Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 7502–7507, June 1999
Microbiology

Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases

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Communicated by Irwin Fridovich, Duke University Medical Center, Durham, NC, May 6, 1999 (received for review March 14, 1999)

ABSTRACT **Periplasmic Cu, Zn-cofactored superoxide dismutase (SodC) protects Gram-negative bacteria from exogenous oxidative damage. The virulent *Salmonella typhimurium* strain ATCC 14028s has been found to contain two discrete periplasmic Cu, Zn-SOD enzymes that are only 57% identical at the amino acid level. SodCI is carried by a cryptic bacteriophage, and SodCII is closely related to the Cu, Zn-superoxide dismutase of *Escherichia coli*. All *Salmonella* serotypes appear to carry the *sodCII* locus, but the phage-associated *sodCI* gene is found only in certain strains belonging to the most highly pathogenic serotypes. Expression of either *sodC* locus appears to be enhanced during stationary phase, but only *sodCII* is regulated by the alternative sigma factor σ^s (RpoS). Mutants lacking both *sodC* genes are less lethal for mice than mutants possessing either *sodC* locus alone, indicating that both Cu, Zn-SOD enzymes contribute to *Salmonella* pathogenicity. The evolutionary acquisition of an additional *sodC* gene has contributed to the enhanced virulence of selected *Salmonella* strains.**

Prokaryotic organisms generally contain one or more superoxide dismutases (SODs) cofactored by manganese (Mn), iron (Fe), or copper and zinc (Cu, Zn). Cu, Zn-SOD, encoded by the *sodC* gene, has been identified in a large number of Gram-negative bacterial genera including *Actinobacillus, Brucella, Caulobacter, Escherichia, Francisella, Haemophilus, Legionella, Neisseria, Pasteurella, Photobacterium,* and *Salmonella* (1–11). Periplasmic localization of Cu, Zn-SOD in bacteria has suggested a role in protection from exogenous oxidative stress, and this has been confirmed in several instances by *in vitro* assays (12–17).

The first Cu, Zn-SOD of *Salmonella typhimurium* was discovered by PCR amplification of genomic DNA using primers derived from consensus *sodC* sequence motifs (8). Sequence analysis of flanking regions revealed bacteriophage structural genes (13, 14), suggesting that the *Salmonella sodC* gene was acquired by bacteriophage-mediated horizontal transfer. Construction of a null mutant showed that Cu, Zn-SOD is required for survival in phagocytes and full virulence in mice (13, 14). Further characterization in immunodeficient mice demonstrated that Cu, Zn-SOD helps *Salmonella* to resist synergistic interactions of the phagocyte NADPH oxidase and nitric oxide synthase (13), possibly by diverting superoxide and limiting peroxynitrite formation.

We now report that some strains of *Salmonella* carry two distinct Cu, Zn-superoxide dismutases. The second *Salmonella* Cu, Zn-SOD was discovered when two-dimensional electrophoretic analysis of *S. typhimurium* was performed to identify acid stress-induced proteins regulated by the alternative sigma factor σ^s. Unexpectedly, the NH_2-terminal sequence of one acid-induced σ^s-dependent protein [(acid shock protein (ASP)71] was found to be highly homologous to the SodC protein of *Escherichia coli*, but clearly distinguishable from the known *S. typhimurium* SodC sequence (8, 13, 14). The new gene has been designated *sodCII,* and the present study describes its sequence analysis, regulation, functional characterization, and implications for the evolution of *Salmonella* pathogenicity.

MATERIALS AND METHODS

Media. Bacterial strains were routinely cultivated and stored in LB broth (10 mg/ml tryptone/5 mg/ml yeast extract/10 mg/ml NaCl)(Difco) at 37°C. Evans blue uranine medium was used to identify pseudolysogen-free transductants (18). M9 minimal medium (7 mg/ml Na_2HPO_4/3 mg/ml KH_2PO_4/0.5 mg/ml NaCl/1 mg/ml NH_4Cl) or xylose-lysine-deoxycholate medium (Difco) was used for selection of recombinant *Salmonella* mutant strains. LB medium with the omission of NaCl and the addition of 6% sucrose was used at 30°C (19) for allelic exchange procedures. Vogel and Bonner E minimal medium (20) supplemented with 0.4% glucose was used for two-dimensional SDS/PAGE studies. Penicillin (250 μg/ml), ampicillin (60 μg/ml), kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), or tetracycline (10 μg/ml in minimal and 20 μg/ml in rich media, respectively), all from Sigma, was added as indicated. Agar (1.5%) was added to solid media.

Strains and Plasmids. Bacterial strains are listed in Table 1. Two-dimensional protein electrophoretic analysis was performed on strains *S. typhimurium* UK1 (wild-type) (21) and JF3912 (UK1 *sodCII*::pRR10[Δ*trfA*]). Overexpression of SodCII for microsequencing was performed in JF2892 (UK1 *mviA*4185::*aph*) (22). β-Galactosidase, chemical susceptibility, and mouse virulence assays were performed by using *S. typhimurium* ATCC 14028s or its isogenic mutant derivatives. Additional *Salmonella* strains analyzed by Southern hybridization are from a published reference collection of *Salmonella enterica* subspecies I strains (23) and from our laboratory collection of clinical isolates. The construction of *rpoS* mutant SF1005 (*rpoS*::pRR10[Δ*trfA*]) is described in ref. 24, and the construction of *sodCI* mutant MF1005 (*sodCI*::pRR10[Δ*trfA*]) is described in ref. 13. The construction of *sodCI* mutant

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Abbreviations: SOD, superoxide dismutase; ASP, acid shock protein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF056931).

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Table 1. Genotypes used

Species	Strain	Relevant genotype	Ref.
S. typhimurium	14028s	Wild-type	American Type Culture Collection
	JF2892	UK1 *mviA* 4185::*aph*	22
	JF3912	UK1 *sodCII*::pRR10(Δ*trf*A)	This work
	MF1005	14028s *sodCI*::pRR10(Δ*trf*A)	13
	MF1006	14028s *sodCI*::*aph*	This work
	MF1007	14028s *sodCII*::pRR10(Δ*trf*A)	This work
	MF1008	14028s *sodCI*::*aph* *sodCII*::pRR10(Δ*trf*A)	This work
	SF1005	14028s *rpoS*::pRR10(Δ*trf*A)	24
	UK1	Wild-type	21
E. coli	S17–1	Tra$^+$ *recA pro thi hsdR chr*::RP4–1	25
	SM10λ*pir*	*thi leu hsd rec*A Km^r RP4–2-Tc::Mu λ*pir*	26

MF1006 (*sodCI*::*aph*), *sodCII* mutants MF1007 and JF3912 (*sodCII*::pRR10[Δ*trfA*]) and *sodCI sodCII* double mutant MF1008 (*sodCI*::*aph sodCII*::pRR10[Δ*trfA*]) is described in *Results*. *E. coli* S17–1 (25) or SM10λ*pir* (26) were used to mobilize suicide vectors pRR10(Δ*trfA*) (24) or pKNG101 (27), respectively. Plasmid pRS1274 was used to construct promoter fusions with *lacZ* (28). Kanamycin-resistant versions of the pRS1274-derived plasmids were constructed by inserting an *aph* cassette (Amersham Pharmacia) into the unique *Pst*I site in the pRS1274 *bla* gene. Plasmid pBluescript KS (Stratagene) was used for routine cloning.

Standard Genetic Procedures. Routine genetic manipulations and PCR amplification were performed by using conventional methods (29). Southern hybridizations were performed by using *Eco*RI- or *Eco*RV-digested chromosomal DNA electrophoresed through 0.7% agarose and transferred onto a nylon membrane. A 790-bp *Eco*RI–*Bgl*II fragment from the *sodCI* locus was used as a probe for the presence of *sodCI*. Primers 5′-ATGAACCTCGTCACGTCGCAAGGGG-TAGGGCAGTC-3′ and 5′-TTGATTTCATCCAGTGATT-TCAGACGAGGCGC-3′ derived from the *E. coli sodC* sequence were used to amplify a 343-bp internal fragment to be used as a probe for the *S. typhimurium sodCII* gene. An internal fragment of the *spvR* coding region was PCR-amplified by using a previously described primer pair (30) and used to probe for the presence of the *spv* plasmid virulence genes. Probes were labeled with ^{32}P or with a chemiluminescent labeling and detection kit (Boehringer Mannheim or NEN). Sequencing was performed on an ABI 1373A automated fluorescent sequencer (Applied Biosystems) or by the Sanger dideoxy method (29) using sequenase (United States Biochemical) and custom primers (BRL).

Pulsed-field gel electrophoresis of *S. typhimurium* 14028s genomic DNA was performed by extracting DNA, embedding it in agarose, and digesting with *Xba*I (New England Biolabs) according to manufacturer's specifications. Plugs containing digested DNA were stored overnight and electrophoresed for 20 hr through 1.2% SeaKem LE agarose (FMC) on a Chef DRIII apparatus (Bio-Rad) with a field strength of 6 V/cm at an included angle of 120°. Initial switch time was 5 sec, and the final switch time was 40 sec. The electrophoresis buffer was 0.5× TBE run at 14°C. The DNA was transferred onto a nylon membrane to allow hybridization.

Two-Dimensional SDS/PAGE. Two dimensional SDS/PAGE was performed as described (31). ASPs were identified by comparing cultures at pH 7.7 to cultures shifted from pH 7.7 to pH 4.4 for 20 min. Typically, the samples were labeled for 5 min with ^{35}S-trans label (40 μCi/ml, ICN; 1 Ci = 37 GBq). After the addition of chloramphenicol, a 1.5-ml sample of cells was pelleted and suspended in SDS lysis buffer. Approximately 5 μg of protein was analyzed for each sample. Basic and acidic proteins are situated to the left and right of the autoradiograph, respectively. The first dimension was obtained by using a pH 5–7 isoelectric-focusing gel containing 1.6% (pH 5–7) and 0.4% (pH 3–10) ampholytes (Bio-Rad), and the second dimension was obtained by using an SDS/12% PAGE gel without stacking gel.

Protein Purification from Two-Dimensional SDS/PAGE. Isolation and purification of the σ^s-dependent ASPs was accomplished by scaling up the standard two-dimensional gel protocol with a few modifications and additions. Minimal E glucose medium (0.5 liters) was inoculated with a 1:100 dilution of an overnight culture of JF2892 (*mviA*). At 0.6 OD_{600}, a portion of the culture (6 ml) was removed for trans ^{35}S labeling (ICN). The labeled and unlabeled cultures were combined and centrifuged, and the pellets were resuspended in 1× sonication buffer. After sonication, the cellular debris was pelleted by low- (12,100 × *g* for 10 min) and high- (380,000 × *g* for 20 min) speed spins. The supernatant was loaded onto a Centricon-100 (Amicon) concentration column with a 100,000 MW protein cutoff. The filtrate from the centrifuged Centricon-100 was then loaded onto a Centricon-30 concentration column. Retentates from both Centricons were processed for two-dimensional gels. After electrophoresis, the polyacrylamide gels were transferred to poly(vinylidene difluoride) (Bio-Rad). The blots containing proteins from the Centricon-30 retentate were sent to The Wistar Institute (Philadelphia) for proteolytic digestion and protein sequencing of ASP71. Analysis of nucleotide and protein sequences were performed by using the Wisconsin Package, Version 8.1-Unix of the Genetics Computer Group (Madison, WI).

β-Galactosidase Assays. Overnight cultures of *S. typhimurium* 14028s (wild-type) or SF1005 (*rpoS*) carrying P_{sodCI}-*lacZ*, P_{sodCII}-*lacZ*, or control plasmids were diluted 1:1000 into fresh LB broth and placed in a shaker-incubator at 275 rpm and 37°C. Samples were assayed at timed intervals for β-galactosidase activity from SDS/chloroform-permeabilized cells with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate (32). Each assay was performed a minimum of two times.

Mouse Virulence Assays. C57BL/6 (*Ity*^{s}, genetically *Salmonella*-susceptible) or C3H/HeN (*Ity*^{r}, genetically *Salmonella*-resistant) mice (The Jackson Laboratory) were administered intraperitoneal inocula of wild-type or *sodC* mutant *S. typhimurium* diluted in 200 μl of PBS. Inocula were determined by dilutional plating, and varied from 600–1,200 organisms. Each virulence assay was performed at least twice by using four to seven mice per group, with similar results.

RESULTS

***S. typhimurium* Has Two sodC Genes.** Previous studies have identified the protein designated ASP71 to be a σ^s-dependent ASP (33). The *mviA* mutant JF2892 overproduces many σ^s-

dependent proteins, apparently because σ^s degradation depends on the *mviA* regulatory locus (22). Consequently, strain JF2892 was subjected to a modified two-dimensional SDS/PAGE protocol to obtain quantities of ASP71 sufficient for microsequencing. Two-dimensional protein gel electrophoresis of total cell lysates from *S. typhimurium* exposed to acid stress had revealed a σ^s-dependent protein of approximately 16 kDa (Fig. 1*B*). NH_2-terminal sequencing of this protein revealed a peptide identical to the mature *E. coli* SodC protein at 20 of 23 residues, suggesting that this protein was in fact a Cu, Zn-SOD. However, the sequence differed from the known corresponding *S. typhimurium sodC* sequence at 16 of 23 residues (Fig. 2), raising the possibility that *S. typhimurium* has two *sodC* genes. Primers derived from the *E. coli sodC* sequence (described in *Materials and Methods*) were subsequently used to amplify a 343-bp internal fragment of the newly identified *S. typhimurium* gene, designated *sodCII*, from genomic DNA of ATCC *S. typhimurium* strain 14028s (34).

The 343-bp *sodCII* fragment was labeled with ^{32}P and used to probe *S. typhimurium* 14028s genomic DNA completely digested with *Bgl*II and *Bam*HI endonucleases. A hybridizing 3.8-kbp chromosomal fragment containing the entire *sodCII* gene was cloned and sequenced (GenBank accession no. AF056931). The nucleotide sequence of *sodCII* revealed a 522-bp gene encoding a predicted 174-aa protein (Fig. 2). The predicted protein sequence is 82% identical to the *E. coli sodC* gene and 54% identical to *S. typhimurium sodCI* (Fig. 2). Like other prokaryotic Cu, Zn-SODs, the SodCII protein possesses a hydrophobic NH_2-terminal sequence consistent with its secretion into the periplasmic space.

sodCI Is Associated with Highly Virulent *Salmonella* Serotypes. Chemiluminescence-labeled *sodCI* and *sodCII* fragments were used to probe an *Xba*I digest of *S. typhimurium* 14028s DNA, a reference collection of chromosomal DNA from 75 strains representing 37 serotypes of *S. enterica* subspecies I (23) and DNA from additional clinical isolates in our laboratory collection (Table 2). The presence of the *Salmonella spvR* plasmid virulence gene was determined for comparison, because the *spv* genes, like *sodCI*, are associated with mobile DNA elements (35). The *sodCI* and *sodCII* loci were found to reside on distinct *Xba*I fragments separated by pulsed-field gel electrophoresis (data not shown); *sodCI* is found on an *Xba*I fragment of ≈750 kbp, whereas *sodCII* is located on an ≈250-kbp fragment.

Although there are more than 2,000 recognized serotypes of *Salmonella*, specific serotypes are overrepresented among patients with invasive extraintestinal infection. *Salmonella*

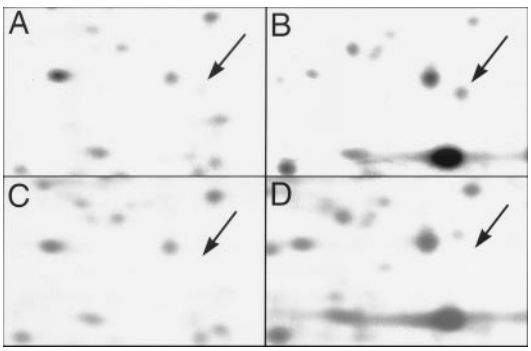

FIG. 1. Two-dimensional SDS/PAGE analysis of SodCII (ASP71) in wild-type *Salmonella* and a *sodCII* mutant. Wild-type UK1 (*A* and *B*) and *sodCII* mutant JF3912 (*C* and *D*) were grown to logarithmic phase at pH 7.7 in minimal EG medium. Cells were either processed directly for two-dimensional SDS/PAGE (*A* and *C*) or acid shocked for 1 hr at pH 4.4 before processing (*B* and *D*). The arrow indicates the position of SodCII. The panels show equivalent small areas of larger gels.

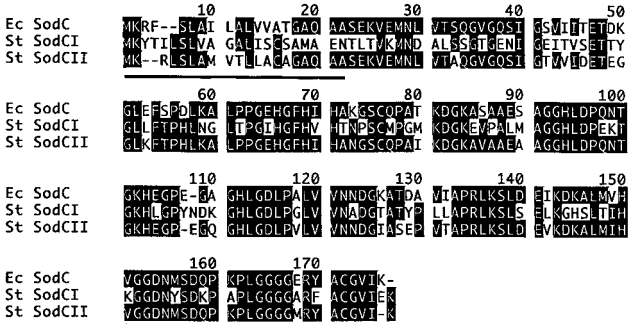

FIG. 2. Sequence Comparison of *E. coli* and *S. typhimurium* SodC proteins. The two *S. typhimurium* SodC proteins share 57% identity. *S. typhimurium* SodCII is 82% identical to *E. coli* SodC, but SodCI is only 54% identical to the *E. coli* protein. Each SodC possesses a typical hydrophobic NH_2 terminus signal sequence (underlined), consistent with secretion of Cu, Zn-SOD into the periplasmic space.

enteritidis, S. typhimurium, Salmonella heidelburg, and *Salmonella dublin* are responsible for nearly 70% of all episodes of *Salmonella* bacteremia in the United States (36). Representative strains from these highly virulent *Salmonella* serotypes were found to have a full complement of the *sodCI, sodCII,* and *spv* virulence-associated genes, whereas serotypes associated with intermediate virulence phenotypes (e.g., *Salmonella newport, Salmonella saintpaul, Salmonella arizona*) have some but not all of these factors, and the majority of serotypes possess only *sodCII*. Serotype was found to correlate imperfectly with the presence of bacteriophage- or plasmid-associated virulence loci *sodCI* and *spvR*, as the presence of these loci was found to vary among isolates of *Salmonella choleraesuis, S. dublin, S. enteritidis, Salmonella gallinarum, S. newport, Salmonella paratyphi* B, *S. paratyphi* C, *S. saintpaul, Salmonella typhisuis*, and *S. typhimurium* (Table 2, Fig. 3). Discrepancies were noted between our results with the *spvR* probe (Fig. 3) and those recently reported by Boyd and Hartl (37) for strains Tm23, De13, Du1, Cs11, Pc1, and De31 of the SARB reference collection (23). These differences might be attributable to the smaller probe used in our studies, or less likely to plasmid loss during laboratory passage of these strains.

Table 2. Virulence genes and serovars carrying them

Virulence gene			
sodCI	***sodCII***	***spvR***	**Serovars**
+	+	+	*S. choleraesuis, S. dublin, S. enteritidis, S. gallinarum, S. heidelberg, S. pullorum, S. typhimurium, S. typhisuis*
+	+		*S. enteritidis, S. gallinarum, S. haifa, S. heidelberg, S. newport, S. paratyphi*B, *S. saintpaul*
	+	+	*S. arizona, S. paratyphi*C
	+		*S. agona, S. anatum, S. barta, S. benta, S. brandenburg, S. cerro, S. choleraesuis, S. decatur, S. derby, S. dublin, S. duisburg, S. emek, S. enteritidis, S. hadar, S. indiana, S. infantis, S. mbandaka, S. miami, S. montevideo, S. muenchen, S. newport, S. panama, S. paratyphi*B, *S. paratyphi*C, *S. reading, S. rubislaw, S. saintpaul, S. schwarzengrund, S. senftenberg, S. stanley, S. stanleyville, S. typhi, S. typhimurium, S. typhisuis, S. wien, S. worthington*

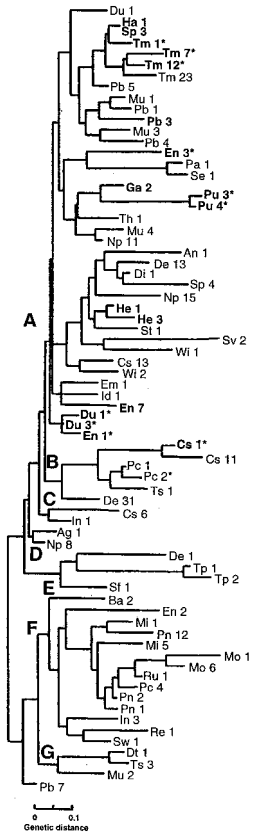

FIG. 3. Presence of *sodCI* and *spvR* in *Salmonella* Reference Collection B Strains. Strains and evolutionary tree are modified from ref. 23, with permission. Strains carrying *sodCI* are indicated in **bold** type. Strains carrying *spvR* are indicated by ∗. Abbreviations of *Salmonella* serotypes: Ag, *S. agona*; An, *S. anatum*; Ba, *S. brandenburg;* Cs, *S. cholerasuis*; De, *S. derby*; Di, *S. duisburg*; Dt, *S. decatur*; Du, *S. dublin*; Em, *S. emek*; En, *S. enteritidis*; Ga, *S. gallinarum*; Ha, *S. haifa*; He, *S. heidelberg*; Id, *S. indiana*; In, *S. infantis*; Mi, *S. miami*; Mo, *S. montevideo*; Mu, *S. muenchen*; Np, *S. newport*; Pa, *S. paratyphi A*; Pb, *S. paratyphi B*; Pc, *S. paratyphi C*; Pn, *S. panama*; Pu, *S. pullorum*; Re, *S. reading*; Ru, *S. rubislaw*; Se, *S. sendai*; Sf, *S. senftenberg*; Sp, *S. saintpaul*; St, *S. stanley*; Sv, *S. stanleyville*; Sw, *S. schwarzengrund*; Th, *S. thompson*; Tm, *S. typhimurium*; Tp, *S. typhi*; Ts, *S. typhisuis*; Wi, *S. wien*. [Reproduced with permission from ref. 23 (Copyright 1993, Society for General Microbiology)].

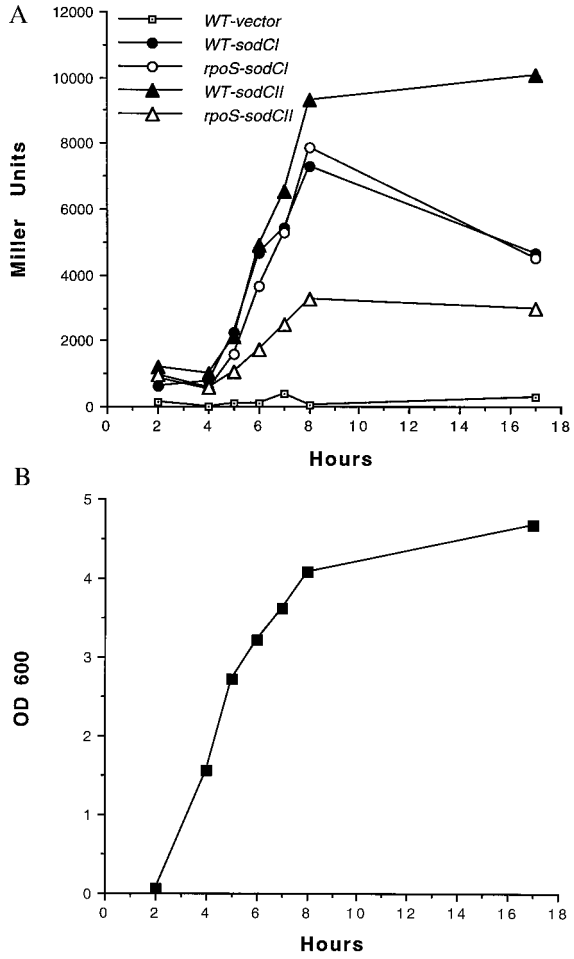

FIG. 4. Effects of growth phase and σ^S on *sodCI* and *sodCII* expression. Expression of both P_{sodC1}-*lacZ* and P_{sodCII}-*lacZ* fusions increases during the transition from exponential growth to stationary phase. However, expression of P_{sodC1}-*lacZ* is σ^S-independent and subsequently declines, whereas σ^S -dependent expression of P_{sodCII}-*lacZ* is sustained. β-Galactosidase activity in Miller units (32) from *S. typhimurium* 14028s (wild-type) or SF1005 (*rpoS*) carrying P_{sodC1}-*lacZ* or P_{sodCII}-*lacZ* fusions is shown in *A*. The OD_{600} of *S. typhimurium* 14028s carrying P_{sodC1}-*lacZ* is shown in *B*; growth curves were highly similar for all strains tested.

sodCI and sodCII Expression Are Differentially Regulated. A transcriptional β-galactosidase fusion with the *sodCI* promoter was constructed by ligating a 277-bp fragment encompassing 81 upstream residues and the initial 196 bp of the *sodCI* coding region into the *Bam*HI and *Eco*RI restriction sites of reporter plasmid pRS1274 (28), and an *aph* cassette (Amersham Pharmacia) was inserted into the unique *Pst*I site of this plasmid to create a kanamycin-resistant derivative. An analogous plasmid carrying the *sodCII* promoter was constructed by using a 584-bp fragment containing 432 upstream residues and the initial 152 bp of *sodCII* coding sequence. The transcriptional fusions were electroporated into *S. typhimurium* 14028s (wild-type) and *S. typhimurium* SF1005 (*rpoS*), and β-galactosidase activity was assayed at various phases of growth in rich medium (Fig. 4). *sodCII* expression is maximal at stationary phase and σ^s-dependent. β-Galactosidase expression from a *sodCI-lacZ* reporter construct also appears to rise during the transition from exponential to stationary phase, but this induction is σ^s-independent and does not appear to be as sustained during stationary phase as that of *sodCII* (Fig. 4*A*).

Both sodC Loci Contribute to *Salmonella* Virulence in Mice. A null mutation was created in *sodCI* by insertion of a kanamycin-resistance cassette between the *Eco*RI restriction site at nucleotide 191 of the *sodCI* coding sequence and the *Cla*I site immediately downstream of the *sodCI* ORF. The interrupted *sodCI* allele was introduced into the chromosome of *S. typhimurium* 14028s by allelic exchange using suicide vector pKNG101 (27), resulting in strain MF1006. A null mutation was created in *sodCII* by cloning the 343-bp internal *sodCII* fragment into the *Eco*RI and *Hin*dIII restriction sites of suicide vector pRR10(Δ*trfA*) (24) and allowing this construct to homologously recombine into the *S. typhimurium* 14028s chromosome, creating strain MF1007. JF3912 was constructed by bacteriophage P22-mediated transduction of the *sodCII* mutation into *S. typhimurium* UK1, and construction of the *sodCI sodCII* double-mutant strain MF1008 was achieved by transduction of the *sodCI* mutation into strain MF1007. Interruption of the *sodCI* and *sodCII* genes was confirmed by using Southern blotting (data not shown). Confirmation that *sodCII* encodes ASP71 was obtained by using two-dimensional SDS/PAGE analysis to compare JF3912 with the parent strain UK1 (Fig. 1).

S. typhimurium strains carrying mutations in either or both *sodC* loci were compared with the isogenic wild-type parent strain for lethality in mice after intraperitoneal inoculation. The *Salmonella*-susceptible *Ity*^{s} C57BL/6 mice each received

600–800 organisms, and the *Salmonella*-resistant *Ity*^{r} C3H/HeN mice each received 700–1,200 organisms. In the *Salmonella*-susceptible *Ity*^{s} C57BL/6 mouse strain, differences in virulence between wild-type *S. typhimurium* and strains carrying mutations in single *sodC* genes were scarcely discernible, but the strain with mutations in both *sodC* loci caused significantly less mortality (Fig. 5*A*). In the more resistant *Ity*^{r} C3H/HeN mouse strain, all of the *sodC* mutant strains were significantly attenuated for virulence (Fig. 5*B*). Thus, the results in the resistant mouse strain demonstrate that each *sodC* locus contributes to virulence, whereas the results in the susceptible mouse strain show that these contributions can be additive.

DISCUSSION

The importance of periplasmic Cu, Zn-SOD in *Salmonella* pathogenesis is underscored by existence of two distinct and unlinked *sodC* genes. The first of these genes to be discovered is now designated *sodCI*, and appears to be encoded on a cryptic λ-like bacteriophage (13, 14). The second locus, *sodCII*, is more closely related to the *E. coli sodC* gene (Fig. 2).

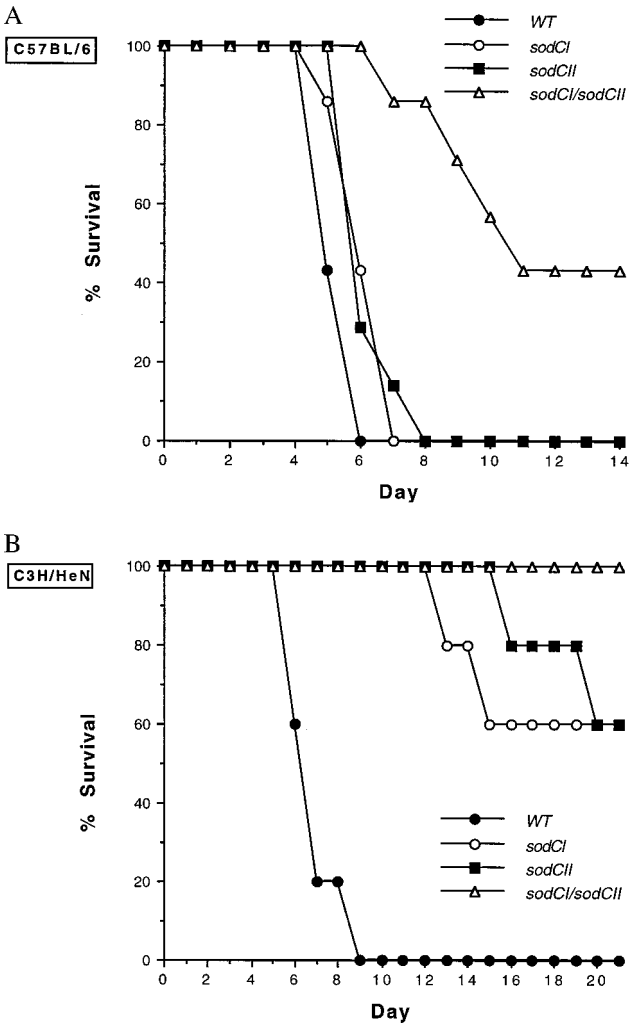

FIG. 5. Mortality of C57BL/6 or C3H/HeN mice infected with *sodC* mutant *S. typhimurium*. Intraperitoneal inocula of wild-type, *sodCI* mutant, *sodCII* mutant, or *sodCI*/*sodCII* double mutant *S. typhimurium* were administered to C57BL/6 (*Ity*^{s}) mice (*A*) or C3H/HeN (*Ity*^{r}) mice (*B*). Mortality was recorded daily. The virulence of *S. typhimurium* carrying mutations in single *sodC* loci was minimally attenuated in susceptible C57BL/6 mice, but the loss of both *sodC* loci had a marked effect on virulence. In contrast, mutations in either or both *sodC* loci significantly reduced virulence in resistant C3H/HeN mice.

Both SodC proteins appear to be functionally important, because a combination of mutations in both *sodC* genes confers a greater reduction in virulence for mice (Fig. 4*A*) than single mutations in either *sodC* gene alone. The *S. typhimurium sodCII* gene is maximally expressed in stationary phase (Fig. 4), similarly to the *sodC* genes of *E. coli* (17), *Caulobacter crescentus* (12), and *Legionella pneumophila* (11). This growth phase-dependent regulation is attributable to dependence on the alternative sigma factor σ^s (17, 24, 33). This observation further establishes σ^s as a coordinate regulator of *Salmonella* virulence determinants, because σ^s has been previously shown to control expression of the *spv* plasmid virulence genes (24). Interestingly, *sodCI* expression may also be enhanced during the growth transition from exponential to stationary phase (Fig. 3), despite its σ^s-independence, but its stationary phase expression does not appear to be as sustained as that of *sodCII*. A growing number of bacterial virulence genes have been found to be induced by nutrient deprivation or depletion (38–42), suggesting that the complex conditions of stationary-phase cultures may mimic the *in vivo* host environment encountered by pathogenic bacteria in some respects.

The contribution of SodC to *Salmonella* virulence is more readily demonstrated in genetically resistant *Ity*^{r} C3H/HeN mice than in genetically susceptible *Ity*^{s} C57BL/6 mice carrying the Nramp1 G169D allele (ref. 43 and 44; Fig. 5). Because Cu, Zn-SOD has been implicated in the resistance of *Salmonella* to products of nitric oxide synthase and the phagocyte NADPH oxidase (13), the reduced phagocyte production of reactive nitrogen and oxygen species associated with the G169D Nramp1 mutation (45–47) is likely to account for the diminished role of Cu, Zn-SOD for *Salmonella* virulence in *Ity*^{s} mice.

Although nearly all nontyphoidal *Salmonella* are considered to represent the single species *S. enterica* (48), it is well recognized that individual *S. enterica* strains can vary widely in their pathogenicity (49–52). Our analysis of the distribution of *sodC* alleles among various *Salmonella* isolates reveals that *sodCII* is widely conserved among *Salmonella* species (Table 2), but *sodCI* is only carried by selected strains belonging to some of the most highly pathogenic serotypes (53), including those associated with extraintestinal infection (49, 51). Thus, *sodCI* joins the *spv* plasmid virulence genes (35, 49, 54) and *sopE* (55) as genetic loci that can help to explain the diversity of *Salmonella* virulence. The association of *sodCI* and *spvR* with mobile genetic elements presumably accounts for their patchy distribution within *Salmonella* strains (Fig. 3).

After its divergence from *E. coli* approximately 100 million years ago, *Salmonella* acquired several "pathogenicity islands" now carried by nearly all strains (52, 55–60). However, additional horizontally acquired determinants carried by bacteriophages or plasmids appear to have enhanced the virulence of many of the strains responsible for *Salmonella*-associated morbidity and mortality in the world today. It is evident from our studies that classical Kaufmann–White serotype identification correlates imperfectly with the presence of important virulence loci. The analysis of the *sodC* genes and their distribution powerfully illustrates the ability of a genotypic perspective to provide important insights into the evolution of bacterial virulence.

This work is supported in part by grants from the National Institutes of Health (A.B., M.A.D., U.O., J.W.F., and F.F.), the United States Department of Agriculture (A.B.), and the James Biundo Foundation (F.F.). The authors are grateful to Andrés Vazquez-Torres for critical review of this manuscript prior to publication, and thank the University of Colorado Health Sciences Center Cancer Center Sequencing Facility for technical support.

1. Martin, J. P., Jr., & Fridovich, I. (1981) *J. Biol. Chem.* **256,** 6080–6089.
2. Bricker, B. J., Tabatabai, L. B., Judge, B. A., Deyoe, B. L. & Mayfield, J. E. (1990) *Infect. Immun.* **58,** 1935–1939.
3. Steinman, H. M. & Ely, B. (1990) *J. Bacteriol.* **172,** 2901–2910.
4. Kroll, J. S., Langford, P. R. & Loynds, B. M. (1991) *J. Bacteriol.* **173,** 7449–7457.
5. Langford, P. R., Loynds, B. M. & Kroll, J. S. (1992) *J. Gen. Microbiol.* **138,** 517–522.
6. Benov, L. T. & Fridovich, I. (1994) *J. Biol. Chem.* **269,** 25310–25314.
7. Kroll, J. S., Langford, P. R., Wilks, K. E. & Keil, A. D. (1995) *Microbiology* **141,** 2271–2279.
8. Canvin, J., Langford, P. R., Wilks, K. E. & Kroll, J. S. (1996) *FEMS Microbiol. Lett.* **136,** 215–220.
9. Lainson, F. A., Thomson, N., Rowe, H. A., Langford, P. R., Aitchison, K. D., Donachie, W. & Kroll, J. S. (1996) *FEMS Microbiol. Lett.* **142,** 11–17.
10. Stevens, M. K., Hassett, D. J., Radolf, J. D. & Hansen, E. J. (1996) *Gene* **183,** 35–40.
11. St. John, G. & Steinman, H. M. (1996) *J. Bacteriol.* **178,** 1578–1584.
12. Schnell, S. & Steinman, H. M. (1995) *J. Bacteriol.* **177,** 5924–5929.
13. DeGroote, M. A. D., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y. & Fang, F. C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 13997–14001.
14. Farrant, J. L., Sansone, A., Canvin, J. R., Pallen, M. J., Langford, P. R., Wallis, T. S., Dougan, D. & Kroll, J. S. (1997) *Mol. Microbiol.* **25,** 785–796.
15. Wilks, K. E., Dunn, K. L., Farrant, J. L., Reddin, K. M., Gorringe, A. R., Langford, P. R. & Kroll, J. S. (1998) *Infect. Immun.* **66,** 213–217.
16. San Mateo, L. R., Hobbs, M. M. & Kawula, T. H. (1998) *Mol. Microbiol.* **27,** 391–404.
17. Gort, A. S., Ferber, D. M. & Imlay, J. (1999) *Mol. Microbiol.* **32,** 179–191.
18. Bochner, B. R. (1984) *BioTechniques* **2,** 234–240.
19. Blomfield, I. C., Vaughn, V., Rest, R. F. & Eisenstein, B. I. (1991) *Mol. Microbiol.* **5,** 1447–1457.
20. Vogel, H. J. & Bonner, D. M. (1956) *J. Biol. Chem.* **218,** 97–106.
21. Curtiss, R. I., Porter, S. B., Munson, M., Tinge, S. A., Hassan, J. O., Gentry-Weeks, C. & Kelly, S. M. (1981) in *Colonization Control of Human Bacterial Enteropathogens in Poultry*, eds. Blankenship, L. C., Bailey, J. H. S., Cox, N. A., Stern, N. J. & Meinersmann, R. J. (Academic, New York), pp. 169–198.
22. Bearson, S. M. D., Benjamin, W. H. J., Swords, W. E. & Foster, J. W. (1996) *J. Bacteriol.* **178,** 2572–2579.
23. Boyd, E. F., Wang, F. S., Beltran, P., Plock, S. A., Nelson, K. & Selander, R. K. (1993) *J. Gen. Microbiol.* **139,** 1125–1132.
24. Fang, F. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J., Harwood, J. & Guiney, D. G. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11978–11982.
25. Simon, R., O'Connell, M., Labes, M. & Pühler, A. (1986) *Methods Enzymol.* **118,** 640–659.
26. Miller, V. L. & Mekalanos, J. J. (1988) *J. Bacteriol.* **170,** 2575–2583.
27. Kaniga, K., Delor, I. & Cornelis, G. R. (1991) *Gene* **109,** 137–141.
28. Simons, R. W., Houman, F. & Kleckner, N. (1987) *Gene* **53,** 85–96.
29. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
30. Mahon, J. & Lax, A. J. (1993) *Epidemiol. Infect.* **111,** 455–464.
31. Spector, M. P., Aliabadi, Z., Gonzalez, T. & Foster, J. W. (1986) *J. Bacteriol.* **168,** 420–424.
32. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
33. Lee, I. S., Lin, J., Hall, H. K., Bearson, B. & Foster, J. W. (1995) *Mol. Microbiol.* **17,** 155–167.
34. Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5189–5193.
35. Ahmer, B. M., Tran, M. & Heffron, F. (1999) *J. Bacteriol.* **181,** 1364–1368.
36. Levine, W. C., Buehler, J. W., Bean, N. H. & Tauxe, R. V. (1991) *J. Infect. Dis.* **164,** 81–87.
37. Boyd, E. F. & Hartl, D. L. (1998) *Genetics* **149,** 1183–1190.
38. DeMaio, J., Zhang, Y., Ko, C., Young, D. B. & Bishai, W. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2790–2794.
39. Badger, J. L. & Miller, V. L. (1998) *J. Bacteriol.* **180,** 793–800.
40. Byrne, B. & Swanson, M. S. (1998) *Infect. Immun.* **66,** 3029–3034.
41. Engelbrecht, F., Dominguez-Bernal, G., Hess, J., Dickneite, C., Greiffenberg, L., Lampidis, R., Raffelsbauer, D., Daniels, J. J., Dreft, J., Kaufmann, S. H., *et al.* (1998) *Mol. Microbiol.* **30,** 405–417.
42. Kullik, I., Giachino, P. & Fuchs, T. (1998) *J. Bacteriol.* **180,** 4814–4820.
43. Vidal, S. M., Malo, D., Vogan, K., Skamene, E. & Gros, P. (1993) *Cell* **73,** 469–485.
44. Vidal, S. M., Pinner, E., Lepage, P., Gauthier, S. & Gros, P. (1996) *J. Immunol.* **157,** 3559–3568.
45. Denis, M., Forget, A., Pelletier, M. & Skamene, E. (1988) *Clin. Exp. Immunol.* **73,** 370–375.
46. Rojas, M., Barrera, L. F., Puzo, G. & Garcia, L. F. (1997) *J. Immunol.* **159,** 1352–1361.
47. Olivier, M., Cook, P., Desanctis, J., Hel, Z., Wojciechowski, W., Reiner, N. E., Skamene, E. & Radzioch, D. (1998) *Eur. J. Biochem.* **251,** 734–743.
48. Le Minor, L. & Popoff, M. Y. (1987) *Int. J. Syst. Bacteriol.* **37,** 465–468.
49. Roudier, C., Krause, M., Fierer, J. & Guiney, D. G. (1990) *Infect. Immun.* **58,** 1180–1185.
50. Fierer, J., Krause, M., Tauxe, R. & Guiney, D. (1992) *J. Infect. Dis.* **166,** 639–642.
51. Ochman, H., Soncini, F. C., Solomon, F. & Groisman, E. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7800–7804.
52. Bäumler, A. J., Tsolis, R. M., Ficht, T. A. & Adams, L. G. (1998) *Infect. Immun.* **66,** 4579–4587.
53. Goldberg, M. B. & Rubin, R. H. (1988) *Infect. Dis. Clin. North. Am.* **2,** 571–598.
54. Gulig, P. A., Danbara, H., Guiney, D. G., Lax, A. J., Norel, F. & Rhen, M. (1993) *Mol. Microbiol.* **7,** 825–830.
55. Hardt, W. D., Urlaub, H. & Galan, J. E. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2574–2579.
56. Galan, J. E. & Curtiss, R. I. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6383–6387.
57. Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. (1995) *Science* **269,** 400–403.
58. Blanc-Potard, A. B. & Groisman, E. A. (1997) *EMBO J.* **16,** 5376–5385.
59. Collazo, C. M. & Galan, J. E. (1997) *Gene* **192,** 51–59.
60. Wong, K. K., McClelland, M., Stillwell, L. C., Sisk, E. C., Thurston, S. J. & Saffer, J. D. (1998) *Infect. Immun.* **66,** 3365–3371.