Oxidative stress activates transcription of *Salmonella* pathogenicity island-2 genes in macrophages

Received for publication, March 29, 2022, and in revised form, June 6, 2022 Published, Papers in Press, June 14, 2022,
https://doi.org/10.1016/j.jbc.2022.102130

Ju-Sim Kim[1], **Lin Liu**[1], **Bennett Davenport**[1], **Sashi Kant**[1], **Thomas E. Morrison**[1], **and Andres Vazquez-Torres**[1,2,*]

From the [1]*Department of Immunology & Microbiology, University of Colorado School of Medicine, Aurora, Colorado, USA;* [2]*Veterans Affairs Eastern Colorado Health Care System, Denver, Colorado, USA*

Edited by Ursula Jakob

The type III secretion system encoded in the *Salmonella* pathogenicity island-2 (SPI-2) gene cluster facilitates intracellular growth of nontyphoidal *Salmonella* by interfering with the maturation of *Salmonella*-containing vacuoles along the degradative pathway. SPI-2 gene products also protect *Salmonella* against the antimicrobial activity of reactive oxygen species (ROS) synthesized by the phagocyte NADPH oxidase 2 (NOX2). However, a potential relationship between inflammatory ROS and the activation of transcription of SPI-2 genes by intracellular *Salmonella* is unclear. Here, we show that ROS engendered in the innate host response stimulate SPI-2 gene transcription. We found that the expression of SPI-2 genes in *Salmonella*-sustaining oxidative stress conditions involves DksA, a protein otherwise known to regulate the stringent response of bacteria to nutritional stress. We also demonstrate that the J and zinc-2-oxidoreductase domains of DnaJ as well as the ATPase activity of the DnaK chaperone facilitate loading of DksA onto RNA polymerase complexed with SPI-2 promoters. Furthermore, the DksA-driven transcription of SPI-2 genes in *Salmonella* experiencing oxidative stress is contingent on upstream OmpR, PhoP, and SsrB signaling events that participate in the removal of nucleoid proteins while simultaneously recruiting RNA polymerase to SPI-2 promoter regions. Taken together, our results suggest the activation of SPI-2 gene transcription in *Salmonella* subjected to ROS produced by the respiratory burst of macrophages protects this intracellular pathogen against NOX2-mediated killing. We propose that *Salmonella* have co-opted inflammatory ROS to induce SPI-2-mediated protective responses against NOX2 host defenses.

The facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium causes countless cases of gastroenteritis around the world annually. Nontyphoidal serovars such as Typhimurium are also responsible for disseminated life-threatening systemic infections in people suffering from HIV or malarial comorbidities (1–5). The pathogenicity of nontyphoidal salmonellae is greatly reliant on the vacuolar remodeling attributes of the type III secretion system encoded within the *Salmonella* pathogenicity island-2 (SPI-2) gene cluster. Induction of SPI-2 genes primes the transition of this enteropathogen from an extracellular location in the gastrointestinal lumen to the intracellular niche of host cells (6, 7). Zoonotic nontyphoidal salmonellae exploit the SPI-2 type III secretion system to grow intracellularly in professional phagocytes and epithelial cells (8–10). SPI-2 transcription is induced in response to elevated proton concentrations and diminished Mg^{2+} levels, both of which are signatures of phagosomes and *Salmonella*-containing vacuoles (11, 12).

Expression of the 40 kb gene cluster encoding the *ssrAB* regulon, secretion apparatus, translocon, chaperones, and effectors, as well as substrate proteins encoded outside the SPI-2 pathogenicity island, imposes a considerable metabolic toll on *Salmonella*. Thus, *Salmonella* must tightly regulate transcription of this pathogenicity island. The combined actions of nucleoid proteins and transcriptional activators result in the timely expression of SPI-2 genes by *Salmonella*. Gene silencing, which is mediated through binding of homologous H-NS, YgdT, and Haa nucleoid proteins along extensive areas of noncoding and coding regions of the *ssrA* regulatory locus, is vital for the contextual expression of SPI-2 genes (13, 14). On the other hand, PhoP–PhoQ, OmpR–EnvZ, and PhoB–PhoR two-component systems responding to acidic pH, osmolarity, or shortages in Mg^{2+} or inorganic phosphorous activate SPI-2 gene transcription (15–17). As is the case for nucleoid proteins, the regulatory activities of PhoP, OmpR, SlyA, or PhoB converge into the *ssrAB* locus encoding a two-component system responsible for global expression of structural and effector SPI-2 gene products (18–21). The SsrB response regulator activates transcription by counteracting H-NS silencing, while simultaneously recruiting RNA polymerase to SPI-2 genes (22).

In addition to the hierarchical control emanating from interactions of traditional transcription factors to regulatory SPI-2 DNA regions, the SPI-2 locus is also operated at the level of transcription initiation. The combined actions of the nucleotide alarmone guanosine tetraphosphate (ppGpp) and the α-helical protein DksA on the kinetics of DNA–RNA polymerase open complexes favor SPI-2 gene transcription (23–25). Historically, DksA and ppGpp have been known for their participation in the stringent response, an adaptation to nutritional stress that is typified by the activation of amino acid biosynthetic genes and the repression of loci encoding

* For correspondence: Andres Vazquez-Torres, Andres.Vazquez-Torres@cuanschutz.edu.

Published by Elsevier Inc on behalf of American Society for Biochemistry and Molecular Biology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

translational machinery (26). Thus, placing SPI-2 transcription under the control of ppGpp and DksA may assist with the adaptation of *Salmonella* to transient nutritional scarcities within host cells.

In addition to responding to nutritional shortages, the stringent response is emerging as a key regulator of antioxidant defense in diverse bacterial pathogens (27–30). Transient amino acid auxotrophies resulting from the oxidative damage of biosynthetic pathways are sensed by the stringent response RelA protein (30, 31). Distorted uncharged tRNAs entering the A-site of the ribosome elicit the ppGpp synthetase activity of RelA (32). Simultaneously, ROS oxidize cysteine residues coordinating a zinc cation in the globular domain of DksA (33, 34). The resulting disulfide bonds unfold the globular domain, triggering interactions of DksA with the DnaJ cochaperone (33). DnaJ first reduces and then presents its unfolded DksA client to DnaK, which catalyzes the nucleation of DksA–RNA polymerase complexes onto DNA that encodes enzymes of amino acid biosynthesis (35). Binding of ppGpp to the resulting ternary DksA–RNA polymerase–DNA complexes activates transcription of amino acid biosynthetic and transport genes in *Salmonella* that sustain oxidative stress (30, 33). Because SPI-2 gene transcription is activated by ppGpp and DksA (23–25) and because *Salmonella* utilize the SPI-2 type III secretion system to combat the phagocyte NADPH oxidase (NOX2) (36–41), herein we studied whether byproducts of the respiratory burst activate DksA-dependent transcription of horizontally acquired SPI-2 genes. Our investigations demonstrate that the DnaJ chaperone enables the ROS-dependent formation of DksA–RNA polymerase–SPI-2-gene ternary complexes and thus fosters the expression of SPI-2 regulatory and effector genes in intracellular *Salmonella* exposed to NOX2 oxidative products.

Results

Oxidative stress stimulates SPI-2 gene transcription

We examined whether *Salmonella* modulate SPI-2 gene transcription after exposure to hydrogen peroxide (H_2O_2), an ROS synthesized by host phagocytes in response to this facultative intracellular pathogen (42). *Salmonella* activated expression of the SPI-2 *sifA::lacZY* reporter construct in response to increasing concentrations of H_2O_2 (Fig. 1*A*). Under the experimental conditions tested, H_2O_2 did not affect *Salmonella* growth (Fig. S1*A*). The concentration of molecular oxygen (O_2) in phagosomes has been estimated at 11 μM (43). Because of the enzymatic activity of both host NOX2 and *Salmonella* terminal cytochromes, it is possible that the cytoplasm of intracellular *Salmonella* is exposed to products of NOX2 under hypoxic or anaerobic conditions. Moreover, a sizable fraction of DksA is oxidized in *Salmonella* cultures grown aerobically in a shaker incubator (33). Taking into account the lines of reasoning, to examine in more detail whether exogenous H_2O_2 can induce DksA-dependent activation of SPI-2 gene transcription, we developed an anaerobic culture system. Anaerobic *Salmonella* failed to grow on N9 medium in the absence of an added terminal electron acceptor (Fig. S1*B*), likely because the growth of Enterobacteriaceae on casamino acids, the sole carbon source in the N9 minimum medium used in our experiments, relies on the electron transport chain (44). Accordingly, the addition of the terminal electron acceptor NO_3^- supported growth of anaerobic *Salmonella* in N9 medium (Fig. S1*C*). Therefore, the N9 medium used in all subsequent anaerobic experiments contained NO_3^-. Compared with aerobic cultures (Figs. S1, *D* and *E*), the downshift of anaerobic *Salmonella* to 8 μM $MgCl_2$ N9 medium, pH 5.8, stimulated modest levels of transcription of both *ssrB* and *sifA* SPI-2 genes (Fig. 1, *B* and *C*). The expression of *ssrB* and *sifA* was stimulated upon treatment of anaerobic cultures grown in 8 μM $MgCl_2$ N9 medium with increasing concentrations of H_2O_2 (Fig. 1, *B* and *C*). The concentrations of H_2O_2 used in our assays are in keeping with the 1 to 17 μM H_2O_2 that are estimated to be synthesized in phagosomes (45, 46). Consistent with the transcriptional findings, H_2O_2 treatment of *Salmonella* in SPI-2-inducing N9 medium in an anaerobic chamber also resulted in the accumulation of SsrB protein (Fig. 1*D*). Collectively, these findings demonstrate that exogenous H_2O_2 triggers expression of SPI-2 genes.

The DnaJ–DnaK chaperone couple facilitates DksA-dependent SPI-2 transcription following oxidative stress

H_2O_2 did not trigger either *ssrB* or *sifA* transcription in *Salmonella* grown anaerobically in 10 mM Mg^{2+} N9 medium, pH 8.0 (Figs. 2*A* and S2*A*). In contrast, H_2O_2 activated significant SPI-2 transcription in anaerobic *Salmonella* grown under 8 μM Mg^{2+}-inducing conditions (Figs. 2*A* and S2*A*). These findings suggest that H_2O_2 potentiates the transcription of SPI-2 genes induced in response to the downshift in the concentration of Mg^{2+}. Next, we determined the molecular mechanism underlying the H_2O_2-induced transcription of SPI-2 genes. Because DksA is a critical determinant in the expression of SPI-2 genes (23, 25) and ROS activate the stringent response (33, 35), we tested whether the H_2O_2-induced transcription of SPI-2 genes involves the DksA protein. According to the latter model, deletion of *dksA* prevented the H_2O_2-stimulated transcription of both *ssrB* and *sifA* (Figs. 2*B* and S2*B*).

Oxidative stress precludes associations of DksA with RNA polymerase (34). The DnaJ–DnaK chaperone couple, nonetheless, can repair oxidized cysteine residues in the zinc finger domain of DksA (35), thereby fostering the formation of enzymatically active DksA–RNA polymerase complexes (33). As noted with amino acid biosynthetic genes (33, 35), Δ*dnaJ* and Δ*dnaK* mutant strains did not support *ssrB* or *sifA* expression in anaerobic *Salmonella* grown in inducing N9 medium after H_2O_2 treatment (Figs. 2, *C*, *D*, S2, *C* and *D*). Plasmids expressing the *dnaJ* and/or *dnaK* genes complemented the *ssrB*- and *sifA*-inducing capacity of Δ*dnaJ* and Δ*dnaKJ Salmonella* strains upon exposure to H_2O_2 (Figs. 2, *E*, *F*, S2, *E* and *F*). To test if the DksA-dependent activation of SPI-2 transcription stimulated by H_2O_2 depends on the oxidoreductase activity of DnaJ (33, 47, 48), we examined SPI-

2 gene transcription in a *Salmonella* strain expressing the DnaJ C186H mutant that disrupts thiol-disulfide exchange activity but preserves the zinc finger fold (Fig. S2*G*; (33)). A strain bearing the *dnaJ* C186H mutant allele was unable to stimulate SPI-2 gene transcription in anaerobic *Salmonella* exposed to H_2O_2 (Figs. 2*G* and S2*H*). Similar results were seen in a *Salmonella* strain bearing the *dnaJ* H33Q allele deficient in the J domain that enables the transferring of clients from DnaJ to DnaK (Figs. 2*H* and S2*I*). Moreover, studies using a *Salmonella dnaK* T199A strain showed that the DnaK-dependent

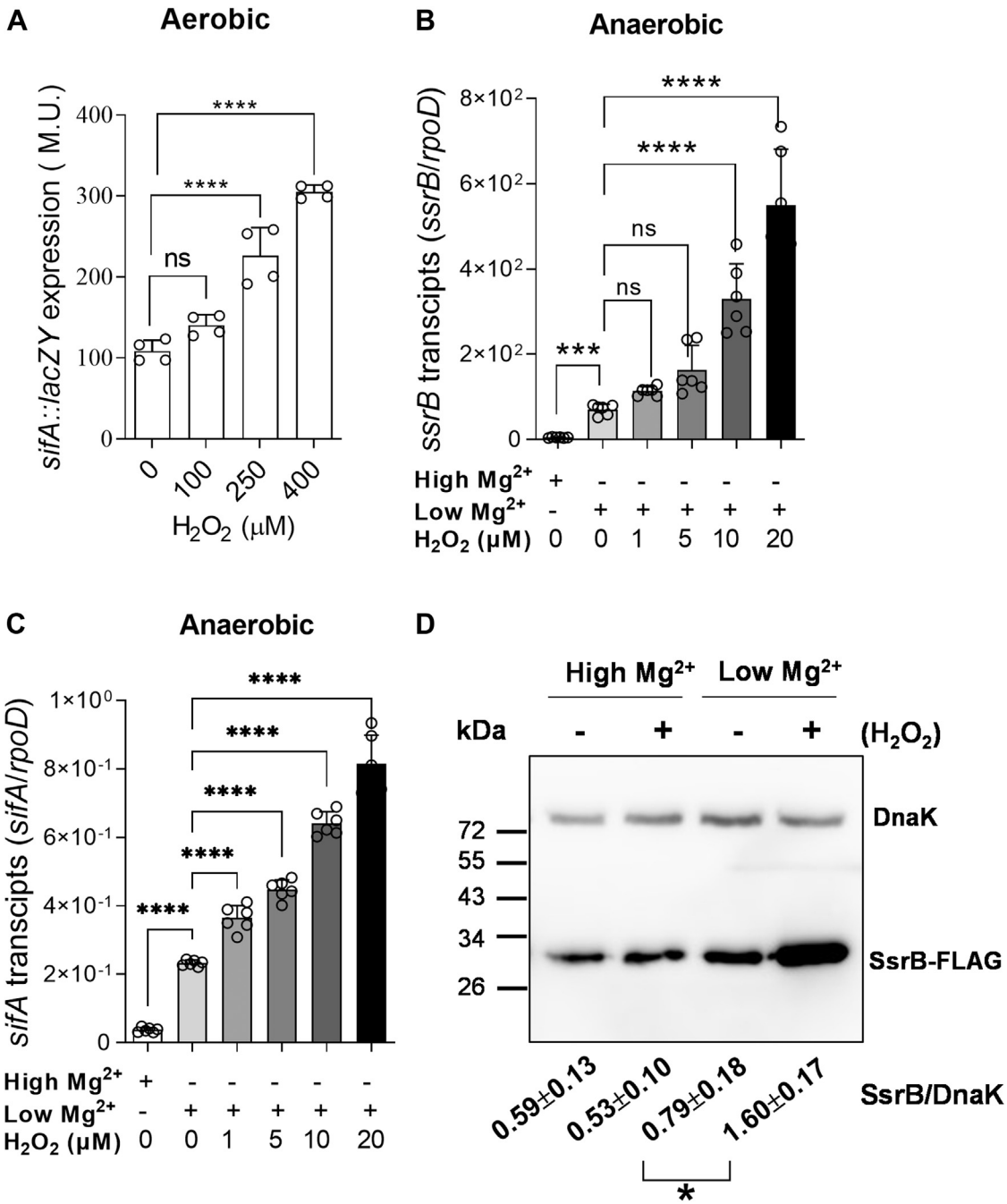

Figure 1. Oxidative stress stimulates *Salmonella* SPI-2 gene expression. *A*, induction of *sifA* gene expression by oxidative stress was determined by measuring β-galactosidase activity of *sifA*::*lacZY*-expressing *Salmonella* grown aerobically in a shaker incubator in N9 low Mg^{2+} minimal medium. H_2O_2 was added at the indicated concentrations for 30 min. The data are the mean ± SD (n = 4) from two independent experiments. ****$p < 0.0001$, as determined by one-way ANOVA. ns when compared with reaction treated with H_2O_2 and untreated. *B* and *C*, the *sifA* and *ssrB* transcripts were induced in wildtype *Salmonella* grown anaerobically in 10 μM Mg^{2+} N9 minimal medium, pH 5.8. Abundance of *ssrB* and *sifA* transcripts was measured by qRT–PCR, using the housekeeping gene *rpoD* as an internal control. ****, $p < 0.0001$, ns, nonsignificant, as determined by one-way ANOVA. *D*, SsrB protein was examined by Western blotting in specimens isolated from anaerobic *Salmonella* treated with or without 10 μM H_2O_2 for 1 h in 10 mM Mg^{2+} N9 media, pH 7.4, or 10 μM Mg^{2+} N9 media, pH 5.8. Density of proteins was measured with ImageJ, and fold change in density was calculated as density of SsrB/density of DnaK controls. The figure is representative of five independent experiments, and band density is shown as means ± SD (n = 5). *$p < 0.05$. H_2O_2, hydrogen peroxide; ns, nonsignificant; qRT–PCR, quantitative RT–PCR; SPI-2, *Salmonella* pathogenicity island-2.

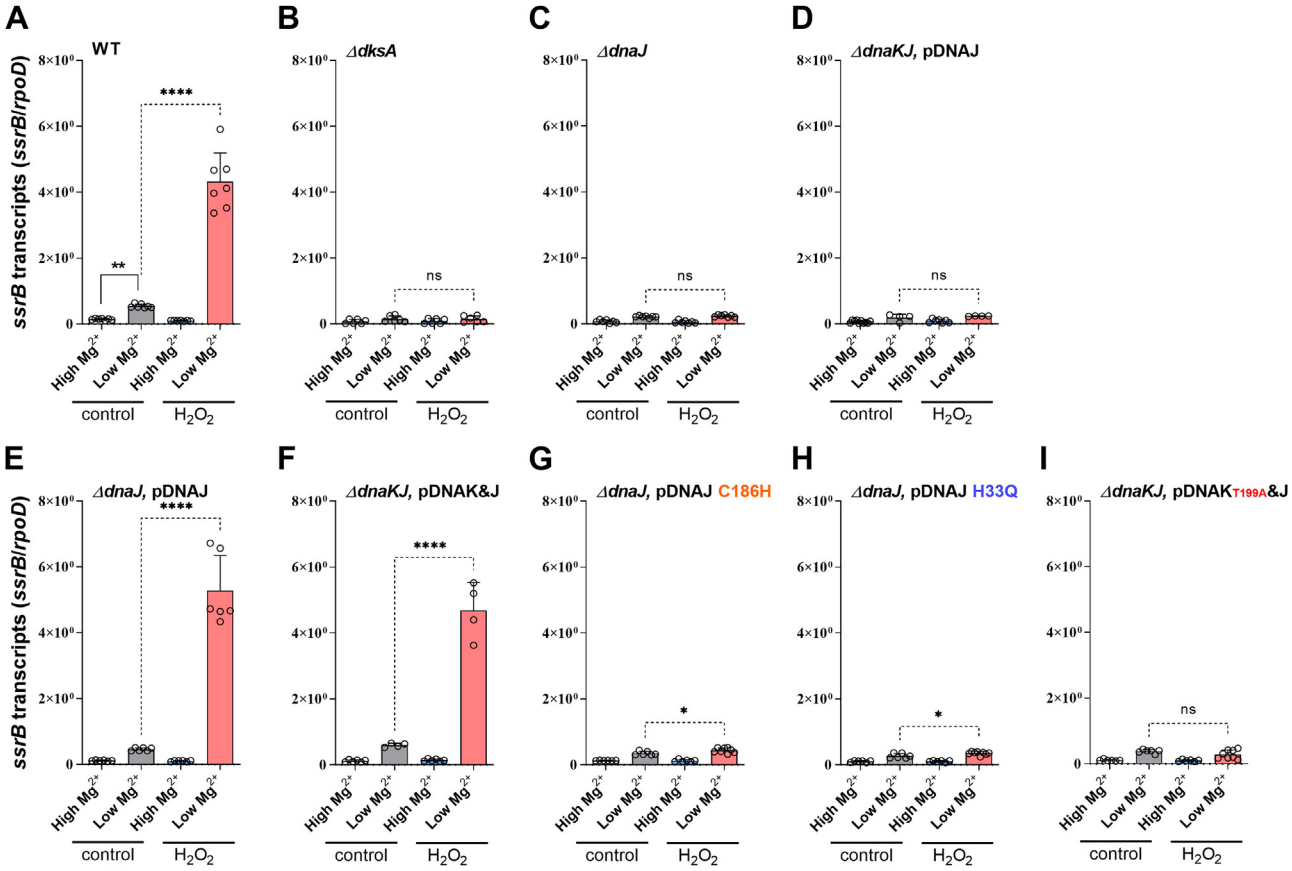

Figure 2. DnaK and DnaJ activate DksA-dependent *ssrB* gene transcription. *A–I*, anaerobic *Salmonella* were grown in N9 high and low Mg^{2+} minimal medium supplemented with 40 mM $NaNO_3^-$. The abundance of *ssrB* transcripts was determined by qRT–PCR in RNA isolated from anaerobic *Salmonella* treated for 30 min with or without 10 μM H_2O_2. The expression of the housekeeping gene *rpoD* was used as internal control. Data are the means ± SD (n = 4–8) from two or three independent experiments. *$p < 0.1$, **$p < 0.01$, ****$p < 0.0001$, as calculated by one-way ANOVA. H_2O_2, hydrogen peroxide; ns, nonsignificant *versus* reactions treated with H_2O_2 and untreated; qRT–PCR, quantitative RT–PCR.

induction of SPI-2 gene transcription in anaerobic *Salmonella* treated with H_2O_2 is supported by the ATPase activity of this chaperone (Fig. 2*I* and S2*J*). Under the conditions used in our experiments, H_2O_2 did not visibly affect the growth of any of the mutants tested (Fig. S3, *A* and *B*). Collectively, this research suggests that the SPI-2 expression recorded in *Salmonella* exposed to H_2O_2 is dependent on DnaJ and DnaK.

The DnaK–DnaJ chaperone couple activates ssrA *in vitro transcription by oxidized DksA*

To directly test the involvement of DnaJ and DnaK on the activation of DksA-mediated SPI-2 transcription in *Salmonella* sustaining oxidative stress, we developed a reconstituted biochemical *ssrA in vitro* transcription system. Reduced DksA stimulated significantly ($p < 0.0001$) more *ssrA in vitro* transcription than oxidized DksA controls (Figs. 3*A* and S4*A*). The addition of neither recombinant DnaJ nor DnaK proteins to RNA polymerase–containing reactions activated *ssrA in vitro* transcription (Figs. 3*A* and S4*B*). Nonetheless, incubation of oxidized DksA with DnaJ and DnaK reconstituted *ssrA in vitro* transcription to levels supported by the reduced DksA protein (Fig. 3*A*). Incubation of reduced DksA with DnaJ and DnaK did not further potentiate the transcriptional activity induced by DksA alone. These findings demonstrate that the DnaJ–DnaK cochaperone system activates SPI-2 *in vitro* transcription by reactivating oxidized DksA.

The addition of DnaJ C186H or DnaJ H33Q recombinant proteins into the *in vitro* transcription system failed to activate *ssrA* expression by oxidized DksA and DnaK proteins (Figs. 3*B* and S4*B*), implicating oxidoreductase and J domains of DnaJ in this activity. The inability of DnaJ C186H or DnaJ H33Q variants to support $DksA^{oxi}$-dependent *ssrA in vitro* transcription cannot be explained by a failure of these variants to bind Zn^{2+} (Fig. S2*G*). A DnaK T119A variant also failed to activate *ssrA* transcription by oxidized DksA and DnaJ proteins (Figs. 3*B* and S4*B*). Together, these investigations demonstrate that the ATPase activity of the DnaK chaperone synergizes with the oxidoreductase- and J-dependent functions of DnaJ to enable *ssrA* transcription by oxidized DksA.

SPI-2 transcription in Salmonella *sustaining oxidative stress requires upstream signaling*

The reconstituted *in vitro* transcription assay demonstrates that the activation of SPI-2 transcription can be achieved by DksA alone. In cells, however, recruitment of RNA polymerase to SPI-2 promoters is hindered by nucleoid proteins bound to

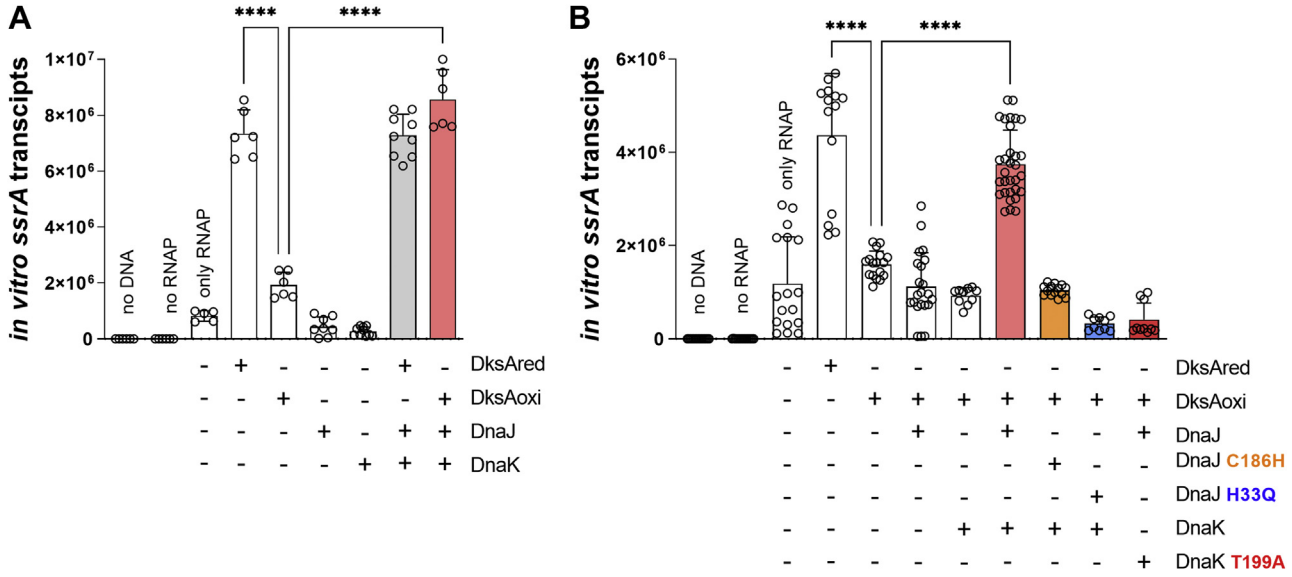

Figure 3. DksA–DnaJ chaperones activate *ssrA in vitro* transcription by oxidized DksA. *A*, activation of *ssrA* transcripts in *in vitro* transcription reaction mixtures containing 2.5 μM oxidized DksA, 50 nM DnaJ, and 500 nM DnaK proteins in the presence of RNA polymerase as determined by qRT–PCR. Reduced DksA (2.5 μM) showed sufficiently activated *in vitro ssrA* transcripts independent of the presence of DnaJ and DnaK proteins. *B*, effect of DnaJ C186H, DnaJ H33Q, or DnaK T199A variants to activate *in vitro ssrA* transcripts by oxidized DksA. The data are the means ± SD (n = 8–32) from at least eight independent experiments. ****$p < 0.0001$, as determined by one-way ANOVA. qRT–PCR, quantitative RT–PCR.

regulatory promoter regions (13, 22). PhoP, OmpR, or SsrB proteins help activate SPI-2 gene transcription by either countersilencing nucleoid proteins or recruiting RNA polymerase to the *ssrAB* locus (13). In the following investigations, we examined if the SPI-2-induced gene expression observed in *Salmonella* treated with H_2O_2 is dependent on upstream signaling steps. As reported previously, the downshift of *Salmonella* to 8 μM $MgCl_2$ N9 medium, pH 5.8, activated modest expression of *ssrB* and *sifA* genes (Figs. 4*A* and S5*A*). Treatment of *Salmonella* with 10 μM H_2O_2 in N9-inducing medium activated the expression of *ssrB* and *sifA* (Figs. 4*A* and S5*A*). H_2O_2 treatment failed to activate transcription in control *Salmonella* strains deficient in *dksA*, *ompR*, *phoP*, or *ssrB* genes (Figs. 4, *B–E* and S5, *B–E*). The addition of H_2O_2 to anaerobic cultures of wildtype, Δ*dksA*, Δ*ompR*, Δ*phoP*, or Δ*ssrB Salmonella* did not affect bacterial growth (Fig. S5, *F* and *G*). Cumulatively, these findings suggest that the activation of DksA-dependent SPI-2 gene transcription in *Salmonella* undergoing oxidative stress is contingent on upstream PhoP, OmpR, and SsrB signaling.

NOX-2-derived ROS activate intracellular SPI-2 gene transcription

We next examined whether ROS produced enzymatically in the respiratory burst of macrophages activate SPI-2 transcription in intracellular *Salmonella* (Fig. 5, *A* and *B*). *Salmonella* expressed *ssrA* and *sifA* SPI-2 genes 9 h after infection of ROS-producing bone marrow–derived macrophages

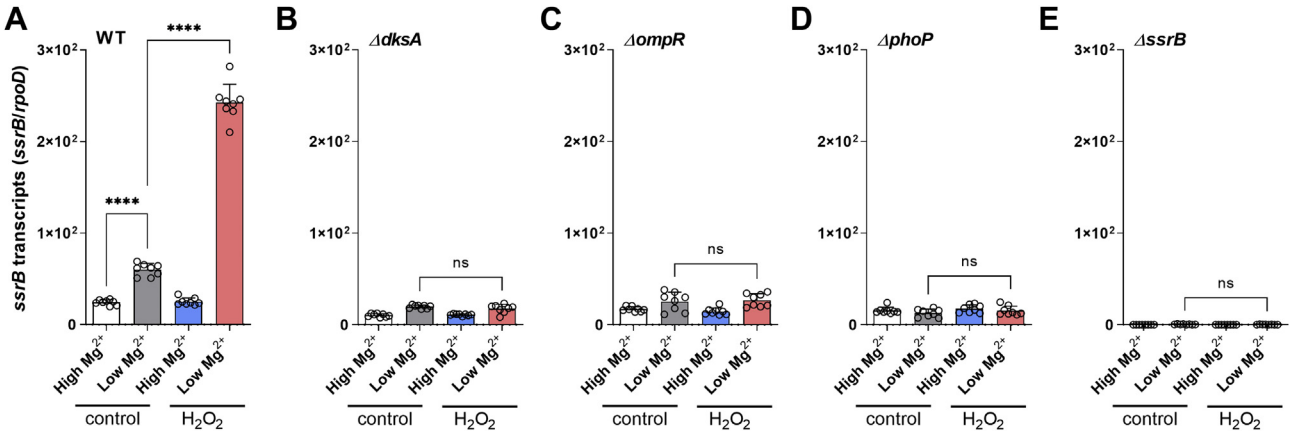

Figure 4. OmpR and PhoP are required for activation of *ssrB* transcripts in *Salmonella* exposed to H_2O_2. *A–E*, the abundance of *ssrB* transcripts was measured by qRT–PCR in RNA isolated from anaerobic *Salmonella* grown in N9 high and low Mg^{2+} minimal medium. The strains were treated with or without 10 μM H_2O_2 for 30 min prior to collection. The expression of the housekeeping gene *rpoD* was used as an internal control. The data are the means ± SD (n = 8) performed on two independent days. ****$p < 0.0001$, as calculated by one-way ANOVA. H_2O_2, hydrogen peroxide; ns, nonsignificant when compared with reactions treated with H_2O_2 or untreated; qRT–PCR, quantitative RT–PCR.

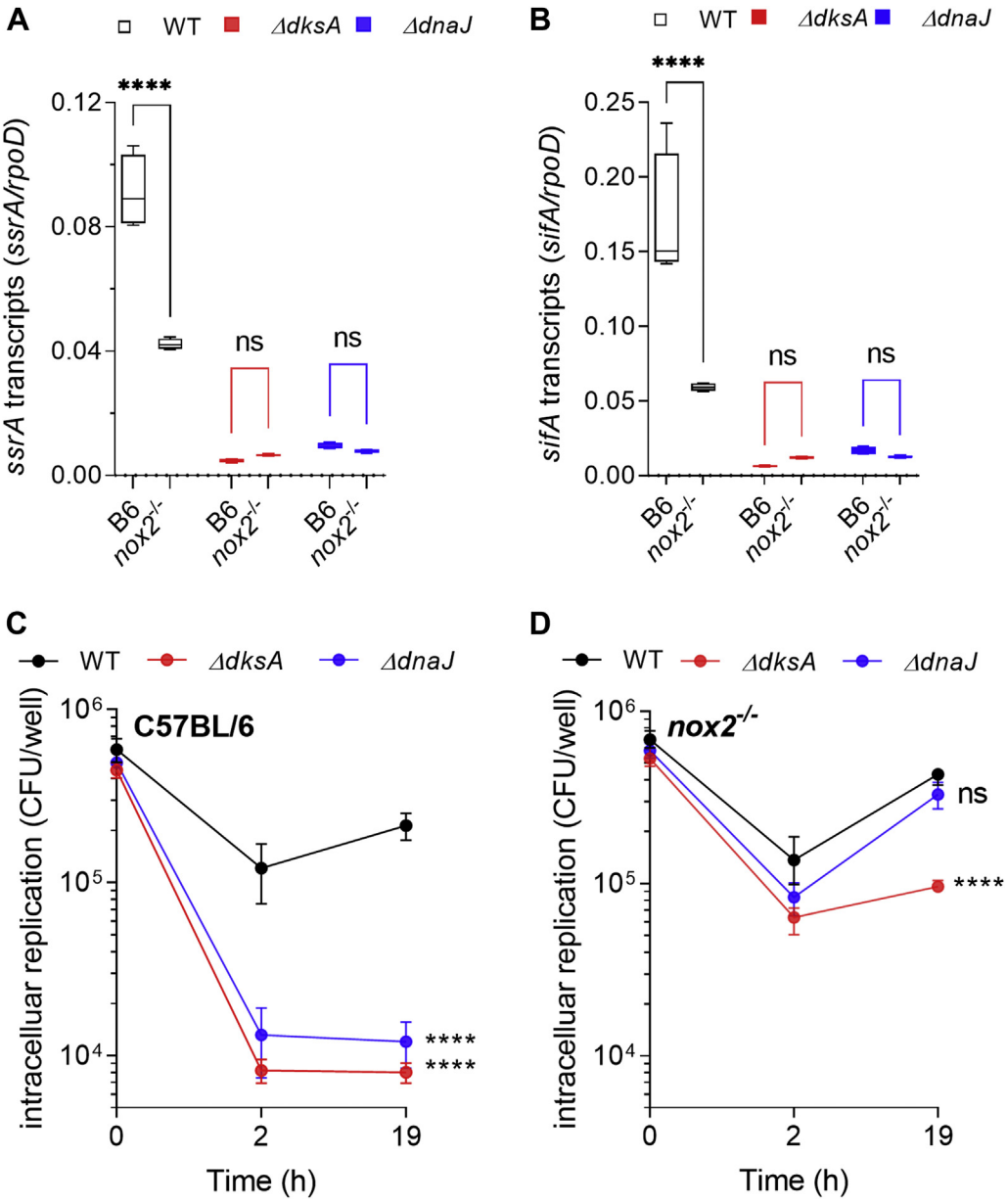

Figure 5. Induction of intracellular SPI-2 gene transcription in response to NOX2 products. *ssrA* (*A*) and *sifA* (*B*) transcripts in RNA isolated from intracellular bacteria 9 h after infection of BMDM was measured by qRT–PCR. The housekeep gene *rpoD* was used as internal control. Bacterial burden in BMDM from C57BL/6 (B6) (*C*) and *nox2*$^{-/-}$ (*D*) mice. The data are mean ± SD (*C*, n = 4; *D*, n = 6). ****$p < 0.0001$ as determined by one-way ANOVA. BMDM, bone marrow–derived macrophage; NOX, NADPH oxidase; ns, not significant; qRT–PCR, quantitative RT–PCR; SPI-2, *Salmonella* pathogenicity island-2.

(BMDMs) from C57BL/6 mice (Figs. 5, *A* and *B* and S6). Compared with wildtype macrophages, the intracellular expression of *ssrA* and *sifA* genes was significantly ($p < 0.0001$) lower in *Salmonella* collected from macrophages derived from *nox2*$^{-/-}$ mice, a population of macrophages that cannot generate superoxide anion (Fig. S6) because of a mutation in the gp91*phox* subunit of the phagocyte NOX2 (Fig. 5, *A* and *B*). These data demonstrate that *Salmonella* activate SPI-2 transcription in response to ROS produced by NOX2 in the innate host response. The intracellular expression of *ssrA* and *sifA* was markedly reduced in Δ*dksA* and Δ*dnaJ Salmonella* (Fig. 5, *A* and *B*), demonstrating that the ROS-dependent intracellular expression of SPI-2 genes is under the positive control of DksA and DnaJ. Transcription of *ssrA* and *sifA* genes by Δ*dksA* and Δ*dnaJ Salmonella* was not recovered in BMDM from *nox2*$^{-/-}$ mice, demonstrating the necessity of DksA and DnaJ signaling for the expression of SPI-2 genes by *Salmonella* in macrophages.

Salmonella deficient in *dksA* or *dnaJ* were similarly hypersusceptible to bactericidal activity emanating from the respiratory burst of BMDM (Fig. 5, *C* and *D*). Although the Δ*dksA Salmonella* strain recovered some growth in *nox2*$^{-/-}$ BMDM, the burden of a Δ*dksA* mutant remained significantly ($p <$ 0.0001) lower in *nox2*$^{-/-}$ BMDM compared with wildtype or Δ*dnaJ Salmonella* controls. We conclude that the DnaJ/DksA-driven expression of SPI-2 genes protects *Salmonella* against

the respiratory burst of macrophages. This research also indicates that DksA and DnaJ play codependent roles at counteracting the antimicrobial activity of NOX2, although independent roles also appear to exist.

Discussion

Our investigations demonstrate that ROS produced in the innate response of macrophages are biologically relevant signals that activate SPI-2 transcription in intracellular *Salmonella*. Induction of SPI-2 genes in *Salmonella* undergoing oxidative stress helps this intracellular pathogen fight the antimicrobial activity of NOX2 flavohemoproteins. The activation of SPI-2 transcription in *Salmonella* exposed to ROS depends on the coordinated regulatory activities of DksA, DnaJ, and DnaK proteins. The oxidoreductase mapping to the zinc-2 domain of DnaJ is necessary for the DksA-dependent activation of SPI-2 transcription observed in *Salmonella* undergoing oxidative stress, possibly by reducing disulfide-bonded cysteine residues in the globular domain of DksA (33). In addition, DnaJ J domain contributes to oxidation-mediated activation of SPI-2 transcription by presenting DksA to the DnaK chaperone. Ultimately, the ATPase catalytic activity of DnaK enables the formation of DksA–RNA polymerase–DNA ternary complexes, thereby activating SPI-2 transcription in *Salmonella* sustaining oxidative stress.

Reduced DksA protein induces SPI-2 *in vitro* transcription as efficiently as the DnaK–DnaJ–$DksA^{oxi}$ complex, and the DnaK and DnaJ proteins do not improve the transcriptional activity of reduced DksA. These findings suggest that the most important role the DnaJ–DnaK chaperone system plays in the expression of SPI-2 genes is to repair DksA molecules oxidized and unfolded in *Salmonella* undergoing oxidative stress. H_2O_2 arising from the univalent reduction of O_2 in redox-active centers in the electron transport chain oxidizes a significant fraction of DksA molecules in aerobic *Salmonella* (33). DksA is also oxidized upon exposure of *Salmonella* to exogenous sources of ROS (34). As little as 1 μM H_2O_2 appears to be sufficient to oxidize DksA in *Salmonella* (33), a concentration of H_2O_2 well within the range achievable in phagosomes (46, 49). A significant fraction of DksA may be oxidized and incapable of activating SPI-2 gene transcription in macrophages supporting a respiratory burst. However, the DnaK–DnaJ chaperone system provides the redox buffering capacity that makes possible the DksA-dependent expression of SPI-2 genes during exposure of *Salmonella* to host-derived oxidants.

The addition of H_2O_2 to *Salmonella* grown under non-inducing conditions fails to stimulate SPI-2 gene expression. In contrast, H_2O_2 induces the expression of *sifA* and *ssrB* genes in anaerobic *Salmonella* grown in acidic and low Mg^{2+}-inducing media. These findings suggest that the induction of SPI-2 gene expression by oxidized DksA protein is contingent on the prior recruitment of RNA polymerase by OmpR, PhoP, or SsrB. Accordingly, H_2O_2 failed to stimulate SPI-2 transcription in Δ*ompR*, Δ*phoP*, or Δ*ssrB Salmonella* lacking key response regulators. SsrB and PhoP partially induce SPI-2 gene transcription by derepressing the actions of nucleoid proteins (22, 50). DksA-mediated activation of SPI-2 transcription must operate downstream of the PhoP- or SsrB-mediated derepression of nucleoid proteins and is probably unrelated to interactions of DksA with nucleoid proteins such as H-NS. This latter idea is supported by two independent lines of reasoning. First, the transcriptional factor DksA interacts with RNA polymerase through the secondary channel, rather than through binding to the promoter regions that are normally footprinted by nucleoid proteins. Second, recombinant DksA protein directly activates SPI-2 *in vitro* transcription, demonstrating direct effects of DksA on transcriptional open complexes.

Previous research linked SPI-2 gene transcription to signaling cascades traditionally associated with the adaptation of bacteria to oxidative stress. For example, RpoE, SoxS, and SlyA activate various SPI-2 genes by either activating *ssrA* transcription or downregulating *hns* expression (13, 51–54). The regulatory proteins RpoE and SlyA increase fitness of *Salmonella* during periods of oxidative stress (55, 56), and the transcriptional activity of the [2Fe–2S] SoxR metalloprotein is stimulated by superoxide anion or redox species (57, 58). It remains unknown whether the activation of SPI-2 gene transcription supported by RpoE, SoxS, or SlyA reflects their responsiveness to ROS. Regardless of their mechanism, the absence of downstream DksA–DnaJ–DnaK signaling prevents the induction of SPI-2 gene transcription in *Salmonella* undergoing oxidative stress.

Aerobic respiration supports higher SPI-2 transcription than nitrate respiration. The differences in the levels of SPI-2 transcription between aerobic and anaerobic *Salmonella* may stem from higher energetic outputs supported by aerobic compared with nitrate respiration. Although not mutually exclusive, ROS produced adventitiously in aerobic metabolism may provide an additional signal for optimal SPI-2 induction. In support of this idea, H_2O_2 signals NO_3^--respiring *Salmonella* to upregulate the expression of SPI-2 genes. These observations could have important implications for SPI-2 expression in *Salmonella* residing in phagocytic cells. ROS synthesized by NOX2 may assume the function normally played by oxidative species produced endogenously in bacterial metabolism, the production of which must be precluded as the O_2 is consumed during the respiratory burst of phagocytic cells.

Acidic pH and nutritional downshifts might be the primary mechanism for the activation of SPI-2 genes *in vivo*. Accordingly, *Salmonella* express SPI-2 genes in epithelial cells and some populations of macrophages unable to sustain the respiratory burst (10, 23). DksA, an effector of the stringent response in starving Gram-negative bacteria, may be a hub in which nutritional signals and oxidative stress are processed for the activation of SPI-2 transcription.

By ameliorating exposure to ROS generated by macrophages, the SPI-2 type III secretion system protects *Salmonella* against the antimicrobial activity of products of cell host NOX2 enzymatic complexes (37–39, 41, 59, 60). SPI-2 gene products decrease the genotoxicity endured by *Salmonella* from exposure to oxidants in macrophages (61). In addition to limiting the

contact of intracellular *Salmonella* to NOX2-derived oxidants, the suppressing apoptotic signals induced by the SPI-2 apparatus shields *Salmonella* from the respiratory burst of inflammatory neutrophils (40). Thus, induction of the SPI-2 type III secretion system by products of the respiratory burst provides a feedback loop to combat the antimicrobial actions of NOX2 enzymatic complexes.

Experimental procedures

Bacterial strains, plasmids, and growth conditions

Tables S1 and S2 list the derivatives of *S. enterica* serovar Typhimurium strain 14032s and *Escherichia coli*, as well as the plasmids used here. Deletion mutants were constructed using the λ-Red homologous recombination system (62). Bacteria were grown in LB broth, N9 high Mg^{2+} medium (100 mM Tris–HCl, 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% casamino acids, and 10 mM $MgCl_2$; pH 8.0), or N9 low Mg^{2+} minimal medium (100 mM Tris–HCl, 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% casamino acids, 8 μM $MgCl_2$; pH 5.8) at 37 °C as described (63). Penicillin, chloramphenicol, kanamycin, and tetracycline were added at final concentrations of 250, 40, 50, and 10 μg/ml, respectively.

β-galactosidase activity

SPI-2 gene expression was performed as previously described (64). Briefly, *Salmonella* expressing the *sifA*::*lac*ZY::Km single-copy chromosomal fusion (64) grown in LB broth overnight were diluted 1:100 in N9 high Mg^{2+} minimal medium and cultured at 37 °C for approximately 4 h to absorbance of 0.5 at 600 nm. Cultures were collected, washed three times with N9 low Mg^{2+} minimal medium, and resuspended in N9 low Mg^{2+} minimal medium to an absorbance at 600 nm of approximately 0.25. Cells were cultured at 37 °C for 2.5 h and then treated for 30 min with or without different concentrations of H_2O_2. β-galactosidase activity was determined in samples with o-nitrophenyl-β-galactoside substrate. Enzyme activity expressed as Miller Units was calculated as absorbance ar 420 nm × 1000/(time [min] × absorbance at 600 nm × cell volume [ml]) (33). Growth was monitored by measuring absorbance at 600 nm after treatment with H_2O_2 for 30 min.

Anaerobic culture and H_2O_2 treatment

Salmonella grown overnight in LB broth at 37 °C in a BACTRON anaerobic chamber were harvested and resuspended in N9 high Mg^{2+} minimal medium supplemented with or without 40 mM $NaNO_3^-$ (Sigma) to an absorbance of 0.1 at 600 nm. After incubation at 37 °C for about 6 h to an absorbance of 0.5 at 600 nm, cultures were harvested, washed with either N9 high Mg^{2+} or N9 low Mg^{2+} minimal medium two times, and resuspended in either N9 high Mg^{2+} or N9 low Mg^{2+} minimal medium supplemented with 40 mM $NaNO_3^-$ to an absorbance of approximately 0.25 at 600 nm. Cells were cultured at 37 °C for 2.5 h and then treated with or without H_2O_2 for 30 min.

Immunoblots

Abundance of SsrB-FLAG protein in *Salmonella* cultures was detected by immunoblot assay. Cells grown anaerobically in either N9 high Mg^{2+} or N9 low Mg^{2+} minimal medium supplemented with 40 mM $NaNO_3^-$ as described previously were collected after treated with 10 μM H_2O_2 for 1 h and disrupted by sonication in PBS. Protein concentrations of cell-free extracts were determined with Bicinchoninic Acid Protein Assay kit (Pierce), and then the samples were loaded onto 12% SDS-PAGE gels. The blots were treated with a 1:500 dilution of anti-FLAG monoclonal antibody (Sigma), followed by a 1:20,000 dilution of goat antimouse immunoglobulin conjugated with horseradish peroxidase (Pierce), and then visualized using the ECL prime Western blotting detection reagent (GE Healthcare Life Sciences) in the ChemiDoc XRS Imaging System (Bio-Rad). DnaK was used as internal control. Protein density was measured by the ImageJ program (National Institutes of Health).

RNA isolation and quantitative RT–PCR

The same culture method described previously was followed to isolate RNA from *Salmonella* cultured aerobically or anaerobically in either N9 high Mg^{2+} or N9 low Mg^{2+} minimal medium. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche). The synthesis of complementary DNA (cDNA) was achieved using 1 μg of purified RNA, TaqMan Gene Expression Master Mix (Thermo Fisher Scientific), and N6 random primers (Thermo Fisher Scientific). Quantitative RT–PCR (qRT–PCR) was performed using specific primers and probes (Table S3) containing 5′ 6-carboxyfluorescein and 3′ black-hole quencher 1 modification in a CFX connect Real-Time System (Bio-Rad). PCR-amplified DNA fragments containing the gene of interest were used to generate standard curves. The abundance of transcripts within each sample was normalized to internal transcripts of the housekeeping gene *rpoD*.

Overexpression and purification of proteins

Protein expression and purification were performed as previously described (35). Briefly, *E. coli* BL21 (DE3) (Thermo Fisher Scientific) (Table S1) grown in LB broth at 37 °C to an absorbance of 0.5 to 0.8 at 600 nm were treated with 0.1 mM isopropyl-β-D-thiogalactopyranoside. After 3 h, the cells were harvested, disrupted by sonication, and centrifuged to obtain cell-free extracts. Glutathione-*S*-transferase (GST) and 6His-tagged fusion proteins were purified using Glutathione-Sepharose 4B (bioWORLD) and TALON metal-affinity chromatography (Clontech), respectively, according to manufacturer's protocols. To purify the DksA proteins, the GST tags were removed from GST-DksA proteins bound to a Glutathione-Sepharose 4B resin. PreScission protease was added to recombinant GST-DksA proteins in PBS containing 10 mM DTT. After overnight incubation at 4 °C, untagged proteins were eluted with PBS. For further purification of DksA protein, size-exclusion chromatography on Superdex 75 (GE Healthcare Life Sciences) was used. Purified DksA

ASBMB

proteins were aliquoted inside a BACTRON anaerobic chamber (Shel Lab). The purity and mass of the recombinant proteins were assessed by SDS-PAGE.

Determination of zinc content

About 5 μM of DnaJ variants were treated with 150 (4-(2-pyridylaso) resorcinol (Sigma) at 37 °C for 1 h in the presence of 8 M urea. The zinc–4-(2-pyridylaso) resorcinol chelate was quantified spectrometrically at an absorbance at 500 nm. Zinc concentrations were calculated from a standard curve with $ZnCl_2$ (65).

In vitro *transcription*

In vitro transcription reactions were quantified by qRT–PCR as previously described (33, 35). Briefly, oxidized or reduced DksA proteins were prepared by treatment with 1 mM H_2O_2 or 1 mM DTT in 40 mM Hepes, pH 7.4, at 37 °C for 1 h. Excess H_2O_2 or DTT was removed using an Amicon Ultra-10 centrifugal filter (Millipore Sigma). Transcription reactions were performed in reaction mixture containing 40 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 60 mM potassium glutamate, 0.1% Nonidet P-40, 200 μM of each ATP, GTP, CTP, and UTP (Thermo Fisher Scientific), 8 U RiboLock RNase inhibitor (Thermo Fisher Scientific), 1 nM *livJ* DNA templates (Table S2), 5 nM *E. coli* holoenzyme RNA polymerase (New England Biolabs), and 2.5 μM of oxidized or reduced DksA proteins. Where indicated, the reactions contained 50 nM DnaJ variants in the presence and absence of 500 nM of DnaK variant proteins. Reactions were incubated at 37 °C for 10 min and then heat-inactivated at 70 °C for 10 min. After DNaseI treatment, template DNA was removed from the reactions with DNA-free DNA Removal Kit (Thermo Fisher Scientific), and the resulting RNA was used as template to generate cDNA using 100 U MMLV reverse transcriptase (Promega), 0.45 μM N6 random hexamer primers (Thermo Fisher Scientific), and 20 U RNase inhibitor (Promega). The amount of cDNA synthesized following 1 h of incubation at 42 °C was quantified by qRT–PCR using gene-specific primers and probes (Table S3). Specific transcripts were normalized to standard curves using known amounts of transcript concentrations.

BMDMs

Bone marrow cells were harvested in Dulbecco's modified Eagle's medium (DMEM) 10% medium (DMEM + 10% fetal bovine serum [FBS] + glutamate) from femurs of 8-week-old wildtype or *nox2*$^{-/-}$ C57BL/6 mice. Bone marrow–derived cells were centrifuged at 500*g* for 5 min and then resuspended 1 ml of ammonium–chloride–potassium lysis buffer for 1 min to lyse red blood cells and quenched after 1 min with 9 ml of DMEM 10% heat-inactivated FBS (Thermo Fisher Scientific). The cells were resuspended in L cell–conditioned DMEM (DMEM supplemented with 10% FBS, 5% horse serum, 1 mM sodium pyruvate, and 20% L cell–conditioned media) at a concentration of 4 × 10^6 cell/ml, and 25 ml of this suspension was seeded into 150-mm plates (Corning) and incubated at 37 °C with 5% CO_2 for 7 days. After 4 days, cell monolayers were rinsed once with prewarmed PBS and cultured in 25 ml of fresh L cell–conditioned DMEM. After 7 days, differentiated BMDMs were collected gently using a cell scraper and adjusted to a final concentration of 3.5 × 10^6 cells/ml in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (Thermo Fisher Scientific). Two milliliters was transferred into each well of a 6-well plate and incubated overnight. Cells in 6-well plates were washed once with RPMI$^+$ medium (Sigma) supplemented with heat-inactivated 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 20 mM Hepes (all reagents from Thermo Fisher Scientific). Collection of murine bone marrow cells was done following protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Infection of BMDMs

The BMDMs were infected with overnight cultures of *Salmonella* at a multiplicity of infection of 2 or 10 in RPMI$^+$ medium for killing assays or mRNA collection, respectively. The infected cells were centrifuged at 3000*g* for 1 min and incubated for 30 min at 37 °C in a 5% CO_2 incubator. Medium was replaced with fresh RPMI medium containing 10 μg/ml gentamicin. The cells were washed once with PBS after 8 h of infection, and the specimens were lysed with 0.1% Triton X-100. The specimens were vortexed for 20 s and centrifuged at 200*g* for 5 min at 4 °C. Intracellular bacteria were collected by centrifugation at 9300*g* for 3 min at 4 °C to be processed for RNA isolation.

Survival of Salmonella *in BMDM*

BMDMs infected with *Salmonella* at a multiplicity of infection of 2 were lysed with 0.1% Triton X-100 after 25 min of challenge (*i.e.*, t = 0). The medium was replaced with fresh RPMI medium containing 10 μg/ml gentamicin. Selected samples were lysed and plated onto LB agar at 2 or 19 h thereafter. The bacterial burden is expressed as colony-forming unit/well.

Intracellular SPI-2 gene transcription

DNA-free RNA was purified from BMDM 9 h after *Salmonella* challenge using a High Pure RNA isolation kit according to the manufacturer's instructions (Roche). cDNA was prepared from 1 μg RNA using a mixture containing 100 U MMLV reverse transcriptase, 0.45 mM N6 random hexamer primers (Thermo Fisher Scientific), and 20 U RNAsin Plus RNase inhibitor (Promega). Reverse transcription was performed for 1 h at 42 °C. qRT–PCR was performed in a CFX connect Real-Time System (Bio-Rad). PCR-amplified DNA fragments containing the *ssrA*, *sifA*, or *rpoD* genes were used to generate standard curves. Reactions were prepared using Lunar Universal qPCR Master Mix (New England Biolabs) and incubated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, and 58 °C for 1 min. Data are expressed as relative expression using the copy number of target gene over copy number of housekeeping gene *rpoD*.

Data availability

All data are contained within the article or supporting information.

Supporting information—This article contains supporting information (23, 33–35, 42, 64, 66–68).

Acknowledgments—We thank Dr Jessica Jones-Carson for providing the mice used in this article. We also thank Drs Jessica Jones-Carson and Siva Uppalapati for critically reading the article. These studies were supported by a Veteran Affairs, United States Merit Grant BX0002073 and National Institutes of Health, United States grants R01 AI54959 and R01 AI136520.

Author contributions—J. S. K. and A. V. T. conceptualization; J. S. K., L. L., B. D., and J. S. K. methodology; J. S. K., A. V. T., J. S. K., and L. L. formal analysis; J. S. K. and L. L. investigation; B. D., J. S. K., T. E. M., and A. V. T. resources; J. S. K. and A. V. T. writing–original draft; A. V. T. supervision; T. E. M. and A. V. T. funding acquisition.

Funding and additional information—The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BMDM, bone marrow–derived macrophage; cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GST, glutathione-*S*-transferase; H_2O_2, hydrogen peroxide; NOX, NADPH oxidase; O_2, molecular oxygen; ppGpp, guanosine tetraphosphate; ROS, reactive oxygen species; SPI-2, *Salmonella* pathogenicity island-2.

References

1. Gordon, M. A., Banda, H. T., Gondwe, M., Gordon, S. B., Boeree, M. J., Walsh, A. L., *et al.* (2002) Non-typhoidal salmonella bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. *AIDS* **16**, 1633–1641
2. Feasey, N. A., Dougan, G., Kingsley, R. A., Heyderman, R. S., and Gordon, M. A. (2012) Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. *Lancet* **379**, 2489–2499
3. Walker, G. T., Yang, G., Tsai, J. Y., Rodriguez, J. L., English, B. C., Faber, F., *et al.* (2021) Malaria parasite infection compromises colonization resistance to an enteric pathogen by reducing gastric acidity. *Sci. Adv.* **7**, eabd6232
4. Mabey, D. C., Brown, A., and Greenwood, B. M. (1987) Plasmodium falciparum malaria and Salmonella infections in Gambian children. *J. Infect. Dis.* **155**, 1319–1321
5. Glaser, J. B., Morton-Kute, L., Berger, S. R., Weber, J., Siegal, F. P., Lopez, C., *et al.* (1985) Recurrent Salmonella typhimurium bacteremia associated with the acquired immunodeficiency syndrome. *Ann. Intern. Med.* **102**, 189–193
6. Perez-Morales, D., Banda, M. M., Chau, N. Y. E., Salgado, H., Martinez-Flores, I., Ibarra, J. A., *et al.* (2017) The transcriptional regulator SsrB is involved in a molecular switch controlling virulence lifestyles of Salmonella. *PLoS Pathog.* **13**, e1006497
7. Brown, N. F., Vallance, B. A., Coombes, B. K., Valdez, Y., Coburn, B. A., and Finlay, B. B. (2005) Salmonella pathogenicity island 2 is expressed prior to penetrating the intestine. *PLoS Pathog.* **1**, e32
8. Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E., and Holden, D. W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**, 400–403
9. Ochman, H., Soncini, F. C., Solomon, F., and Groisman, E. A. (1996) Identification of a pathogenicity island required for Salmonella survival in host cells. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 7800–7804
10. Luk, C. H., Valenzuela, C., Gil, M., Swistak, L., Bomme, P., Chang, Y. Y., *et al.* (2021) Salmonella enters a dormant state within human epithelial cells for persistent infection. *PLoS Pathog.* **17**, e1009550
11. Rathman, M., Sjaastad, M. D., and Falkow, S. (1996) Acidification of phagosomes containing Salmonella typhimurium in murine macrophages. *Infect. Immun.* **64**, 2765–2773
12. Cunrath, O., and Bumann, D. (2019) Host resistance factor SLC11A1 restricts Salmonella growth through magnesium deprivation. *Science* **366**, 995–999
13. Fass, E., and Groisman, E. A. (2009) Control of Salmonella pathogenicity island-2 gene expression. *Curr. Opin. Microbiol.* **12**, 199–204
14. Coombes, B. K., Wickham, M. E., Lowden, M. J., Brown, N. F., and Finlay, B. B. (2005) Negative regulation of Salmonella pathogenicity island 2 is required for contextual control of virulence during typhoid. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 17460–17465
15. Jiang, L., Wang, P., Li, X., Lv, R., Wang, L., Yang, B., *et al.* (2020) PagR mediates the precise regulation of Salmonella pathogenicity island 2 gene expression in response to magnesium and phosphate signals in Salmonella Typhimurium. *Cell. Microbiol.* **22**, e13125
16. Kim, C. C., and Falkow, S. (2004) Delineation of upstream signaling events in the salmonella pathogenicity island 2 transcriptional activation pathway. *J. Bacteriol.* **186**, 4694–4704
17. Deiwick, J., Nikolaus, T., Erdogan, S., and Hensel, M. (1999) Environmental regulation of Salmonella pathogenicity island 2 gene expression. *Mol. Microbiol.* **31**, 1759–1773
18. Linehan, S. A., Rytkonen, A., Yu, X. J., Liu, M., and Holden, D. W. (2005) SlyA regulates function of Salmonella pathogenicity island 2 (SPI-2) and expression of SPI-2-associated genes. *Infect. Immun.* **73**, 4354–4362
19. Lee, A. K., Detweiler, C. S., and Falkow, S. (2000) OmpR regulates the two-component system SsrA-ssrB in Salmonella pathogenicity island 2. *J. Bacteriol.* **182**, 771–781
20. Bijlsma, J. J., and Groisman, E. A. (2005) The PhoP/PhoQ system controls the intramacrophage type three secretion system of Salmonella enterica. *Mol. Microbiol.* **57**, 85–96
21. Feng, X., Oropeza, R., and Kenney, L. J. (2003) Dual regulation by phospho-OmpR of ssrA/B gene expression in Salmonella pathogenicity island 2. *Mol. Microbiol.* **48**, 1131–1143
22. Walthers, D., Li, Y., Liu, Y., Anand, G., Yan, J., and Kenney, L. J. (2011) Salmonella enterica response regulator SsrB relieves H-NS silencing by displacing H-NS bound in polymerization mode and directly activates transcription. *J. Biol. Chem.* **286**, 1895–1902
23. Tapscott, T., Kim, J. S., Crawford, M. A., Fitzsimmons, L., Liu, L., Jones-Carson, J., *et al.* (2018) Guanosine tetraphosphate relieves the negative regulation of Salmonella pathogenicity island-2 gene transcription exerted by the AT-rich ssrA discriminator region. *Sci. Rep.* **8**, 9465
24. Song, M., Kim, H. J., Ryu, S., Yoon, H., Yun, J., and Choy, H. E. (2010) ppGpp-mediated stationary phase induction of the genes encoded by horizontally acquired pathogenicity islands and cob/pdu locus in Salmonella enterica serovar Typhimurium. *J. Microbiol.* **48**, 89–95
25. Rice, C. J., Ramachandran, V. K., Shearer, N., and Thompson, A. (2015) Transcriptional and post-transcriptional modulation of SPI1 and SPI2 expression by ppGpp, RpoS and DksA in Salmonella enterica sv typhimurium. *PLoS One* **10**, e0127523
26. Gourse, R. L., Chen, A. Y., Gopalkrishnan, S., Sanchez-Vazquez, P., Myers, A., and Ross, W. (2018) Transcriptional responses to ppGpp and DksA. *Annu. Rev. Microbiol.* **72**, 163–184
27. Henard, C. A., Bourret, T. J., Song, M., and Vazquez-Torres, A. (2010) Control of redox balance by the stringent response regulatory protein promotes antioxidant defenses of Salmonella. *J. Biol. Chem.* **285**, 36785–36793
28. Holley, C. L., Zhang, X., Fortney, K. R., Ellinger, S., Johnson, P., Baker, B., *et al.* (2015) DksA and (p)ppGpp have unique and overlapping contributions to Haemophilus ducreyi pathogenesis in humans. *Infect. Immun.* **83**, 3281–3292

29. Chou, W. K., and Brynildsen, M. P. (2019) Loss of DksA leads to multi-faceted impairment of nitric oxide detoxification by Escherichia coli. *Free Radic. Biol. Med.* **130**, 288–296
30. Kim, H. Y., Go, J., Lee, K. M., Oh, Y. T., and Yoon, S. S. (2018) Guanosine tetra- and pentaphosphate increase antibiotic tolerance by reducing reactive oxygen species production in vibrio cholerae. *J. Biol. Chem.* **293**, 5679–5694
31. Carlioz, A., and Touati, D. (1986) Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life? *EMBO J.* **5**, 623–630
32. Agirrezabala, X., Fernandez, I. S., Kelley, A. C., Carton, D. G., Ramakrishnan, V., and Valle, M. (2013) The ribosome triggers the stringent response by RelA *via* a highly distorted tRNA. *EMBO Rep.* **14**, 811–816
33. Kim, J. S., Liu, L., Fitzsimmons, L. F., Wang, Y., Crawford, M. A., Mastrogiovanni, M., *et al.* (2018) DksA-DnaJ redox interactions provide a signal for the activation of bacterial RNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E11780–E11789
34. Henard, C. A., Tapscott, T., Crawford, M. A., Husain, M., Doulias, P. T., Porwollik, S., *et al.* (2014) The 4-cysteine zinc-finger motif of the RNA polymerase regulator DksA serves as a thiol switch for sensing oxidative and nitrosative stress. *Mol. Microbiol.* **91**, 790–804
35. Kim, J. S., Liu, L., and Vazquez-Torres, A. (2021) The DnaK/DnaJ chaperone system enables RNA polymerase-DksA complex formation in Salmonella experiencing oxidative stress. *mBio* **12**, e03443-20
36. Vazquez-Torres, A., and Fang, F. C. (2001) Salmonella evasion of the NADPH phagocyte oxidase. *Microbes Infect.* **3**, 1313–1320
37. Gallois, A., Klein, J. R., Allen, L. A., Jones, B. D., and Nauseef, W. M. (2001) Salmonella pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane. *J. Immunol.* **166**, 5741–5748
38. van der Heijden, J., Bosman, E. S., Reynolds, L. A., and Finlay, B. B. (2015) Direct measurement of oxidative and nitrosative stress dynamics in Salmonella inside macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 560–565
39. Noster, J., Chao, T. C., Sander, N., Schulte, M., Reuter, T., Hansmeier, N., *et al.* (2019) Proteomics of intracellular Salmonella enterica reveals roles of Salmonella pathogenicity island 2 in metabolism and antioxidant defense. *PLoS Pathog.* **15**, e1007741
40. Hiyoshi, H., English, B. C., Diaz-Ochoa, V. E., Wangdi, T., Zhang, L. F., Sakaguchi, M., *et al.* (2022) Virulence factors perforate the pathogen-containing vacuole to signal efferocytosis. *Cell Host Microbe* **30**, 163–170.e166
41. Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D. W., Lucia, S. M., Dinauer, M. C., *et al.* (2000) Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* **287**, 1655–1658
42. Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H., and Fang, F. C. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. *J. Exp. Med.* **192**, 227–236
43. James, P. E., Grinberg, O. Y., Michaels, G., and Swartz, H. M. (1995) Intraphagosomal oxygen in stimulated macrophages. *J. Cell Physiol.* **163**, 241–247
44. Jones-Carson, J., Yahashiri, A., Kim, J. S., Liu, L., Fitzsimmons, L. F., Weiss, D. S., *et al.* (2020) Nitric oxide disrupts bacterial cytokinesis by poisoning purine metabolism. *Sci. Adv.* **6**, eaaz0260
45. Burton, N. A., Schurmann, N., Casse, O., Steeb, A. K., Claudi, B., Zankl, J., *et al.* (2014) Disparate impact of oxidative host defenses determines the fate of Salmonella during systemic infection in mice. *Cell Host Microbe* **15**, 72–83
46. Imlay, J. A. (2009) Oxidative stress. *EcoSal Plus* **3**. https://doi.org/10.1128/ecosalplus.5.4.4
47. de Crouy-Chanel, A., Kohiyama, M., and Richarme, G. (1995) A novel function of Escherichia coli chaperone DnaJ. Protein-disulfide isomerase. *J. Biol. Chem.* **270**, 22669–22672
48. Linke, K., Wolfram, T., Bussemer, J., and Jakob, U. (2003) The roles of the two zinc binding sites in DnaJ. *J. Biol. Chem.* **278**, 44457–44466
49. Winterbourn, C. C., Hampton, M. B., Livesey, J. H., and Kettle, A. J. (2006) Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: implications for microbial killing. *J. Biol. Chem.* **281**, 39860–39869
50. Choi, J., and Groisman, E. A. (2020) Horizontally acquired regulatory gene activates ancestral regulatory system to promote Salmonella virulence. *Nucl. Acids Res.* **48**, 10832–10847
51. Feng, X., Walthers, D., Oropeza, R., and Kenney, L. J. (2004) The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at Salmonella pathogenicity island 2. *Mol. Microbiol.* **54**, 823–835
52. Osborne, S. E., and Coombes, B. K. (2009) RpoE fine tunes expression of a subset of SsrB-regulated virulence factors in Salmonella enterica serovar Typhimurium. *BMC Microbiol.* **9**, 45
53. Li, J., Overall, C. C., Nakayasu, E. S., Kidwai, A. S., Jones, M. B., Johnson, R. C., *et al.* (2015) Analysis of the Salmonella regulatory network suggests involvement of SsrB and H-NS in sigma(E)-regulated SPI-2 gene expression. *Front. Microbiol.* **6**, 27
54. Wang, P., Zhang, H., Liu, Y., Lv, R., Liu, X., Song, X., *et al.* (2020) SoxS is a positive regulator of key pathogenesis genes and promotes intracellular replication and virulence of Salmonella Typhimurium. *Microb. Pathog.* **139**, 103925
55. Buchmeier, N., Bossie, S., Chen, C. Y., Fang, F. C., Guiney, D. G., and Libby, S. J. (1997) SlyA, a transcriptional regulator of Salmonella typhimurium, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. *Infect. Immun.* **65**, 3725–3730
56. Testerman, T. L., Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Libby, S. J., and Fang, F. C. (2002) The alternative sigma factor sigmaE controls antioxidant defences required for Salmonella virulence and stationary-phase survival. *Mol. Microbiol.* **43**, 771–782
57. Demple, B. (1996) Redox signaling and gene control in the Escherichia coli soxRS oxidative stress regulon–a review. *Gene* **179**, 53–57
58. Zheng, M., and Storz, G. (2000) Redox sensing by prokaryotic transcription factors. *Biochem. Pharmacol.* **59**, 1–6
59. Vazquez-Torres, A., Fantuzzi, G., Edwards, C. K., 3rd, Dinarello, C. A., and Fang, F. C. (2001) Defective localization of the NADPH phagocyte oxidase to Salmonella-containing phagosomes in tumor necrosis factor p55 receptor-deficient macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 2561–2565
60. Berger, S. B., Romero, X., Ma, C., Wang, G., Faubion, W. A., Liao, G., *et al.* (2010) SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages. *Nat. Immunol.* **11**, 920–927
61. Suvarnapunya, A. E., and Stein, M. A. (2005) DNA base excision repair potentiates the protective effect of Salmonella pathogenicity Island 2 within macrophages. *Microbiology* **151**, 557–567
62. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 6640–6645
63. Fitzsimmons, L. F., Liu, L., Kant, S., Kim, J. S., Till, J. K., Jones-Carson, J., *et al.* (2020) SpoT induces intracellular Salmonella virulence programs in the phagosome. *mBio* **11**, e03397-19
64. McCollister, B. D., Bourret, T. J., Gill, R., Jones-Carson, J., and Vazquez-Torres, A. (2005) Repression of SPI2 transcription by nitric oxide-producing, IFNgamma-activated macrophages promotes maturation of Salmonella phagosomes. *J. Exp. Med.* **202**, 625–635
65. Crawford, M. A., Tapscott, T., Fitzsimmons, L. F., Liu, L., Reyes, A. M., Libby, S. J., *et al.* (2016) Redox-active sensing by bacterial DksA transcription factors is determined by cysteine and zinc content. *mBio* **7**, e02161-02115
66. Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557–580
67. Husain, M., Jones-Carson, J., Song, M., McCollister, B. D., Bourret, T. J., and Vázquez-Torres, A. (2010) Redox sensor SsrB Cys203 enhances Salmonella fitness against nitric oxide generated in the host immune response to oral infection. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 14396–14401
68. Wang, R. F., and Kushner, S. R. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**, 195–199

Ju-Sim Kim is an assistant research professor in the Department of Immunology and Microbiology at University of Colorado School of Medicine. She has been studying cellular antioxidant mechanisms from diverse bacteria including Gram positive and negative strains; *Streptomyces griseus, Rhodobacter sphaeroides, Synechocystis, Vibrio vulnificus, and Corynebacterium diphtheria*. With extensive experimental knowledge, she is investigating the molecular mechanisms of antioxidant defenses against oxidative/nitrosative stress to protect *Salmonella* against inflammatory mediators of the innate host response.