Redox sensor SsrB Cys[203] enhances *Salmonella* fitness against nitric oxide generated in the host immune response to oral infection

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We show herein that the *Salmonella* pathogenicity island 2 (SPI2) response regulator SsrB undergoes S-nitrosylation upon exposure of *Salmonella* to acidified nitrite, a signal encountered by this enteropathogen in phagosomes of macrophages. Mutational analysis has identified Cys[203] in the C-terminal dimerization domain of SsrB as the redox-active residue responding to nitric oxide (NO) congeners generated in the acidification of nitrite. Peroxynitrite and products of the autooxidation of NO in the presence of oxygen, but not hydrogen peroxide, inhibit the DNA-binding capacity of SsrB, demonstrating the selectivity of the reaction of Cys[203] with reactive nitrogen species (RNS). These findings identify the two-component response regulator SsrB Cys[203] as a thiol-based redox sensor. A C203S substitution protects SsrB against the attack of RNS while preserving its DNA-binding capacity. When exposed to SPI2-inducing conditions, *Salmonella* expressing the wild-type *ssrB* allele or the *ssrB* C203S variant sustain transcription of the *sifA*, *sspH2*, and *srfJ* effector genes. Nonetheless, compared with the strain expressing a redox-resistant SsrB C203S variant, wild-type *Salmonella* bearing the NO-responsive allele exhibit increased fitness when exposed to RNS in an NRAMP[R], C3H/HeN murine model of acute oral infection. Given the widespread occurrence of the wild-type allele in *Salmonella enterica*, these findings indicate that SsrB Cys[203] increases *Salmonella* virulence by serving as a redox sensor of NO resulting from the host immune response to oral infection.

macrophages | pathogenesis | two-component regulatory system

Nitric oxide (NO) and its congeners react with metal prosthetic groups, organic and inorganic radicals, lipids, and DNA molecules. This rich biochemistry probably mediates the broad-spectrum antimicrobial activity of reactive nitrogen species (RNS) against phylogenetically diverse microorganisms (1). Diarrheagenic *Salmonella* elicit a burst of NO in rectal content in infected humans (2) and stimulate the accumulation of iron-nitrosyl adducts in affected viscera of experimental animals (3). Mounting evidence indicates that these RNS are not mere signatures of the infection but are active components of the anti-*Salmonella* host arsenal. The gross susceptibility of mice unable to synthesize NO in response to experimentally induced salmonellosis is arguably the most dramatic proof of the role for RNS in the host response to *Salmonella* (3, 4).

Despite being a radical, NO exhibits remarkable specificity in its reactions with organic and inorganic molecules. Most direct effects of NO on biological systems stem from its interaction with transition metals in terminal cytochromes of the electron transport chain and [4Fe-4S] prosthetic groups in dehydratases (5, 6). The selectivity of NO for metalloproteins also applies to *Salmonella*, as shown by the nitrosylation of the *bd* quinol cytochrome oxidase (7); in that case, NO is a positive signal that stimulates the expression of antioxidant defenses. The biochemistry of this diatomic radical is enriched through its interactions with other radicals. The reaction of NO and superoxide ($O_2^{\bullet -}$), species, which are concomitantly produced by professional phagocytes (8), yields peroxynitrite ($ONOO^-$). $ONOO^-$ is a potent oxidizing species with high affinity for tyrosine residues and the solvent-exposed Feα of [4Fe-4S] clusters (9, 10). The autooxidation products nitrogen dioxide ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3) further enrich the biological chemistry of NO (11). N_2O_3, which can arise independently from the condensation of acidified nitrite (NO_2^-), oxidizes and nitrosylates redox-active thiol groups. *Salmonella* are likely to be exposed to N_2O_3 in the context of IFNγ-primed macrophages or during transit through the stomach (12–14). In turn, microorganisms sense NO through the nitrosylation of either iron or sulfhydryl metal groups in response regulators, thereby engaging signaling pathways that promote the enzymatic and nonenzymatic detoxification of RNS. By avoiding contact with inducible NOS (iNOS)-containing vesicles, the *Salmonella* pathogenicity island 2 (SPI2) type III secretion system has been shown to contribute to the antinitrosative defenses of *Salmonella* (15).

Both positive and negative regulation of SPI2 are critical for *Salmonella* pathogenesis (16). The capacity of *Salmonella* to survive within host phagocytes and proliferate within epithelial cells relies on SPI2-dependent remodeling of the *Salmonella*-containing vesicle. *Salmonella* reside intracellularly within vacuoles that establish contact with the secretory pathway but minimize fusion with late endosomes and lysosomes (17, 18). By doing so, SPI2 decreases contact of *Salmonella* with the hydrolytic enzymes of lysosomes as well as reactive oxygen species (ROS) and RNS generated by NADPH oxidase and iNOS (17, 19). Nonetheless, the overexpression of SPI2 can have adverse effects on *Salmonella* pathogenicity in vivo. In fact, negative regulation of SPI2 by YdgT is needed for *Salmonella* virulence at late stages of the infection (20). RNS also appear to exert negative control of SPI2 transcription (13, 21). RNS repress genes within and outside the SPI2, including those encoding for chaperones, the type III secretion apparatus, translocon, and effectors. Collectively, the widespread repression of SPI2 loci suggests that RNS inhibit a critical signaling pathway upstream of or involving the SsrAB two-component regulatory system. The goals of the work presented here are to test whether the SsrB response regulator is a target of RNS and to

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establish the extent to which the interaction of SsrB and RNS contributes to *Salmonella* pathogenesis.

Results

S-Nitrosylation of the Redox-Active Cys^{203} Thiol in the SsrB Response Regulator. We first sought to determine possible targets of NO-mediated inhibition of SPI2. *In silico* analysis revealed the presence of three cysteines in SsrB, raising the possibility that these residues could be targets of RNS. To determine whether SsrB contains redox-active cysteines, strain AV07104 expressing a C-terminal 3xFLAG-tagged SsrB variant was constructed using the λ Red system. This strain proved to be as virulent as its parent control when tested in an i.p. model of acute infection in C57BL/6 mice (Fig. S1). Having established its full virulence potential, we exposed strain AV07104 to nitrosative stress generated by acidified NO_2^-, which at low pH is protonated to its acid conjugate nitrous acid (HNO_2). Two molecules of HNO_2 in turn condensate to form the strong oxidative and nitrosative species N_2O_3. Acidified NO_2^- is a relevant source of nitrosative stress in our system because it adds to the RNS of *Salmonella*-infected macrophages (14). Strain AV07104 expressing an SsrB::3xFLAG was cultured for 6 h in EG medium, pH 5.5, containing 750 μM NO_2^- or NO_3^-. The amount of SsrB protein was similar in NO_2^-- and NO_3^--treated *Salmonella* (Fig. 1*A*, *Upper*). The cytoplasmic extracts were tested for the presence of S-nitrosylated SsrB after derivatization of S-nitrosylated proteins in a biotin switch assay. Immunoblots of NeutrAvidin-purified, biotinylated proteins demonstrated the S-nitrosylation of SsrB in *Salmonella* cultures exposed to acidified NO_2^- but not in controls grown in NO_3^- (Fig. 1*A*). To test the hypothesis that Cys^{203} is a redox-active cysteine modifiable by RNS, we took advantage of the C-terminal position of this residue to engineer a serine substitution by the λ Red system. The C203S substitution is a conservative mutation, because the redox thiol is exchanged for a nonreactive hydroxyl group. Wild-type and SsrB C203S variants expressed by AV07104 and AV08171 strains, respectively, were grown for 6 h in EG medium, pH 5.5, in the presence of 750 μM NO_2^-. Fig. 1*B* shows that *Salmonella* strains AV07104 and AV08171 harbor similar concentrations of the SsrB protein. However, SsrB is solely S-nitrosylated in the wild-type *Salmonella* strain AV07104 but not in its C203S-expressing isogenic control. The lack of S-nitrosylated SsrB in the *ΔssrB::FRT* strain AV0321 attests to the specificity of the assay. Collectively, these data demonstrate that Cys^{203} not only is redox active but also can be S-nitrosylated by the RNS generated by NO_2^- at an acid pH normally found in *Salmonella*-containing phagosomes.

DNA-Binding Activity of SsrB Is Selectively Inactivated by NO Congeners. The effects that RNS have on the DNA-binding capacity of SsrB were used to gain insights into the biological relevance of the redox-active Cys^{203}. We directed our efforts to the study of the DNA-binding, SsrB C-terminal domain (SsrBc) that encompasses amino acids 137–212, including Cys^{203}. SsrBc was found to bind in a concentration-dependent manner to the *ssrA* promoter (Fig. S2*A*). Binding was abrogated with 100- to 200-fold excess of unlabeled *ssrA* or *srfH* SPI2 promoters but not by a similar-sized DNA fragment from the *rpoD* gene (Fig. S2*B*). The specificity in the interaction between SsrBc and the *ssrA* DNA was demonstrated further by the supershift noted when antibodies to the His-tag were added to the reaction (Fig. S2*C*). Because the affinity of SsrBc was higher for *ssrA* than for *srfH*, the DNA fragment containing the *ssrA* promoter was used subsequently as a readout to measure the sensitivity of SsrB to oxidative and nitrosative stress. The binding activity of SsrBc to its *ssrA* cognate promoter was inhibited by 100 μM $ONOO^-$ but not by 1 mM H_2O_2 (Fig. 2*A*). These data suggest that SsrB is modified selectively by $ONOO^-$ but not H_2O_2, both of which are produced by *Salmonella*-infected macrophages (8). That SPI2 transcription can be inhibited in gp91*phox*-deficient macrophages unable to synthesize $ONOO^-$ (8) indicates that other RNS also may inhibit SsrB binding to DNA. Consequently, NO itself was tested for its ability to modify the DNA-binding capacity of SsrBc. Addition of 100 μM authentic NO prevented the formation of an SsrBc-*ssrA* complex (Fig. 2*B*). In the absence of O_2, however, NO did not affect the binding of SsrBc to *ssrA* (Fig. 2*C*). These results indicate that nitrosative and/or oxidative species such as $NO_2^\bullet$ and N_2O_3 generated during the autooxidation of NO in the presence of O_2 can inhibit the DNA-binding activity of SsrBc.

Oxidation of Cys^{203} Inactivates the DNA-Binding Capacity of SsrB. Mass spectrometry revealed that $ONOO^-$ oxidizes Cys^{203} to a mixture of sulfenic (−SOH), sulfinic ($-SO_2^-$), and sulfonic ($-SO_3^-$) groups (Fig. S3). It therefore is possible that the RNS-mediated oxidation of the thiol group in Cys^{203} inhibits binding of SsrBc to DNA. In support of the notion that oxidation of the redox-active thiol group of Cys^{203} can alter the function of SsrB, the thiol-specific oxidizer iodoacetate effectively inhibited SsrB DNA binding (Fig. 3*A*). Based on the oxidation patterns seen in the mass spectrometric analysis, genetic studies were performed to evaluate whether the oxidized, negatively charged Cys^{203} could compromise the DNA-binding capacity of SsrB. A C203D substitution was generated to mimic the negative charge of the SO_2^- and SO_3^- modifications of $ONOO^-$-treated SsrBc. An SsrBc C203D variant failed to bind to the *ssrA* promoter (Fig. 3*B*). The failure of SsrBc C203D to bind to *ssrA* does not appear to reflect overall changes in secondary structure, because circular dichroism spectra revealed an α-helical pattern similar to the wild-type protein (Fig. 3*C*). Nor does an SsrBc C203S variant affect the α-helical structure of SsrBc (Fig. 3*C*). In contrast to the

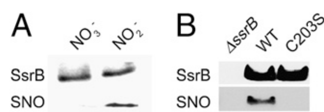

Fig. 1. SsrB Cys^{203} is S-nitrosylated in *Salmonella* cultures grown in acidified NO_2^-. (*A*) *Salmonella* were grown in EG medium, pH 5.5, in the presence of 750 μM NO_3^- or NO_2^-. S-nitrosothiols were derivatized in the biotin switch assay. SsrB::3xFLAG was detected in unfractionated bacterial cytoplasmic extracts (*Upper*) or in affinity-purified, biotinylated fractions (*Lower*). (*B*) S-nitrosylated SsrB was determined in *Salmonella* strains AV0321 (*ΔssrB*), AV07194 (WT; *ssrB*::3xFLAG), or AV08171 (SNO; C203S; *ssrB* C203S::3xFLAG) grown for 6 h in 750 μM NO_2^- EG medium, pH 5.5. Representative data from two or three independent experiments are shown.

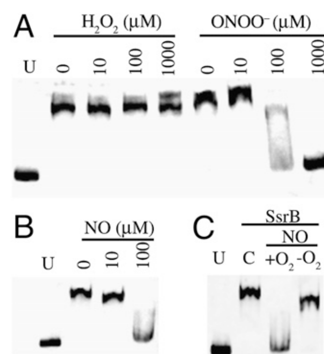

Fig. 2. Effect of ROS and RNS on the ability of SsrBc to bind to its *ssrA* cognate promoter. Effect of increasing concentrations of authentic H_2O_2, $ONOO^-$, (*A*) or NO (*B*) on the binding of SsrBc to *ssrA* DNA. (*C*) Effects of NO autooxidation products on the binding of SsrBc to the *ssrA* promoter. The blot shows the binding of SsrBc with *ssrA* in untreated (U), control (C), or NO-treated samples in the presence or absence of O_2. Representative data from two independent experiments are shown.

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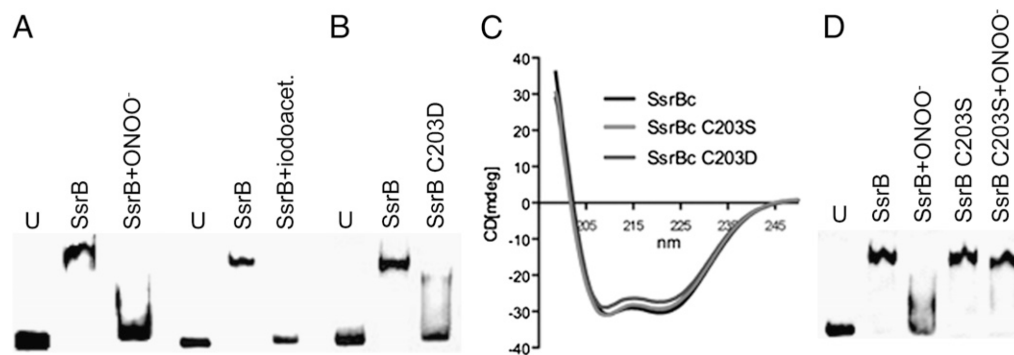

Fig. 3. Effects of the oxidation of Cys[203] on SsrB DNA binding. (*A*) Binding of 300 ng SsrBc to 20 fmol of its cognate *ssrA* promoter after treatment with 1 mM $ONOO^-$ or 25 mM iodoacetate. The unbound, biotinylated *ssrA* DNA probe (U) is shown for comparison. (*B* and *D*) DNA binding of the SsrBc C203D or C203S alleles. Where indicated, selected samples in *D* were treated with 1 mM $ONOO^-$ before binding to the DNA. (*C*) Circular dichroic (CD) spectra of 0.2 mg/mL SsrBc recombinant variants. Representative data from two or three independent experiments are shown.

SsrB C203D allele, the C203S variant bound to the *ssrA* promoter with affinity apparently similar that of the wild-type protein (Fig. 3*D*). These findings demonstrate that the cysteine in SsrBc is not required for DNA binding. Nonetheless, Cys[203] provides a mechanism for redox sensing, because the SsrBc variant bearing the C203S allele retained its ability to bind to the *ssrA* promoter after the protein was treated with 1 mM $ONOO^-$ (Fig. 3*D*). Together, these data suggest that Cys[203] serves as a redox sensor of RNS, modulating the binding of SsrB to DNA.

SsrB C203S Variant Sustains SPI2 Expression. We tested whether the SsrBc C203S variant can stimulate SPI2 expression. As expected, the *sifA::lacZY*, *srfJ::lacZY*, and *sspH2::lacZY* transcriptional fusions were induced in wild-type *Salmonella* grown in 8 μM $MgCl_2$ N salts medium, pH 6.9 (Fig. 4*A*). *Salmonella* expressing the SsrB C203S variant also supported the expression of the *sifA::lacZY*, *srfJ::lacZY*, and *sspH2::lacZY* transcriptional fusions. Of note, the expression of *sifA::lacZY* and *sspH2::lacZY* was slightly higher in *Salmonella* bearing the *ssrB* C203S allele. Because of their wild-type nitrosative capacity but inability to kill *Salmonella* (8, 14), macrophages lacking the gp91*phox* subunit of the NADPH oxidase were used to determine the contribution of the redox-active Cys[203] to the transcriptional activity of SsrB in *Salmonella* exposed to host-derived RNS. Macrophages deficient in gp91*phox* iNOS hemoproteins unable to sustain nitrosative stress were used as controls. The intracellular expression of a *sifA::lacZY* transcriptional fusion was higher in the strain AV08270 expressing SsrB C203S than in the AV08267 control bearing the wild-type *ssrB* allele (Fig. 4*B*). The lack of iNOS abrogated the differences in *sifA::lacZY* expression in these two strains, suggesting that the presence of a redox-active Cys[203] modulates the transcriptional activity of SsrB in response to RNS produced in the innate response of macrophages.

Redox-Active Wild-Type SsrB Allele Increases *Salmonella* Fitness in a Model of Acute Intestinal Infection. Given that the RNS-resistant SsrB C203S allele sustains SPI2 transcription (Fig. 4), it seems paradoxical that diverse populations of *S. enterica* adapted to a wide range of vertebrate hosts maintain a redox-sensitive wild-type *ssrB* allele in their genome. We reasoned that the presence of a redox-sensitive cysteine in the dimerization domain of SsrB may prove advantageous at some point during the association of this enteropathogen with its vertebrate host. Because *Salmonella* is transmitted primarily through contaminated food and water, we tested the virulence of wild-type and its *ssrB* C203S variant in a model of acute gastrointestinal infection. C3H/HeN mice were selected in these studies because the presence of the wild-type $NRAMP1^R$ locus is associated with increased NO production by macrophages (22). Strain AV07104 expressing the wild-type *ssrB* allele exhibited higher virulence than its isogenic *ssrB* C203S var-

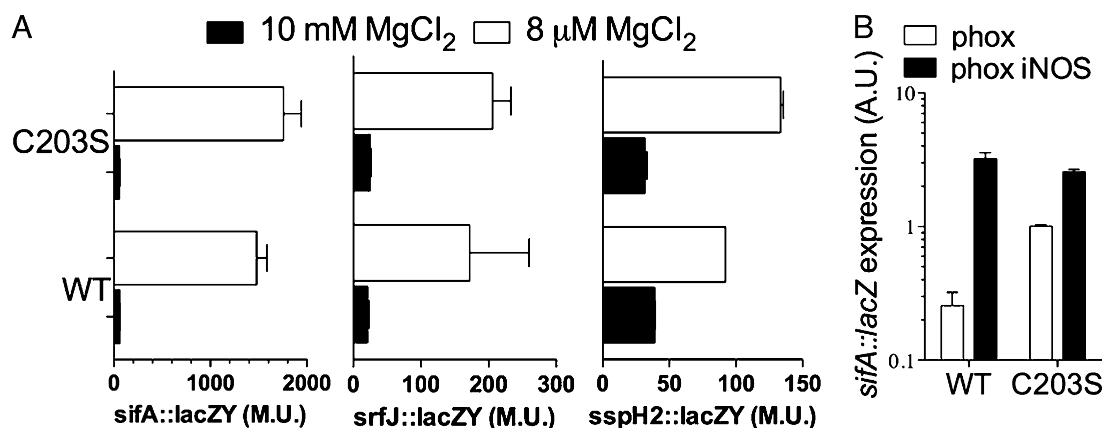

Fig. 4. Regulatory functions of SsrB C203S. (*A*) SPI2 expression was induced in wild-type and *ssrB* C203S-expressing *Salmonella* by shifting the bacteria grown to an OD_{600} of 0.5 in 10 mM $MgCl_2$ N9 medium into 8 μM $MgCl_2$ N9 medium. Expression of SPI2 genes quantified as β-galactosidase of the *lacZY* transcriptional fusions is expressed in Miller Units (M.U.) 3 h after the shift. The data represent eight observations from three independent experiments. (*B*) Transcriptional *sifA::lacZY* activity also was measured in gp91*phox* (phox) and gp91*phox* iNOS-deficient (phox iNOS) macrophages as described in *SI Materials and Methods*. Data are represented as arbitrary units of light/10^6 bacteria (A.U.) ± SEM from four independent observations.

iant when tested in an NRAMP1^R C3H/HeN murine model of acute oral infection (Fig. 5*A*). The *Salmonella* burden in mesenteric lymph nodes, liver, and spleen was similar for the wild-type and the SsrB C203S variant expressing bacteria 5 d after oral infection (Fig. 5*C*, *Left*). However, 10^3–10^5 times more wild-type bacteria were isolated from most mice 10 d after oral infection (Fig. 5*C*, *Right*). Hepatomegaly and splenomegaly were evident 10 d after C3H/HeN mice had been infected with wild-type *Salmonella* (Fig. 5*D*), probably reflecting increases in bacterial burden (Fig. 5*C*) and the inflammatory response. In fact, abscesses were noted on the surface of livers and spleens of C3H/HeN mice 10 d after infection with wild-type bacteria (Fig. 5*D*). The virulence of *Salmonella* bearing the wild-type *ssrB* allele in a C3H/HeN mouse model of acute oral infection appears to be related to the RNS-sensing capability of this response regulator, because bacteria expressing the RNS-resistant SsrB C203S allele regained full pathogenicity in C3H/HeN mice treated with 500 μg/mL of the iNOS-specific inhibitor N^6-(1-iminoethyl)-L-lysine, dihydrochloride (L-NIL) administered daily in drinking water (Fig. 5*E*). In contrast to the differences noted when the bacteria were tested in an oral model of infection, wild-type and *ssrB* C203S-expressing *Salmonella* exhibited identical virulence ($P = 1.0$, log-rank Martel–Cox test) when tested in an acute i.p. C3H/HeN model of systemic infection (Fig. 5*B*).

Discussion

Thiol groups can undergo oxidation and/or S-nitrosylation in bacteria exposed to RNS (23, 24). Our investigations have identified the two-component response regulator SsrB Cys^{203} as a thiol-based, redox sensor. A wild-type SsrB bearing a redox-active thiol in the Cys^{203} is advantageous to the interactions of *Salmonella* with its host, as demonstrated by the increased fitness of wild-type *Salmonella* over an isogenic strain expressing the RNS-resistant SsrB C203S variant in a C3H/HeN murine model of acute oral salmonellosis. The full recovery of virulence of *Salmonella* expressing the RNS-resistant SsrB C203S variant upon L-NIL treatment argues that Cys^{203} is a redox sensor of reactive species generated by the iNOS hemoprotein. The redox-sensing capabilities of SsrB appear to be dispensable for virulence of *Salmonella* in a model of acute systemic infection that is dominated by the antimicrobial activity of the NADPH phagocyte oxidase. This observation is in keeping with the fact that Cys^{203} in SsrB is relatively resistant to oxidation by H_2O_2.

Alignment of the *ssrB* allele of *Salmonella* isolates logged in the GenBank database shows that most typhoidal and nontyphoidal *Salmonella* strains encode for a conserved cysteine at position 203. The only exception is an isolate of *S*. Typhimurium strain St. Paul, which instead encodes for a tyrosine. Similar to cysteine, tyrosine is a common target of $ONOO^-$ (9). Also, in analogy to cysteine oxidation, the nitrotyrosine formed upon $ONOO^-$ treatment can disrupt protein function. The expression of cysteine or tyrosine residues at position 203 of SsrB raises the intriguing possibility that a redox-active moiety at this position provides *Salmonella* with a selective advantage. Data presented herein indicate that the fitness afforded by Cys^{203} is manifested in the context of RNS generated by the host response to *Salmonella* encountered in an oral infection. The expression of the SPI2 type III secretion system is essential for *Salmonella* pathogenesis. Nonetheless, its overexpression attenuates the virulence of *Salmonella* at later time points of the infection (20). In this context, the transcriptional control of SPI2 expression through the RNS-mediated modifications of SsrB Cys^{203}, anal-

Fig. 5. The wild-type, RNS-sensitive SsrB allele provides a selective advantage in an acute model of oral salmonellosis. The virulence of wild-type (WT) *Salmonella* strain AV07104 or its isogenic SsrB C203S variant strain AV08171 (C203S) was investigated in acute intragastric and i.p. models of infection. (*A*) Survival of NRAMP1^R C3H/HeN mice challenged *per os* (p.o.) with 10^7 cfu *Salmonella*. (*B*) NRAMP1^R C3H/HeN mice were inoculated i.p. with 3×10^3 cfu of *Salmonella*. (*C*) The bacterial burden was recorded in mesenteric lymph nodes (MLN), liver, and spleen 5 and 10 d after C3H/HeN mice were inoculated p.o. with 10^7 cfu of *Salmonella*. (*D*) Gross pathological findings of C3H/HeN mice infected p.o. with *Salmonella* for 10 d. Liver (L) and spleen (S) are identified; white arrows indicate hepatic and splenic abscesses. (*E*) Effect of 500 μg/mL of the iNOS-inhibitor L-NIL administered in drinking water on survival of C3H/HeN mice infected p.o. with 10^7 cfu of *Salmonella*. Data represent two independent experiments (*n* =5–11 mice per group).

ogous to the importance that YdgT- and Hha-mediated silencing, is important for *Salmonella* virulence (20, 25).

Chemical production of RNS by the acidification of NO_2^- in the gastric lumen and enzymatic synthesis of NO from the oxidation of L-arginine by epithelial, inflammatory, and stromal cells are likely sources of NO in the gastrointestinal tract (2, 13). The nitrosative chemistry generated by acidified NO_2^- not only inhibits SPI2 expression (13) but also, as shown herein, S-nitrosylates SsrB. The advantage of *Salmonella* expressing the wild-type, RNS-responsive *ssrB* allele is evident in mice on an $NRAMP1^R$ background, indicating that sensing of RNS by Cys^{203} must take place within macrophages where the NRAMP1 metal transporter is expressed (26). Accordingly, the increased bacterial burden of *Salmonella* expressing the wild-type *ssrB* locus was most evident after 10 d of infection. A Cys^{203} residue could be advantageous for *Salmonella* in at least three different ways. First, the RNS-dependent repression of SPI2 is concomitant with the transcriptional up-regulation of the SPI1 type III secretion system, flagella, and fimbrial operons (13). Thus, RNS may favor the expression of *Salmonella* virulence factors that promote invasion, inflammation, adhesion, and motility in the gastrointestinal phase of the infection. The effects of RNS on these *Salmonella* virulence factors are likely to occur in the gastrointestinal lumen, before the NRAMP1-dependent, RNS-mediated effects take place. Therefore, we do not favor the idea that SsrB Cys^{203} helps *Salmonella* sense RNS in the gastrointestinal lumen. Second, RNS inhibit *Salmonella*-induced apoptosis (3), in which the SPI2 type III secretion system plays a critical role (27). In this context, the inhibition of phagocyte cell death by the RNS-dependent repression of SPI2 may be advantageous later in the course of infection. Third, redox control of SsrB may down-regulate intracellular SPI2 expression and thereby minimize detection by T and B lymphocytes. The second and third models are not necessarily mutually exclusive.

Biochemical analyses have revealed that Cys^{203} can undergo several covalent modifications after *Salmonella* or recombinant SsrBc protein is exposed to a variety of NO congeners. The thiol group of Cys^{203} is S-nitrosylated in *Salmonella* challenged with acidified NO_2^-, whereas recombinant SsrBc is oxidized upon exposure to $ONOO^-$. The range of covalent modifications induced by NO congeners unequivocally demonstrates the redox nature of Cys^{203}. Structural studies have shown that the Cys^{203} side chain contacts Leu^{192} in the homodimer interface (28). The negatively charged modifications of $ONOO^-$-treated SsrB may block these interactions and thereby inhibit the formation of dimers necessary for the binding of SsrB to SPI2 promoters. Binding of SsrB to DNA also is inhibited by NO autooxidation products. N_2O_3-mediated oxidation of the Cys^{203} thiol group may have mechanistic consequences for binding of SsrB to DNA, similar to those seen in $ONOO^-$-treated protein or an SsrB Asp^{203} variant. It also is possible that S-nitrosylation of Cys^{203} may itself interfere with the binding of SsrB with DNA or may promote formation of mixed disulfides with glutathione or other small molecular weight thiols, thereby increasing the complexity of the side chain of Cys^{203}.

Redox-active Cys^{203} is not equally responsive to ROS and RNS. The DNA-binding activity of SsrB is preserved after treatment with H_2O_2 or NO but is inhibited after exposure to $ONOO^-$, oxidative byproducts of NO, or the thiol oxidizer iodoacetate. The apparent susceptibility of the Cys^{203} thiol group to $ONOO^-$-mediated oxidation is reminiscent of the preferential reactivity of $ONOO^-$ over H_2O_2 with thiols in serum albumin ($k = 2,700$ $M^{-1}s^{-1}$ vs. $k = 2.26$ $M^{-1}s^{-1}$, respectively) (29). Our observation also is consistent with the fact that most protein sulfhydryls in *Escherichia coli* are differentially affected by RNS and H_2O_2 (24). Functionally, the preservation of DNA binding in H_2O_2-treated SsrB may help explain why SPI2 protects *Salmonella* from NADPH phagocyte oxidase-derived oxyradicals (19, 30). The resistance of SsrB to biologically relevant ROS also may explain the comparable virulence of *Salmonella* strains expressing the wild-type or the redox-resistant SsrB C203S variant in the i.p. model of infection, because this phase is dominated by the antimicrobial activity of the NADPH phagocyte oxidase.

The number of sensors of nitrosative stress in bacteria continues to increase. The transcriptional regulators [2Fe-2S] SoxR and [4Fe-4S] Fnr serve as examples (31, 32). To the best of our knowledge, OxyR is the only identified bacterial regulator in which sulfhydryls have been co-opted as sensors of nitrosative stress (33). In this respect, Cys^{203} in the C-terminal H4 helix of the dimerization domain of SsrB serves a function analogous to that of OxyR Cys^{199}. There are, nonetheless, significant differences between these two bacterial regulators. The ability of OxyR to sense nitrosative stress appears to be secondary to its recognition of endogenous H_2O_2. In contrast, as discussed above, SsrB appears to be more specific for RNS than ROS. Furthermore, S-nitrosylation of Cys^{203} is manifested readily in wild-type *Salmonella,* whereas the RNS-dependent modifications of OxyR are best noted in *E. coli* lacking the reductive power of glutathione (33). Another important difference between these two regulators lies in the activation of DNA binding by OxyR in response to nitrosative stress, whereas RNS inhibit the formation of SsrB-DNA complexes. In this sense, Cys^{203} of SsrB serves a function analogous to that of Cys^{13} of the *Staphylococcus* global regulator SarZ, whose DNA-binding activity is disrupted upon oxidation with H_2O_2 or organic hydroperoxides (34).

In conclusion, Cys^{203} in the C-terminal dimerization domain of the SsrB response regulator is a sensor of RNS. RNS-mediated posttranslational modifications of Cys^{203} repress the binding of SsrBc to DNA. This redox-active cysteine provides *Salmonella* with a selective advantage in the context of nitrogen oxides encountered when the infection takes place through the oral mucosa. The widespread occurrence of a cysteine at this position in SsrB of medically important *Salmonella* isolates indicates that RNS exert a positive selective pressure in the effector domain of SsrB in both typhoidal and nontyphoidal strains of *Salmonella enterica*.

Materials and Methods

Bacterial Strains. *Salmonella enterica* serovar Typhimurium strain ATCC 14028s was used as wild type and as the background for the construction of *ssrB* mutations and *lacZY*-transcriptional fusions (Table S1). Bacterial strains were constructed using primers in Table S2 following the λ Red method as described in *SI Materials and Methods*.

EMSA. EMSAs were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific,) as described in *SI Materials and Methods*.

In Vivo Detection of S-Nitrosylated SsrB Protein. *S.* Typhimurium strains AV07104 and AV08171 expressing 3xFLAG-tagged SsrB and 3xFLAG-tagged SsrB C203S variants were grown overnight in LB medium. Strain AV321 (*ΔssrB::FRT*) was used as a negative control. The bacteria were subcultured at an OD_{600} of 0.3 in EG medium, (0.2 g/L $MgSO_4$, 2 g/L $C^6H_8O_7$-H_2O, 10 g/L K_2HPO_4, 3.5 g/L $Na(NH_4)HPO_4$-4 H_2O, and 4 g/L D-glucose) pH 5.5, with 750 μM $NaNO_2^-$ or $NaNO_3^-$ at 37 °C for 6 h, with shaking. Bacterial soluble lysates were obtained by sonication in 250 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine (HEN) buffer, pH 7.7, and the protein concentration in the clear lysates adjusted to 0.8 mg/mL. S-nitrosylation was detected by the biotin switch method (35) as described in *SI Materials and Methods*.

Circular Dichroism Spectroscopy. Circular dichroism spectroscopy was performed on a Jasco-810 spectrometer as described in *SI Materials and Methods*.

Transcriptional Analysis. Transcription of SPI2 genes was induced in vitro by culturing *Salmonella* in 8 μM $MgCl_2$ N salts medium (36). SPI2 transcriptional analysis also was studied in gp91*phox* and gp91*phox* iNOS-deficient macrophages exhibiting different nitrosative capacities. The methods are described in *SI Materials and Methods*.

Virulence of *Salmonella* in Murine Models of Infection. C3H/HeNcrl/Br mice (8–10 wk old) were used to assess oral virulence of *Salmonella* as described in *SI Materials and Methods*.

Statistical Analysis. Data are presented as means ± SEM or SD. The statistical significance was calculated with a two-way ANOVA, followed by a Bonferroni post test. Differences in mouse survival after *Salmonella* infection were determined by a log-rank Martel–Cox test. Data were considered statistically significant when $P < 0.05$.

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