Supporting Information

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SI Materials and Methods

Bacterial Strains. *Salmonella enterica* serovar Typhimurium strain ATCC 14028s was used as wild type and as a background for the construction of *ssrB* mutations and *lacZY*-transcriptional fusions (Table S1). The chromosomal ssrB::3xFlag fusion and its C203S substitution variant were prepared using the λ Red method as described (1). Primers (Table S2) encoding 60 nucleotides homologous to a target gene followed by 20 nucleotides homologous to the pSUB11 template plasmid were used for the PCR amplification of the Flp recombinant target (FRT)-flanked kanamycin resistance cassette with 3×Flag. The amplicons were DpnI digested and electroporated into *S*. Typhimurium strain TT22236 carrying the pTP2223 plasmid that expresses the λ Red recombinase under Ptac control. Mutations were moved into *S.* Typhimurium strain 14028s by P22-mediated transduction, and pseudolysogens were eliminated by streaking on Evans blue uranine agar plates. In-frame deletions were generated by recombining the two FRT sites flanking the kanamycin resistance cassette with the Flp recombinase encoded in the pCP20 plasmid (2). The *ssrB*::3×Flag or *ssrB* C203S::3×Flag was verified by sequencing analysis of a PCR fragment generated from genomic DNA. The *sspH2::*, *srfH::*, and *sifA::lacZY* transcriptional fusions were transduced from strains AV0208, AV0209, and AV0210 (3) into strains AV07104 and AV08171 expressing the *ssrB::3xFLAG* and *ssrB C203S::3xFLAG* alleles, respectively.

Biotin Switch Assay. Free thiols were blocked with four volumes of 250 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine (HEN) buffer containing 20 mM methyl methanethiosulfonate and 2.5% (wt/vol) SDS at 50 °C with frequent vortexing. After 20 min, the cytoplasmic proteins were precipitated and washed with ice-cold acetone. The proteins were solubilized in 1% (wt/vol) SDS HEN buffer, and the nitrosothiols present in the specimens were reduced with 1 mM ascorbate. The exposed thiol groups were derivatized with 16.66 mM biotin {N-[6-(Biotinamido)hexyl]-3′-(2’-pyridyldithio)-propionamide} (Thermo Scientific) for 1 h at room temperature. After biotinylation of the S-nitrosothiols, the proteins were precipitated and washed with ice-cold acetone. The protein pellets were solubilized in 1% (wt/vol) SDS HEN buffer and mixed with two volumes of low-salt neutralization buffer (1 mM EDTA, 0.1 mM neocuproine, 100 mM NaCl, 0.5% (vol/vol) Triton X-100, 250 mM Hepes-NaOH, pH 7.7). A NeutrAvidin-agarose resin (Thermo Scientific) was added, and the mixture was incubated at 4 °C overnight. The resin was washed with high-salt neutralization buffer (1 mM EDTA, 0.1 mM neocuproine, 600 mM NaCl, 0.5% (vol/vol) Triton X-100, 250 mM Hepes-NaOH, pH 7.7), and the proteins were eluted after boiling the resin in 2× SDS sample buffer containing β-mercaptoethanol. The proteins were resolved on 12% (vol/vol) SDS/PAGE and electroblotted onto a nitrocellulose membrane. The blots were probed for the SsrB::3xFLAG protein with the M2 monoclonal antibody (Sigma-Aldrich).

Expression and Purification of the SsrB Carboxyl-Terminal Domain Protein. A 5′ fragment of *ssrB* encoding residues 137–212 (SsrBc) was cloned in pET14b and expressed in *Escherichia coli* strain BL21(DE3), yielding *E. coli* strain AV0505. Expression of SsrBc was induced for 5 h by adding 1 mM isopropyl-β-D-thiogalactopyranoside to cultures of AV0505 grown in LB medium to an OD_{600} of 0.5. The cells were harvested by centrifugation, freeze/thawed at −80 °C, and incubated on ice for 20 min in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole lysis buffer, pH 8.0, containing 1 mg/mL chicken white lysozyme. The cells were lysed by sonication. The cleared lysate was mixed with a Ni^{++}-nitrilotriacetic acid resin (Qiagen) for 1 h at 4 °C. The resin was washed with 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole buffer, pH 8.0, and the His-tagged SsrBc protein was eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole buffer, pH 8.0. The protein was stored at −80 °C after glycerol was added to a final concentration of 5%.

EMSA. EMSAs were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific). Briefly, the SsrBc protein was affinity purified as described above. SsrBc was incubated at room temperature in 10 mM Tris-HCl, 50 mM KCl, 2.5% (vol/vol) glycerol, 5 mM $MgCl_2$, 50 ng/μL poly(dI-dC), and 0.05% (vol/vol) Nonidet P-40 binding buffer, pH 7.5. After 10 min, 20 fmol of a biotin-labeled *ssrA* DNA fragment containing SsrB binding sites was added to the equilibrated SsrBc protein. After 20 min incubation at room temperature, the samples were resolved on a 5% nondenaturing polyacrylamide gel prepared in 45 mM Tris-borate and 1 mM EDTA (TBE) buffer. The specimens were electrotransferred onto a 0.45-μm Biodyne B nylon membrane (Pall Corporation) at 380 mA for 30 min at 4 °C, and crosslinked to the membrane using a TL-2000 UV Translinker (Ultra-Violet Products). The blots were developed using Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Scientific).

Circular Dichroism Spectroscopy. The His6-SsrB protein obtained by Ni^{++}-nitrilotriacetic acid affinity chromatography was purified further at 4 °C on a Superdex-75 size-exclusion FPLC column equilibrated with 200 mM NaCl, 10% (vol/vol) glycerol, 50 mM NaH_2PO_4 buffer, pH 8.0. Positive fractions were pooled and concentrated to 10 mg/mL using Centricon filter devices. Circular dichroism spectroscopy was performed on a Jasco-810 spectrometer with constant nitrogen flushing (Jasco). Circular optical cells with a path length of 0.05 cm were used to determine the spectra of proteins in PBS (100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.4) over a wavelength of 195–250 nm in 1-nm increments. Each spectrum is the average of six wavelength scans.

Transcriptional Analysis. Transcription of SPI2 genes was induced in vitro by culturing *Salmonella* in 8 μM $MgCl_2$ N salts medium (4). Briefly, *Salmonella* strains harboring *lacZY* transcriptional fusions grown overnight in LB broth were subcultured in high Mg^{2+} N salts medium [5 mM KCl, 7.5 mM $(NH_4)SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 10 mM $MgCl_2$, 38 mM glycerol, 0.1% (wt/vol) casamino acids, 100 mM Tris-HCl, pH 7.6]. The bacteria were grown in high Mg^{2+} N salts medium at 37 °C in a shaker incubator until they reached an OD_{600} of 0.5. SPI2 transcription was induced by switching the bacteria to 8 μM $MgCl_2$ N salts medium, pH 6.9, for 3 h. The expression of the *lacZY* transcriptional fusions was quantified spectrophotometrically as β-galactosidase enzymatic activity using the substrate *o*-nitrophenyl-β-D-galactopyranoside. β-galactosidase activity is expressed in Miller Units using the equation: $1{,}000 \times [(OD_{420} - 1.75 \times OD_{550})/(T_{(min)} \times V_{(mL)} \times OD_{600})]$.

Macrophages. Peritoneal macrophages were harvested from B6.129S6-*Cybb*^{tm1din}/J (gp91*phox*$^{-/-}$) (5) or gp91*phox*$^{-/-}$ iNOS$^{-/-}$ (6, 7) mice 4 d after i.p. inoculation of 1 mg/mL sodium periodate (8). The peritoneal exudate cells were resuspended in RPMI 1640 cell culture medium (Mediatech) supplemented with 10% heat-inactivated FBS (BioWhittaker), 15 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich) (RPMI$^+$ medium)

containing 100 U/mL penicillin and 100 mg/mL streptomycin (Mediatech). The peritoneal exudate cells were seeded at 2×10^5 cells/mL in 96-well plates (BD Biosciences) for β-galactosidase activity assays. The macrophages were enriched by adherence after 48 h of culture at 37 °C in a 5% CO_2 incubator.

Measurement of Intracellular Transcription of *sifA*. The β-galactosidase activity of *S.* Typhimurium strains harboring a *sifA::lacZY* transcriptional fusion was studied in gp91*phox*$^{-/-}$ or gp91*phox*$^{-/-}$ iNOS$^{-/-}$ macrophages. Macrophages were challenged at an multiplicity of infection of 2 with *lacZY*-expressing *S.* Typhimurium strains opsonized with 10% normal mouse serum for 20 min as described (9). Extracellular bacteria were removed from the macrophage monolayers 25 min after challenge by washing with prewarmed RPMI$^+$ medium containing 12 μg/mL gentamicin (Sigma-Aldrich). *S.* Typhimurium-infected macrophages were lysed with 0.25% (wt/vol) deoxycholate 8 h after challenge. β-galactosidase activity was measured in an Lmax luminometer (Molecular Devices) with the Galacto-Light Plus chemiluminescent reporter gene assay system according to the manufacturer's instructions (Applied Biosystems). Intracellular β-galactosidase expression is represented as mean arbitrary units of light/10^6 bacteria (A.U.) ± SEM.

Virulence of *Salmonella* in Murine Models of Infection. C3H/HeNcrl/Br mice (8–10 wk old) were purchased from Charles River Laboratories. NRAMP1^R C3H/HeNCrl mice were challenged *per os* with ∼10^7 cfu or i.p. with 3×10^3 cfu/mouse of *ssrB* C203S::3xFLAG *S.* Typhimurium (strain AV08171) or its isogenic control *ssrB*::3xFLAG (strain AV07104). Survival of *Salmonella*-infected mice was scored over time. Some of the mice were killed 5 or 10 d after infection. The spleens, livers, and mesenteric lymph nodes were excised and macerated in sterile PBS for plating on LB agar plates.

1. Uzzau S, Figueroa-Bossi N, Rubino S, Bossi L (2001) Epitope tagging of chromosomal genes in Salmonella. *Proc Natl Acad Sci USA* 98:15264–15269.
2. Cherepanov PP, Wackernagel W (1995) Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158:9–14.
3. McCollister BD, Bourret TJ, Gill R, Jones-Carson J, Vázquez-Torres A (2005) Repression of SPI2 transcription by nitric oxide-producing, IFNγ-activated macrophages promotes maturation of Salmonella phagosomes. *J Exp Med* 202:625–635.
4. Deiwick J, Nikolaus T, Erdogan S, Hensel M (1999) Environmental regulation of Salmonella pathogenicity island 2 gene expression. *Mol Microbiol* 31:1759–1773.
5. Pollock JD, et al. (1995) Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet* 9:202–209.
6. MacMicking JD, et al. (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 81:641–650.
7. Shiloh MU, et al. (1999) Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* 10:29–38.
8. Ding AH, Nathan CF, Stuehr DJ (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol* 141: 2407–2412.
9. McCollister BD, et al. (2008) N_2O_3 enhances the nitrosative potential of IFNγ-primed macrophages in response to *Salmonella*.. *Immunobiology* 212:759–769.

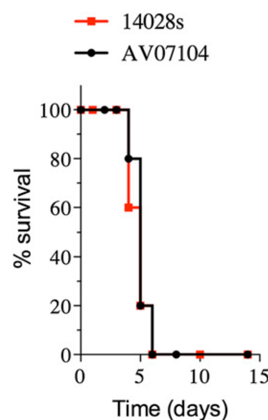

Fig. S1. Survival of C57BL/6 mice after i.p. inoculation of about 200 cfu of wild-type *Salmonella* strains ATCC 14028s or AV07104.

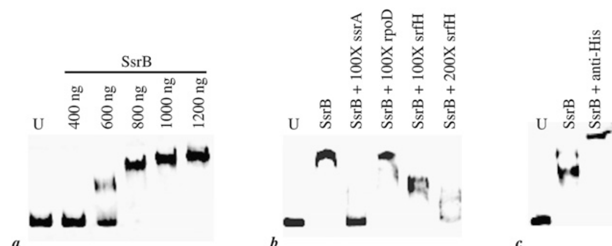

Fig. S2. (*A*) Gel shift of increasing concentrations of recombinant SsrBc and a biotinylated DNA fragment containing the *ssrA* promoter. U, unbound (control *ssrA* samples). (*B*) Binding of 300 ng SsrBc and 20 fmol biotinylated *ssrA* promoter in presence of 100- to 200-fold excess of unlabeled *ssrA*, *rpoD*, or *srfH* DNA. (*C*) Effect of 2 μg anti-6× His tag polyclonal antibody on the electrophoretic migration of His-tagged SsrBc and its cognate *ssrA* promoter.

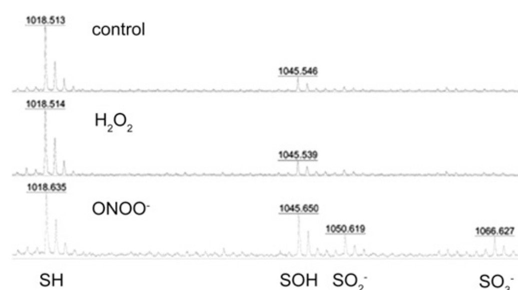

Fig. S3. Selective oxidation of the thiol group of SsrBc C203 by $ONOO^-$ but not H_2O_2. Oxidation of C203 on SsrBc was analyzed by mass spectrometry after treatment of recombinant SsrBc with 100 μM $ONOO^-$ or 1 mM H_2O_2. Untreated SsrBc was used as control.

Table S1. Bacterial strains and plasmids

Strain	Relevant characteristic	Source
E. coli strain BL21(DE3)	F-ompT hsdB (r-Bm-B) gal dcm(DE3) pLysS (Cm^R)	Novagen
Salmonella Typhimurium strain 14028s	Wild type	ATCC
AV0208	*sspH2::lacZY*	(1)
AV0209	*srfH::lacZY*	(1)
AV0210	*sifA::lacZY*	(1)
AV0321	Δ*ssrB::FRT*	This study
AV0505	pET14b::*ssrB* (aa137-212)	This study
AV0529	pET14b::*ssrB* (aa137-212) C203S	This study
AV0628	pET14b::*ssrB* (aa137-212) C203E	This study
AV07185	pET14b::ssrB (aa137-212) C203D	This study
AV07260	pGEX-6P-1::*ssrB* (aa137-212)	This study
AV0866	pGEX-6P-1::*ssrB* (aa137-212) C203D	This study
AV07104	*ssrB*::3xFLAG	This study
AV08171	*ssrB* C203S::3xFLAG	This study
AV08267	*ssrB*::3xFLAG *sifA::lacZY*	This study
AV08268	*ssrB*::3xFLAG *srfJ::lacZY*	This study
AV08269	*ssrB*::3xFLAG *sspH2::lacZY*	This study
AV08270	*ssrB* C203S::3xFLAG *sifA::lacZY*	This study
AV08271	*ssrB* C203S::3xFLAG *srfJ::lacZY*	This study
AV08272	*ssrB* C203S::3xFLAG *sspH2::lacZY*	This study
Plasmids		
pCP20	bla cat cI857 lPR flp pSC101 oriTS	(2)
pKD13	bla FRT ahp FRT PS1 PS4 oriR6K	(3)
pWSK29	bla lacZa oripSC101	(4)
pSUB11	3xFLAG FRT ahp FRT bla R6KoriV	(5)
pCE36	ahp FRT lacZY+ this oriR6K	(6)
pET14b	His-tag expression vector with T7 promoter	Novagen
pGEX-6P-1	GST expression vector with tac promoter	GE Healthcare

1. McCollister BD, Bourret TJ, Gill R, Jones-Carson J, Vázquez-Torres A (2005) Repression of SPI2 transcription by nitric oxide-producing, IFNγ-activated macrophages promotes maturation of Salmonella phagosomes. *J Exp Med* 202:625–635.
2. Cherepanov PP, Wackernagel W (1995) Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158:9–14.
3. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645.
4. Wang RF, Kushner SR (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli. *Gene* 100:195–199.
5. Uzzau S, Figueroa-Bossi N, Rubino S, Bossi L (2001) Epitope tagging of chromosomal genes in Salmonella. *Proc Natl Acad Sci USA* 98:15264–15269.
6. Ellermeier CD, Janakiraman A, Slauch JM (2002) Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. *Gene* 290:153–161.

Table S2. Primers

Gene	Primers	Region encoded from translational start site
srfH	F5′-gaaggtggcctcatctgaaa-3′	−440 −205
	R5′-gctttccacgatgaccagat-3′	
ssrA	F5′-accttgtcacaggcgattct-3′	−291 +104
	R5′-aagcacagatagcagccaaa-3′	
rpoD	F5′-gtggcttgcaattccttgat-3′	+1331 +1553
	R5′-agcatctggcgagaaatacg-3′	
ssrB::3xFLAG	F5′-tgtgcccgaagaatgaggttaatagagtatgactacaaagaccatgacgg-3′*	
	R5′-atccgtatggattttgtcgatgatgagcaatactgcgtgcatatgaatatcctccttag-3′[†]	
ssrB C203S::3xFLAG	F5′-gttacttaactctgcccgaagaatgaggttaatagagtatgactacaaagaccatgac-3′	

*The sequences for pSUB11 are underlined.
[†]The reverse primer was used for the construction of both ssrB::3xFLAG and ssrB C203S::3xFLAG alleles.