bacteria from extracellular oxidative stress (6), but its precise functional significance has not been established.

In the present study, the facultative intracellular pathogen *Salmonella typhimurium* was used to examine the role of Cu/Zn-SOD in defense against oxygen-dependent antimicrobial systems of mammalian phagocytes. The complete *S. typhimurium* *sodC* gene (4) encoding Cu/Zn-SOD and its flanking regions were cloned and sequenced. Superoxide dismutase activity of the *SodC* protein was confirmed, and insertion inactivation of the gene was achieved by homologous recombination. The *SodC*-deficient mutant strain and its isogenic parent were subsequently evaluated in chemical susceptibility studies, macrophage survival assays, and mouse virulence experiments to assess the functional importance of bacterial Cu/Zn-SOD both in *vitro* and *in vivo*.

MATERIALS AND METHODS

Media. Bacteria were routinely grown in Luria–Bertani medium (10 mg/ml tryptone, 5 mg/ml yeast extract, 10 mg/ml NaCl) at 37°C, with penicillin (250 μg/ml) added as required. Chemical killing assays were performed in M9 minimal medium (7 mg/ml Na₂HPO₄, 3 mg/ml KH₂PO₄, 0.5 mg/ml NaCl, 1 mg/ml NH₄Cl, 5 μg/ml thiamine, 0.12 mg/ml MgSO₄, 0.015 mg/ml CaCl₂) or PBS as indicated. Xylose-lysine-deoxycholate medium (Difco) was used for selection of recombinant *Salmonella* mutants. Agar (1.5%) was added to solid medium. Macrophages were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM Hepes buffer, and 1 mM sodium pyruvate.

Bacterial Strains and Plasmids. Chemical susceptibility assays, macrophage killing assays, and virulence studies used wild-type *Salmonella typhimurium* ATCC14028s or its *sodC* mutant derivative MF1005. *S. typhimurium* XF1001 (katE/katG) is described in ref. 8. Escherichia coli S17–1 (9) was used to mobilize suicide vector pRR10(Δσα) (10). Plasmid pBluescript KS (Stratagene) was used for construction of a *S. typhimurium* genomic library, and plasmid pET23a (Novagen) in E. coli BL21 (DE3) (11) was used for *sodC* overexpression under the control of the T7 RNA Polymerase.

*sodC* Mutant Construction. A 262-bp internal *sodC* fragment (nucleotides 228–489) was amplified by PCR using oligonucleotide primers 5'-TATGCGCGGAATAGAAAGAC-3' and 5'-CAGTGGACAGTTATCGGGATT-3' derived from the published partial *S. typhimurium sodC* sequence (4). The fragment was first cloned into the Smal site of pBluescript (Stratagene), then subcloned into the EcoRI and BamHI sites of suicide vector pRR10(Δσα) (10), and mobilized from *E. coli* S17–1 into *S. typhimurium* 14028s, with selection of transconjugants on xylose-lysine-deoxycholate agar containing penicillin 250 μg/ml. Homologous recombination of the plasmid into the *Salmonella* chromosome created an interruption of *sodC*, which resulted in deletion of 14 aa from the carboxyl terminus of the *SodC* protein; *sodC* interruption was confirmed by Southern hybridization (not shown) (12). The *sodC* mutant *S. typhimurium* strain was designated MF1005.

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: SOD, superoxide dismutase; ATCC, American Type Culture Collection; SIN-1, 3-morpholinosydnonimine hydrochloride; SPER/NO, 2,2'-hydroxynitrosohydroxrazono)bisethanamine; IFN-γ, interferon γ; PMA, phorbol 12-myristate 13-acetate; MMA, N²-(i-monomethyl arginine; AV, acetovanillone; ko, knock-out; *tv*, genetic locus conferring immunity to *Salmonella typhimurium* (NRAMP1).

Data deposition: The sequence reported in this paper (*S. typhimurium sodC*) and flanking regions have been deposited in the GenBank database (accession no. AF007380).

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Cloning of the S. typhimurium sodC Gene. The S. typhimurium sodC gene fragment described above (4) was used as a probe to identify a 3.3-kbp sodC-containing chromosomal fragment (GenBank accession no. AF007380) from an S. typhimurium 14028s genomic library. Sequencing was performed on an ABI373A automated fluorescent sequencer (Applied Biosystems).

Detection of Superoxide Dismutase Activity. Sonicated cell extracts from E. coli overexpressing the S. typhimurium sodC gene from plasmid pET2a-sodC or carrying the pET vector alone were subjected to 15% PAGE under nondenaturing conditions at neutral pH. SOD activity was demonstrated as an achrmonic zone after photoactivation of riboflavin and reaction with nitroblue tetrazolium (13). Sodium cyanide (1 mM) was added to confirm that S. typhimurium SodC is a copper, zinc-containing superoxide dismutase (14).

Chemical Susceptibility Assays. Disk diffusion susceptibility assays (15) were performed with 106 stationary phase bacteria on M9–0.2% glucose agar using 15 μl of 3% hydrogen peroxide, 1.9% methyl viologen, 500 mM S-nitrosoglutathione (GSNO), or 500 mM SIN-1 (3-morpholinosydnonimine hydrochloride) (all reagents from Sigma); GSNO was synthesized as described (15). Other killing assays were performed with 0.1 unit/ml xanthine oxidase (Sigma) and 250 μM hypoxanthine added to overnight cultures diluted 1:100 in PBS. Serial dilutions were plated in triplicate at timed intervals for quantitation of colony-forming units.

Macrophage Killing Assays. Murine peritoneal exudate cells were harvested in RPMI 1640 medium with 10% fetal calf serum were plated in 96-well flat-bottomed microtiter plates and allowed to grow for 24 h. Cells were resuspended in PBS and lysed with 0.5% deoxycholate. Serial dilutions were plated for enumerating colony-forming units. 0.5% deoxycholate. Serial dilutions were plated for enumerating colony-forming units.

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Macrophage Killing Assays. Murine peritoneal exudate cells were harvested in RPMI 1640 medium with 10% fetal calf serum 4 days after intraperitoneal injection of 5 mM sodium periodate (Sigma), which stimulates both the phagocyte respiratory burst and inducible NO synthase (16). Cells were plated in 96-well flat-bottomed microtiter plates and allowed to adhere overnight in the presence of 20 units/ml interferon γ (IFN-γ). S. typhimurium opsonized with normal mouse serum were spun onto adherent cells at 101 multiplicity of infection (moi), internalized for 15 min, and washed with medium containing 6 μg/ml gentamicin to kill extracellular bacteria. N6,N6-dimethyl arginine (250 μM) (control, Sigma), 250 μM N6-l-monomethyl arginine (NO synthase inhibitor, Sigma), or 250 μM acetoxyanilone (apocynin, 4-hydroxy-3-methoxyacetophenone; NADPH-oxidase inhibitor, Aldrich) were added to wells at 0 h as indicated. In separate experiments, 250 μM N6-l-monomethyl arginine was found to effectively block NO2- production by IFN-γ-stimulated macrophages as measured by the Griess reaction (17), 250 μM acetoxyanilone was found to abrogate stimulation of H2O2 production (data not shown). At 2-, 6-, and 24-h time points, cells were resuspended in PBS and lysed with 0.5% deoxycholate. Serial dilutions were plated for enumeration of colony-forming units.

Mouse Virulence Assays. C57BL/6 (Ity+, genetically Salmo nella-susceptible) mice, congenic phox-91 ko (knock-out) mice (19), or C3H/HeN (Ity+, genetically Salmonella-resistant) mice were intraperitoneally challenged with wild-type or mutant S. typhimurium diluted in 200 μl PBS. Inocula were determined by dilutional plating. Mutant bacteria were recovered from dead mice to confirm in vivo stability of insertion mutations. Each virulence assay was performed at least twice using five mice per experimental group, with identical results.

RESULTS

Sequence Analysis of S. typhimurium sodC and Flanking Regions. Analysis of sodC and flanking regions revealed an adjacent countertranscribed ORF encoding a predicted protein with 20–64% homology to members of a family of outer membrane proteins that includes Lom of bacteriophage λ (20) and Ail of Yersinia enterocolitica (21), as well other ORFs encoding proteins highly homologous (53–86%) to bacteriophage λ tail proteins (Fig. 1A). The predicted full-length SodC protein is only 55% identical to the E. coli SodC protein (22) (Fig. 1B), and a typical hydrophobic NH2 terminus signal sequence was identified, consistent with secretion of SodC into the periplasmic space. Hybridization studies using the internal sodC fragment described in Materials and Methods as a probe demonstrated the presence of the sodC gene in Salmonella enteritidis, S. choleraesuis, S. Newport, S. heidelberg, and S. typhimurium (not shown).

Demonstration of SOD Activity. Overexpression of the cloned S. typhimurium sodC gene revealed SOD activity (13) (Fig. 2), inhabitable by sodium cyanide as is characteristic of copper, zinc-containing superoxide dismutases. E. coli Cu,ZnSOD activity is not visible in extracts from cells carrying the plasmid vector alone, reflecting its low concentration relative to that of Fe-SOD and Mn-SOD (5).

Susceptibility to Chemically Generated Reactive Oxygen and Nitrogen Species. The chemical susceptibility of sodC mutant S. typhimurium was compared with that of isogenic wild-type bacteria by disk diffusion assay (15). Wild-type and sodC mutant S. typhimurium were found to have identical susceptibility to hydrogen peroxide, methyl viologen (paraquat), GSNO, and SIN-1 (data not shown). However, the sodC mutant has increased (29±3-fold) susceptibility to extracellular superoxide generated by xanthine oxidase (Fig. 3). Susceptibility to xanthine oxidase but not the intracellular superoxide-generating methyl viologen is consistent with earlier observations indicating that the inner membrane of Gram-negative bacteria is impermeable to superoxide (6, 23). The sodC mutation was observed to dramatically increase (2,700×) susceptibility to synergistic killing by xanthine oxidase (Fig. 3) and the NO donor SPER/NO, although SPER/NO by itself has no significant antimicrobial activity against either wild-type or sodC mutant Salmonella.

Fig. 1. (A) Map of S. typhimurium sodC region. An ORF encoding a predicted protein with homology to the Ail protein of Yersinia enterocolitica and ORFs highly homologous to genes that encode bacteriophage λ tail proteins are designated. (B) Sequence alignment of S. typhimurium and E. coli SodC proteins. The full-length SodC proteins from S. typhimurium and E. coli (22) are 54% identical at the amino acid level. The S. typhimurium sodC gene encodes a protein of 18.3 kDa predicted size. The protein possesses a typical hydrophobic NH2 terminus signal sequence (underlined); cleavage would produce a mature protein of 16.3 kDa predicted size.
Susceptibility to Macrophage Killing. The sodC S. typhimurium mutant has increased susceptibility to killing by activated murine macrophages (Fig. 4), but this hypersusceptibility requires the presence of both the respiratory burst and nitric oxide synthase. Enhanced killing can be blocked by either the NO synthase inhibitor NG-L-monomethyl arginine (24) or by the respiratory burst inhibitor acetovanillone (18). Macrophage killing assays were performed three times with samples in triplicate, with identical results.

Murine Virulence. Further evidence of the biological relevance of bacterial Cu,Zn-SOD was obtained in murine virulence studies. Six-week-old C57BL/6(1ty) and congenic phox-91 ko mice (19) were challenged with 50 intraperitoneal wild-type or sodC mutant S. typhimurium organisms. Attenuated virulence of the sodC mutant was completely restored by abrogation of the respiratory burst (Fig. 5A). In a parallel experiment, 6-week-old C3H/HeN (1ty)r mice were challenged with 2 × 10^3 intraperitoneal wild-type, sodC mutant, or catalase-deficient katE katG mutant (8) S. typhimurium. Separate groups of mice were administered 2.5% (wt/vol) aminoguanidine (Sigma), an inhibitor of inducible NO synthase (25). Attenuated virulence of the sodC mutant strain was restored by inhibition of NO synthase (Fig. 5B). In contrast, the catalase-deficient strain was fully virulent, and its virulence was not significantly affected by coadministration of aminoguanidine. Mouse virulence studies were performed a minimum of two times each; results of representative experiments are shown.

DISCUSSION

Both clinical observations in patients with chronic granulomatous disease and experimental studies with murine phagocytes indicate that the phagocyte respiratory burst with production of superoxide is a critical component of host defense against salmonellosis (26, 27), as it is in many other infections (28). Studies in experimental animals also suggest a beneficial role of inducible NO synthase during Salmonella infection (25), and elevated production of NO has been detected in patients with bacterial gastroenteritis (29). The present observations indicate that synergistic antimicrobial activity can result from the combination of the phagocyte respiratory burst and NO synthase in vivo as well as in vitro. This study also demonstrates a critical role of periplasmic Cu,Zn-SOD encoded by the sodC gene in defending Salmonella from products of both the respiratory burst NADPH-oxidase and inducible NO synthase. This contrasts with the function of the more abundant cytosolic Mn-SOD enzyme, which is not required for Salmonella virulence despite its importance in detoxification of intracellular superoxide (30) or limiting production of intracellular peroxynitrite generated from SIN-1 (15).
and respiratory burst-deficient mice. C57BL/6 in addition to S. typhimurium adjustment for multiple comparisons. 

C57BL/6 the effect of NO synthase inhibition. C3H untreated mice challenged with 25 (AG), an inhibitor of inducible NO synthase. The survival of received drinking water treated with 2.5% (wt y katEG sodC intraperitoneally challenged with wild-type, sodC wild-type and sodC mutant (MF1005) were intraperitoneally challenged with wild-type.

sodC mutant (MF1005), or katEG mutant (XF1001) S. typhimurium. Separate groups of mice received drinking water treated with 2.5% (wt/vol) aminoguanidine (25) (AG), an inhibitor of inducible NO synthase. The survival of untreated mice challenged with sodC mutant organisms was significantly different from the other experimental groups (P < 0.01) after adjustment for multiple comparisons.

We have detected sodC in other virulent Salmonella serovars in addition to S. typhimurium, and this gene has also been detected in bacterial pathogens such as E. coli, Brucella, Actinobacillus, Pseudomonas, Haemophilus, Pasteurella, Neisseria, and Legionella (2, 4, 5, 31). Flanking sequences with homology to bacteriophage genes indicate a possible mechanism for horizontal gene transfer of the sodC gene and its counterpart in P. leioigathi (55% identity) (33), a non-enteric member of the Vibrionaceae.

Damage to periplasmic or inner membrane proteins and lipids, potentially could be mediated by superoxide, hydrogen peroxide, hydroxyl radical formed by the metal-catalyzed Haber–Weiss reaction, or a product of NO and superoxide, such as peroxynitrite (15, 34–36) (Fig. 6). Hydrogen peroxide and peroxynitrite can penetrate cell membranes and may also act on intracellular targets. However, the requirement of periplasmic SOD but not catalase for virulence suggests against a critical role of either hydrogen peroxide or hydroxyl. The restoration of virulence to a sodC mutant by abrogation of either NO synthase or the respiratory burst, as well as the profoundly increased susceptibility of sodC mutant Salmonella to the combination of xanthine oxidase and NO, implicate a product of NO and superoxide and, to a lesser extent, superoxide alone in bacterial killing. Cu,Zn-SOD appears most likely to protect Salmonella by detoxifying superoxide and reducing periplasmic formation of peroxynitrite or other reactive species (35) produced from superoxide and NO, although alternative mechanisms of synergistic interaction, as proposed for hydrogen peroxide and NO (37), cannot be excluded.

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