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RESEARCH ARTICLE

# Redox-Active Sensing by Bacterial DksA Transcription Factors Is Determined by Cysteine and Zinc Content

**Matthew A. Crawford,[a] Timothy Tapscott,[b] Liam F. Fitzsimmons,[a] Lin Liu,[a] Aníbal M. Reyes,[c] Stephen J. Libby,[d] Madia Trujillo,[c] Ferric C. Fang,[d,e] Rafael Radi,[c] Andrés Vázquez-Torres[a,b,f]**

Department of Immunology and Microbiology[a] and Molecular Biology Program,[b] University of Colorado School of Medicine, Aurora, Colorado, USA; Departamento de Bioquimica and Center for Free Radical and Biomedical Research, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay[c]; Department of Laboratory Medicine[d] and Department of Microbiology,[e] University of Washington School of Medicine, Seattle, Washington, USA; Veterans Affairs Eastern Colorado Health Care System, Denver, Colorado, USA[f]

**ABSTRACT** **The four-cysteine zinc finger motif of the bacterial RNA polymerase regulator DksA is essential for protein structure, canonical control of the stringent response to nutritional limitation, and thiol-based sensing of oxidative and nitrosative stress. This interdependent relationship has limited our understanding of DksA-mediated functions in bacterial pathogenesis. Here, we have addressed this challenge by complementing Δ*dksA Salmonella* with *Pseudomonas aeruginosa dksA* paralogues that encode proteins differing in cysteine and zinc content. We find that four-cysteine, zinc-bound (C4) and two-cysteine, zinc-free (C2) DksA proteins are able to mediate appropriate stringent control in *Salmonella* and that thiol-based sensing of reactive species is conserved among C2 and C4 orthologues. However, variations in cysteine and zinc content determine the threshold at which individual DksA proteins sense and respond to reactive species. In particular, zinc acts as an antioxidant, dampening cysteine reactivity and raising the threshold of posttranslational thiol modification with reactive species. Consequently, C2 DksA triggers transcriptional responses in *Salmonella* at levels of oxidative or nitrosative stress normally tolerated by *Salmonella* expressing C4 orthologues. Inappropriate transcriptional regulation by C2 DksA increases the susceptibility of *Salmonella* to the antimicrobial effects of hydrogen peroxide and nitric oxide, and attenuates virulence in macrophages and mice. Our findings suggest that the redox-active sensory function of DksA proteins is finely tuned to optimize bacterial fitness according to the levels of oxidative and nitrosative stress encountered by bacterial species in their natural and host environments.**

**IMPORTANCE** **In order to cause disease, pathogenic bacteria must rapidly sense and respond to antimicrobial pressures encountered within the host. Prominent among these stresses, and of particular relevance to intracellular pathogens such as *Salmonella*, are nutritional restriction and the enzymatic generation of reactive oxygen and nitrogen species. The conserved transcriptional regulator DksA controls adaptive responses to nutritional limitation, as well as to oxidative and nitrosative stress. Here, we demonstrate that each of these functions contributes to bacterial pathogenesis. Our observations highlight the importance of metabolic adaptation in bacterial pathogenesis and show the mechanism by which DksA orthologues are optimized to sense the levels of oxidative and nitrosative stress encountered in their natural habitats. An improved understanding of the conserved processes used by bacteria to sense, respond to, and limit host defense will inform the development of novel strategies to treat infections caused by pathogenic, potentially multidrug-resistant bacteria.**

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Address correspondence to Andrés Vázquez-Torres, Andres.Vazquez-Torres@ucdenver.edu.

DksA is a conserved RNA polymerase (RNAP) regulatory protein that orchestrates the control of intermediary metabolism in Gram-negative bacteria. Upon nutritional limitation, DksA, often together with the nucleotide alarmone guanosine tetraphosphate (ppGpp), directs a transcriptional program known as the stringent response (1). These adaptive changes have been observed in nearly every bacterial species, implying their widespread physiological importance (2). Yet DksA also controls more confined, organism-specific transcriptional programs, suggesting that this key regulator has been finely tuned to promote fitness according to the challenges associated with particular microbial lifestyles (3).

DksA affects gene transcription by interacting directly with RNAP (4). DksA orthologues maintain a highly conserved coiled-coil domain that has been demonstrated to access the secondary channel of RNAP and interact with the trigger loop domain of the $\beta'$ subunit of the polymerase (5, 6). Although the mechanism(s) by which DksA regulates transcription is multifaceted and remains unsettled, it appears that the interaction of DksA with RNAP affects the stability of the open promoter complex and influences transcriptional initiation (4, 7). In addition to the coiled-coil domain, DksA contains a globular domain that is separated from the C-terminal $\alpha$-helix by a variable hinge region. In most annotated DksA orthologues, this critical interface contains a

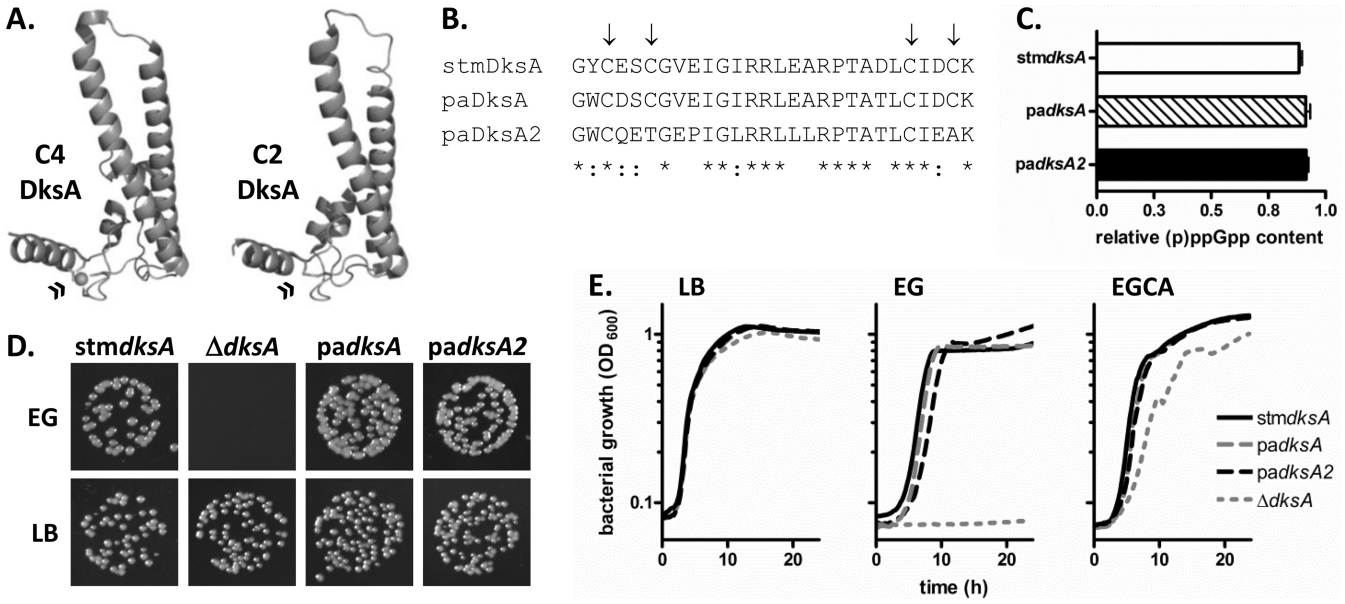


**FIG 1** Functional complementation of the stringent response by paDksA and paDksA2 in *Salmonella*. (A) In C4 DksA proteins, the interdomain hinge region (») contains a four-cysteine zinc finger motif; in C2 proteins, this region contains two conserved cysteine residues and no $Zn^{2+}$ cofactor. The structures shown were previously reported for *E. coli* DksA (C4; Protein Data Bank [PDB] code 1TJL) and *P. aeruginosa* DksA2 (C2; PDB code 4IJJ) (5, 8). (B) The primary amino acid sequence of the hinge region is shown for stmDksA, paDksA, and paDksA2. Cysteine residues ( ↓ ), similarities (:), and identities (*) are indicated. (C) Guanosine pentaphosphate and tetraphosphate [(p)ppGpp] were measured in nucleotide extracts (see Fig. S3B in the supplemental material) prepared from bacteria treated with or without 0.4 mg/ml SHX. Data, expressed as the ratio of (p)ppGpp/[(p)ppGpp + GTP], are the means ± standard errors of the means (SEM) ($n = 3$). (D) Bacteria were grown on LB or EG agar. Images shown are representative of 4 experiments. (E) The optical density at 600 nm ($OD_{600}$) of bacteria grown in LB, EG, or EGCA medium was measured over time. Data are the means ± SEM ($n = 3$). $P < 0.001$ when Δ*dksA Salmonella* grown in EG or EGCA medium were compared to stm*dksA*-expressing controls.

four-cysteine (C4) zinc finger motif proposed to serve a structural purpose essential to protein function (5). The recently resolved structure of a two-cysteine, zinc-deficient (C2) DksA orthologue, however, indicates that the zinc finger motif is not needed for protein structure or canonical regulation of the stringent response (8). Indeed, four- and two-cysteine DksA orthologues each access the secondary channel of RNAP, mediate similar mechanisms of transcriptional regulation, and functionally complement one another in bacteria experiencing nutritional limitation (8, 9). Variations in cysteine and zinc content, however, may distinctly impact the reported capacity of DksA to sense reactive oxygen species (ROS) and reactive nitrogen species (RNS) (10).

ROS and RNS, prominent antimicrobial effectors of innate host defense, damage an array of biomolecules in the microbial cell, causing disruptions in metabolism, protein synthesis, and the maintenance of DNA integrity (11). Many of the bacterial tactics used to counter oxidative and nitrosative stress are controlled by redox-active transcriptional regulators that induce the expression of protective detoxification, repair, and metabolic programs (12, 13). Recent work by our laboratory has demonstrated that cysteine thiol groups (R-SH) in the zinc finger motif of the DksA of *Salmonella enterica* serovar Typhimurium (stmDksA) sense ROS and RNS (10). Oxidative or nitrosative modification of stmDksA sensory thiols triggers a loss of protein α-helicity and release of the coordinated zinc ion (10). The transcriptional response mediated by oxidized stmDksA *in vivo* is characterized by the down-regulation of genes encoding translational machinery, as well as amino acid biosynthesis and transport (10, 14). DksA has also been shown to regulate the synthesis of glutathione (GSH) and support NAD(P)H/$NAD(P)^+$ redox balance through regulatory control of discrete steps in the pentose phosphate pathway and tricarboxylic acid cycle (15). This regulatory profile is distinct from the stringent response, independent of ppGpp, and reversible by the reducing agent dithiothreitol (DTT) (14, 15). Collectively, adaptive changes mediated by oxidized stmDksA appear to promote redox buffering and biomolecule repair, thereby supporting *Salmonella* resistance to oxidative and nitrosative stress.

We hypothesized that the capacity of DksA to sense ROS and RNS is conserved among disparate DksA orthologues and that variations in cysteine and zinc content differentially influence this function. Site-directed mutagenesis of sensory cysteine residues has been used to distinguish functional roles in a number of thiol-based sensors. This approach, however, cannot reasonably be used to examine DksA function, as mutagenesis of cysteine residues in the hinge region of DksA abolishes transcriptional control and results in Δ*dksA* phenotypic traits (4, 9). Here, we have taken advantage of the natural variation among DksA orthologues to test our hypothesis. This approach has provided a unique opportunity to examine the conservation of redox-active sensing among DksA orthologues, investigate the role of the thiol microenvironment in sensing reactive species, and apply these insights to the ability of DksA to promote bacterial pathogenesis in macrophage and murine models of infection.

## RESULTS

**Functional complementation of stringent control by C4 and C2 DksA orthologues in *Salmonella*.** Four-cysteine, zinc-binding (C4) and two-cysteine, zinc-deficient (C2) orthologues of DksA

maintain analogous structures (Fig. 1A) and interact similarly with RNAP (5, 8, 9). *Pseudomonas aeruginosa* encodes both a C4-type DksA (paDksA) and a C2-type DksA (paDksA2) paralogue (8, 9). These proteins display similarity to stmDksA (see Fig. S1 in the supplemental material), and each mediates stringent control *in vivo* (9); however, differences in thiol content and zinc coordination within the hinge region of these proteins (Fig. 1B) are likely to influence the sensing of reactive species. To examine this possibility, we placed codon-optimized pa*dksA* and pa*dksA2* (see Fig. S2) under the control of the native stm*dksA* promoter and cloned these alleles into the chromosome of Δ*dksA Salmonella*. The expression of pa*dksA* and pa*dksA2* by *Salmonella* was indistinguishable from that of stm*dksA* in both the logarithmic and stationary phases of growth (see Fig. S3A). The production of guanosine pentaphosphate and tetraphosphate in response to the amino acid analog serine hydroxymate (SHX), which elicits stringent control (16), was also equal among stm*dksA*-, pa*dksA*-, and pa*dksA2*-expressing *Salmonella* (Fig. 1C). Moreover, complementation with either pa*dksA* or pa*dksA2* relieved amino acid auxotrophy in Δ*dksA Salmonella* grown on minimal E salts glucose (EG) agar (Fig. 1D, top) or liquid medium (Fig. 1E, middle). Bacterial growth in Luria-Bertani (LB; Fig. 1E, left) or EG medium supplemented with 0.1% Casamino Acids (EGCA; Fig. 1E, right) was also indistinguishable among *Salmonella* expressing stm*dksA*, pa*dksA*, or pa*dksA2*. These data demonstrate that both paDksA and paDksA2 mediate appropriate stringent control in *Salmonella* experiencing nutritional limitation and are consistent with similar observations made in *Escherichia coli* and *P. aeruginosa* that have shown that variations in cysteine and zinc content do not affect regulatory control of the stringent response (9).

**Susceptibility of *Salmonella* expressing C4 or C2 DksA orthologues to oxidative and nitrosative stress.** We next investigated the abilities of pa*dksA*- and pa*dksA2*-expressing *Salmonella* to resist oxidative killing by hydrogen peroxide ($H_2O_2$) (Fig. 2A) and the bacteriostatic effects of nitric oxide (·NO) (Fig. 2B). *Salmonella* expressing stm*dksA* or pa*dksA* displayed comparable levels of resistance to oxidative and nitrosative stress, indicating that the ability of DksA to promote bacterial defense against ROS and RNS is conserved among C4 orthologues. In contrast, *Salmonella* expressing pa*dksA2* were hypersusceptible to the antimicrobial effects of $H_2O_2$ and the ·NO donor (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)-amino]diazen-1-ium-1,2-diolate (DETA NONOate [dNO]). We previously reported that Δ*dksA Salmonella* exhibit diminished levels of the cellular antioxidant GSH (15). Consistent with this determination, Δ*dksA Salmonella* demonstrated a more oxidized intrabacterial redox environment in the absence of oxidative stress, as determined by using the redox-sensitive green fluorescent protein roGFP2 (Fig. 2C) (17). In contrast, *Salmonella* expressing stm*dksA*, pa*dksA*, or pa*dksA2* displayed comparably reduced redox potentials. This observation indicates that the increased susceptibility of pa*dksA2*-expressing *Salmonella* to ROS and RNS is due to flawed antioxidative and antinitrosative responses, not deficiencies in basal reducing power.

**Thiol reactivity among C4 and C2 DksA orthologues.** Redox-active sensory cysteine residues are typically maintained as an unprotonated thiolate ($R^-S^-$) that is stabilized via interactions with specific features of the protein microenvironment, including hydrogen bond networks and metal cofactors (18, 19). To determine whether paDksA2 is capable of sensing reactive species, we examined thiol reactivity toward the ROS $H_2O_2$ and the RNS peroxynitrite, the sum of peroxynitrous acid (ONOOH) and peroxynitrite anion ($ONOO^-$). Each of these molecules has been shown previously to modify cysteine thiols in stmDksA (10).

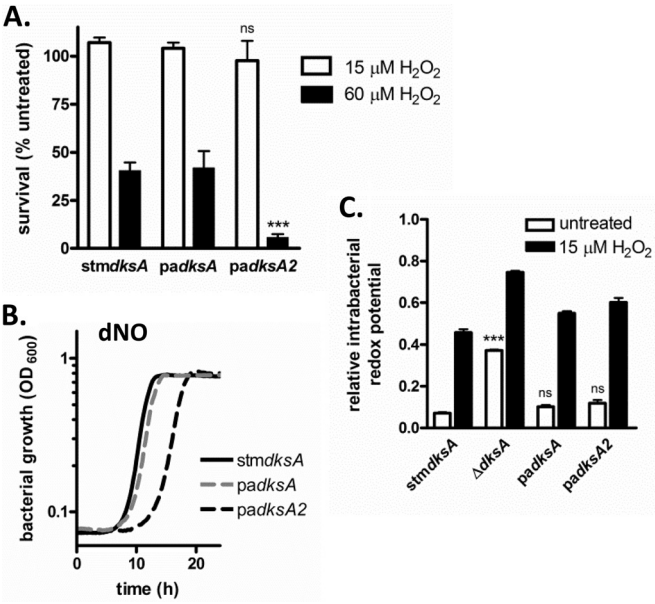


**FIG 2** Bacterial susceptibility to the antimicrobial effects of ROS and RNS. (A) Bacteria were treated with $H_2O_2$, and survival was measured by CFU determination. Data, expressed as the percentage of the result for the respective untreated control, are the means ± SEM ($n = 3$). ***, $P < 0.001$, and ns, nonsignificant, compared to the results for analogously treated stm*dksA*-expressing *Salmonella*. (B) Bacteria were treated with 300 μM of the ·NO donor dNO, and $OD_{600}$ was measured over time. Data are the means ± SEM ($n = 3$). $P < 0.001$ when the results for pa*dksA2*- and stm*dksA*-expressing *Salmonella* were compared. (C) Relative intrabacterial redox potential was determined using roGFP2 and is expressed as the normalized ratios of the emission signals at 510 nm obtained by excitation at 405 and 480 nm; increased ratiometric fluorescence indicates a more oxidizing intrabacterial environment. Data are the means ± SEM ($n = 3$). ***, $P < 0.001$, and ns, nonsignificant, compared to the results for untreated, stm*dksA*-expressing *Salmonella*.

Thiol oxidation with $H_2O_2$ was quantified over time for recombinant stmDksA, paDksA, and paDksA2 (Fig. 3A). The pseudo–first-order rate constant ($k_{obs}$) was plotted against the $H_2O_2$ concentration (see Fig. S4A in the supplemental material) to obtain the second-order rate constant ($k$) for each protein. The preceding kinetic analysis demonstrated paDksA2 ($k = 65.1 \pm 2.5\ M^{-1}\ s^{-1}$ [mean ± 95% confidence interval]) to be more readily modified with $H_2O_2$ than stmDksA ($k = 0.64 \pm 0.03\ M^{-1}\ s^{-1}$) and paDksA ($k = 1.33 \pm 0.05\ M^{-1}\ s^{-1}$). The reactivities of paDksA and paDksA2 toward peroxynitrite were also assessed and showed paDksA ($k = 2{,}420 \pm 60\ M^{-1}\ s^{-1}$) and paDksA2 ($k = 1{,}430 \pm 95\ M^{-1}\ s^{-1}$) to react similarly with peroxynitrite (see Fig. S4B). Interestingly, the extent of thiol modification mediated by equimolar treatment with peroxynitrite was observed to be greater for paDksA2 than for zinc-bound stmDksA and paDksA (Fig. 3B). These data demonstrate that cysteine thiols in the hinge region of paDksA2 are modifiable by ROS and RNS. This conclusion indicates that thiol-based sensing of ROS and RNS is a conserved function of DksA regardless of zinc coordination.

The increased sensitivity of paDksA2 thiols toward reactive species compared to the sensitivities of the zinc-coordinating thiols of stmDksA and paDksA raises the possibility that the $Zn^{2+}$

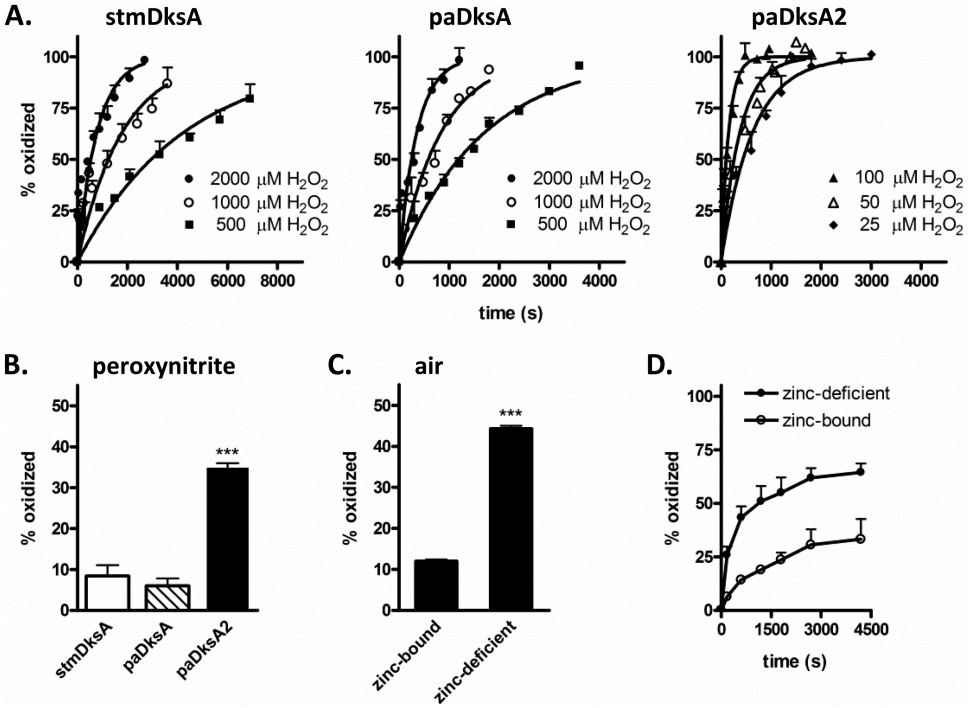


**FIG 3** Thiol reactivities of stmDksA, paDksA, and paDksA2 toward $H_2O_2$ and peroxynitrite. Thiol oxidation in the indicated recombinant DksA proteins (50 $\mu$M) at 37°C and pH 7.4 was determined by the loss of DTNB labeling. The percentage of total thiol content is expressed relative to the results for DTT-reduced controls. (A) DksA proteins were treated with $H_2O_2$, and thiol consumption was measured over time. Data are the means ± SEM ($n = 3$ or 4). The data were fitted to an exponential function, and the $k_{obs}$ values obtained were used to determine rate constants (see Fig. S4A in the supplemental material). (B) DksA proteins were treated with equimolar concentrations of peroxynitrite for 5 min. Data are the means ± SEM ($n = 3$). ***, $P < 0.001$ compared to the results for stmDksA. (C) Reduced zinc-bound and zinc-deficient stmDksA proteins were exposed to air for 1 h at 37°C. Data are the means ± standard deviations (SD) ($n = 2$). ***, $P < 0.001$ compared to the results for zinc-bound stmDksA. (D) Reduced zinc-bound and zinc-deficient stmDksA proteins were treated with 200 $\mu$M $H_2O_2$ inside an anaerobic chamber (5% $CO_2$, 5% $H_2$, 90% $N_2$). Data are the means ± SEM ($n = 3$). $P < 0.001$ when zinc-bound and zinc-deficient stmDksA proteins were compared.

cofactor acts as an antioxidant that increases the threshold of thiol reactivity in C4 DksA orthologues. To directly test this notion, we compared thiol oxidation between zinc-bound and zinc-deficient stmDksA. The $Zn^{2+}$ cofactor of stmDksA was removed by treatment with the reversible thiol-reactive agent *S*-methyl methanethiosulfonate (MMTS). Treatment of stmDksA with MMTS resulted in the complete release of coordinated zinc (see Fig. S4C in the supplemental material). Subsequent reduction with DTT restored the original free thiol content, yielding reduced, zinc-deficient stmDksA (see Fig. S4D). Importantly, we have previously shown, by circular dichroism, that reduced, zinc-deficient stmDksA retains the secondary structure of the zinc-bound protein (10). Exposure of zinc-deficient stmDksA to air resulted in considerable thiol oxidation (Fig. 3C). The thiols of zinc-deficient stmDksA were also hypersensitive to oxidation with $H_2O_2$ as measured in an anaerobic chamber (Fig. 3D). Taken together, these investigations demonstrate that the coordination of zinc in C4 DksA orthologues mediates an antioxidant function that increases the threshold of thiol modification.

**Transcriptional regulation by C4 and C2 DksA orthologues in *Salmonella* experiencing oxidative or nitrosative stress.** That *Salmonella* expressing paDksA2 are more susceptible to the antimicrobial effects of ROS and RNS, despite the ability of this C2 DksA orthologue to detect reactive species, suggests that paDksA2 misregulates the adaptive response of *Salmonella* experiencing oxidative or nitrosative stress. To investigate this prospect, we examined changes in gene expression in response to ROS and RNS using quantitative real-time PCR (qPCR). These analyses focused on transcriptional changes among *livJ*, which encodes a component of the branched-chain amino acid transport system, as well as *rplU* and *rpsM*, which encode ribosomal proteins. Each of these genes has been shown to be down-regulated in a DksA-dependent fashion in *Salmonella* experiencing oxidative or nitrosative stress (10, 15).

As *Salmonella* expressing paDksA2 are hypersusceptible to the bactericidal effects of $H_2O_2$, we examined the transcriptional changes that occur in response to 15 $\mu$M $H_2O_2$. At this concentration of $H_2O_2$, stm*dksA*-, pa*dksA*-, and pa*dksA2*-expressing *Salmonella* each experienced a comparable level of oxidative stress (Fig. 2C; see also Fig. S5A in the supplemental material) and maintained viability (Fig. 2A). Transcriptional analysis showed that pa*dksA2*-expressing *Salmonella* underwent significantly greater down-regulation of *livJ*, *rplU*, and *rpsM* upon exposure to $H_2O_2$ than did stm*dksA*- and pa*dksA*-expressing bacteria (Fig. 4A). *Salmonella* expressing stm*dksA* or pa*dksA* responded similarly to one another upon treatment, and changes in gene expression were consistent with the use of suboptimal levels of $H_2O_2$ for triggering responses from zinc-bound C4 DksA orthologues. As anticipated, treatment of stm*dksA*- and pa*dksA*-expressing *Salmonella* with higher levels of $H_2O_2$ (100 $\mu$M) resulted in the down-regulation of *livJ*, *rplU*, and *rpsM* expression to levels comparable to those mediated by paDksA2 at 15 $\mu$M $H_2O_2$ (see Fig. S5C). To determine

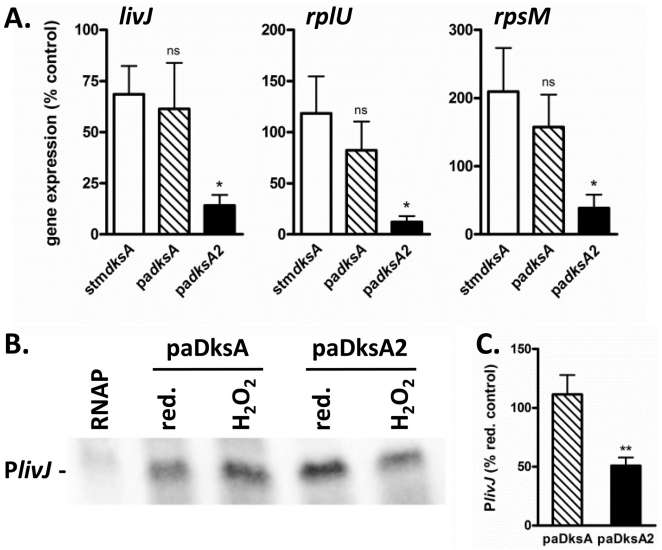


**FIG 4** DksA-dependent transcriptional responses to ROS *in vivo* and *in vitro*. (A) Target gene expression levels, normalized to the expression of the housekeeping gene *rpoD*, in bacteria treated with 15 $\mu$M $H_2O_2$ are expressed as the percentage of the result for the respective untreated control. Data are the means ± SEM ($n = 3$). *, $P < 0.05$, and ns, nonsignificant, compared to the results for stm*dksA*-expressing *Salmonella*. Representative transcript levels for untreated and $H_2O_2$-treated bacteria are reported in Fig. S5B in the supplemental material. (B) *In vitro* transcription from P*livJ* was measured in reaction mixtures containing RNAP and the indicated reduced (red.) or 50 $\mu$M $H_2O_2$-treated DksA proteins. The image shown is representative of the results of 3 experiments. (C) *In vitro* differences in regulatory control were quantified by densitometry. Data [(oxidized/reduced) × 100] are the means ± SEM ($n = 3$). **, $P < 0.01$ compared to the results for paDksA.

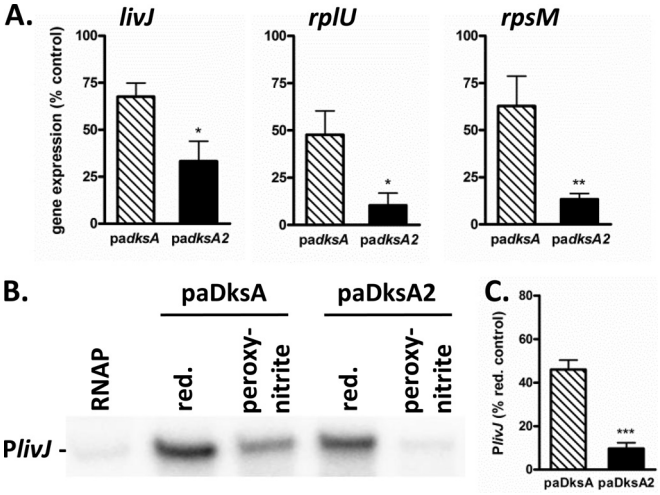


**FIG 5** DksA-dependent transcriptional response to RNS *in vivo* and *in vitro*. (A) Target gene expression levels, normalized to that of the housekeeping gene *rpoD*, in bacteria treated with 25 $\mu$M sNO are expressed as the percentage of the result for the respective untreated control. Data are the means ± SEM ($n = 3$). *, $P < 0.05$, and **, $P < 0.01$, compared to the results for pa*dksA*-expressing *Salmonella*. Representative transcript levels for untreated and sNO-treated bacteria are reported in Fig. S6C in the supplemental material. (B) *In vitro* transcription from P*livJ* was measured in reaction mixtures containing RNAP and the indicated reduced (red.) or 25 $\mu$M peroxynitrite-treated DksA proteins. The image shown is representative of the results of 3 experiments. (C) *In vitro* differences in regulatory control were quantified by densitometry. Data [(oxidized/reduced) × 100] are the means ± SEM ($n = 3$). ***, $P < 0.001$ compared to the results for paDksA.

whether the disparate transcriptional responses mediated by C4 and C2 DksA orthologues to $H_2O_2$ reflect direct effects on transcription, we compared the ability of reduced or oxidized paDksA and paDksA2 to regulate *in vitro* transcription from the *livJ* promoter (P*livJ*) (Fig. 4B and C). As previously reported (10), RNAP alone was unable to initiate transcription from P*livJ*. The inclusion of reduced paDksA or paDksA2 activated transcription, consistent with the ability of each of these proteins to direct stringent control *in vivo*. Upon oxidation with 50 $\mu$M $H_2O_2$, paDksA2 but not paDksA mediated a marked reduction in P*livJ* transcription.

*In vivo* and *in vitro* transcriptional analyses were also performed to define RNS-mediated changes in gene expression. Exposure to 25 $\mu$M of the ·NO donor spermine NONOate (sNO) did not affect the growth of either pa*dksA*- or pa*dksA2*-expressing *Salmonella* (see Fig. S6A in the supplemental material). The expression of *livJ*, *rplU*, and *rpsM* was down-regulated in pa*dksA2*-expressing *Salmonella* upon treatment with sNO (Fig. 5A). Bacteria expressing pa*dksA* also exhibited reduced transcription of *livJ*, *rplU*, and *rpsM*; however, these effects were milder than those observed for pa*dksA2* organisms. Similar results were observed when dNO was used as the ·NO donor (see Fig. S6B). *In vitro* transcription from P*livJ* was also examined for paDksA and paDksA2 in response to the RNS peroxynitrite (Fig. 5B and C). Peroxynitrite treatment of these DksA orthologues resulted in transcriptional down-regulation, particularly by paDksA2. Cumulatively, our investigations demonstrate that C4 and C2 DksA orthologues facilitate similar regulatory outputs in response to ROS and RNS; however, the C2 DksA orthologue paDksA2 does so at lower levels of oxidative or nitrosative stress. These findings are consistent with the notion that the thiol microenvironment of DksA governs the oxidative/nitrosative threshold at which DksA-dependent transcriptional responses are triggered and that differences in the hinge region of disparate DksA orthologues directly influence this quality.

**Virulence of *Salmonella* expressing C4 or C2 DksA orthologues.** The ability of *Salmonella* to survive and replicate in macrophages following phagocytosis is an essential aspect of pathogenesis and requires that *Salmonella* respond effectively to nutritional limitation (20). To test the capacity of paDksA and paDksA2 to support intracellular survival and replication, we infected J774 macrophage-like cells with Δ*dksA*-, stm*dksA*-, pa*dksA*-, and pa*dksA2*-expressing *Salmonella*. Importantly, under the experimental conditions examined here, the amount of superoxide ($O_2^{\cdot -}$) produced by J774 cells in response to bacterial infection or treatment with phorbol 12-myristate 13-acetate is under the limit of detection by lucigenin-mediated chemiluminescence (see Fig. S7A in the supplemental material). The absence of a respiratory burst permits the specific study of nutritional challenges encountered by *Salmonella* within host cells, independent of oxidative stress. As expected due to their inability to mediate stringent control, Δ*dksA* bacteria were incapable of intracellular growth in J774 cells (Fig. 6A). *Salmonella* expressing stm*dksA*, pa*dksA*, or pa*dksA2* survived and replicated intracellularly, validating the presence of a functional stringent response and the ability to overcome intracellular nutritional limitation. We next examined bacterial survival within primary peritoneal macrophages isolated from immunocompetent C57BL/6 mice and capable of generating ROS and RNS or from congenic gp91phox$^{-/-}$

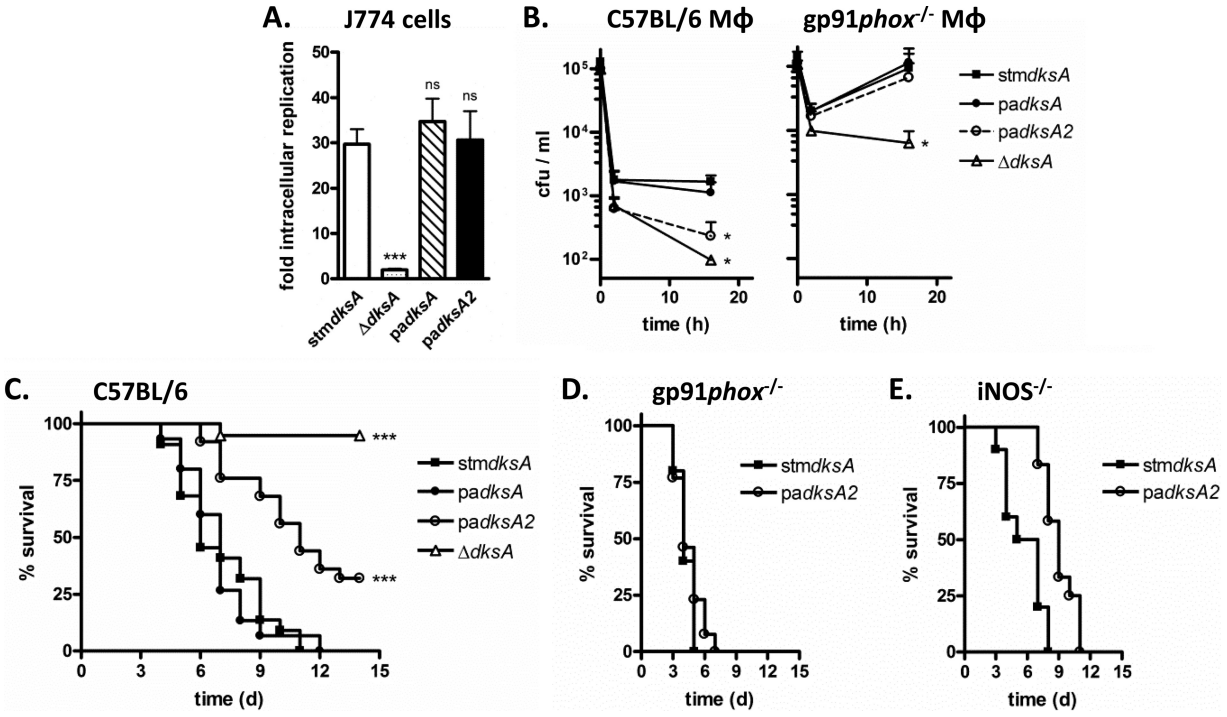


**FIG 6** ROS- and RNS-dependent disparities in the abilities of C4 and C2 DksA orthologues to functionally complement *Salmonella* pathogenesis *in vivo*. (A) Intracellular replication in J774 cells 20 h postinfection as measured by CFU determination. Data, expressed as fold change compared to the number of intracellular bacteria determined 2 h after infection, are the means ± SEM ($n = 3$). ***, $P < 0.001$, and ns, nonsignificant, compared to the results for stm*dksA*-expressing *Salmonella*. (B) Intracellular survival and replication of *Salmonella* in C57BL/6 and gp91phox$^{-/-}$ primary macrophages was determined by CFU determination. Data, expressed as CFU/ml, are the means ± SEM ($n = 2$ or 3). *, $P < 0.05$ compared to the results for stm*dksA*-expressing *Salmonella*. (C) Mortality among C57BL/6 mice challenged with the indicated bacteria. Percent survival represents combined mortality from 12 to 15 mice per group; ***, $P < 0.001$ compared to the results for stm*dksA*-expressing *Salmonella*. (D and E) Mortality among gp91phox$^{-/-}$ and iNOS$^{-/-}$ mice represents combined data from 6 to 8 mice per group. $P = 0.49$ and $P < 0.001$ when pa*dksA2*- and stm*dksA*-expressing *Salmonella* were compared in gp91phox$^{-/-}$ and iNOS$^{-/-}$ mice, respectively.

animals unable to generate $O_2^{\cdot-}$ (see Fig. S7B and C). Survival within immunocompetent macrophages was significantly reduced among Δ*dksA* and pa*dksA2*-expressing *Salmonella* compared to the survival of stm*dksA*- and pa*dksA*-expressing bacteria (Fig. 6B). In the absence of phagocyte-derived $O_2^{\cdot-}$, *Salmonella* expressing pa*dksA2* were able to survive and replicate. In contrast, while Δ*dksA* bacteria were able to survive in gp91phox$^{-/-}$ macrophages, these bacteria were still unable to replicate, likely as a consequence of their inability to overcome the nutritional restrictions imposed by the host cells.

We also examined the virulence of Δ*dksA*, stm*dksA*-, pa*dksA*-, and pa*dksA2*-expressing *Salmonella* in a murine model of acute infection (Fig. 6C). *Salmonella* deficient in DksA cannot respond appropriately to nutritional limitation or oxidative/nitrosative stress and were observed to be severely attenuated. *Salmonella* expressing paDksA, which is capable of appropriately mediating both nutritional and oxidative/nitrosative responses in the context of *Salmonella*, demonstrated virulence equivalent to that of stm*dksA*-expressing bacteria. The virulence of *Salmonella* expressing paDksA2 was intermediate to the preceding phenotypes. This observation is in agreement with the ability of *Salmonella* expressing paDksA2 to respond appropriately to nutritional stress, but not to oxidative and nitrosative stress. Indeed, the attenuation observed for *Salmonella* expressing paDksA2 in immunocompetent mice was fully relieved in gp91phox$^{-/-}$ mice (Fig. 6D). Attenuation was also diminished in iNOS$^{-/-}$ mice (Fig. 6E). The incomplete restoration of virulence by *Salmonella* expressing paDksA2 in iNOS$^{-/-}$ mice is likely owed to the increased production of $O_2^{\cdot-}$ observed in this host background during infection (21). Taken together, the results of these investigations indicate that Gram-negative bacteria harbor DksA orthologues that are finely tuned to sense and respond to the levels of oxidative and nitrosative stress encountered in their particular environmental niches.

## DISCUSSION

The bacterial RNAP regulator DksA orchestrates metabolic adaptation in response to antimicrobial pressures encountered in natural and host environments. Although highly conserved in Gram-negative bacteria, DksA proteins maintain hinge regions that vary in cysteine and zinc content. Our investigations have revealed that these variations differentially govern the threshold at which DksA proteins respond to reactive species, suggesting that the natural disparity inherent among DksA orthologues reflects a mechanism to influence the redox-active sensory function of DksA while preserving canonical stringent control. In particular, the absence of the $Zn^{2+}$ cofactor in paDksA2, or its removal from stmDksA, is associated with increased thiol reactivity, demonstrating that zinc serves an antioxidant function in C4 DksA orthologues. This function is similar to the role of zinc in the redox-sensitive anti-sigma factor RsrA (22) and supports theoretical considerations that have suggested that zinc coordination by thiol groups limits nucleophi-

licity, thereby raising the free energy barrier of oxidative modification and dampening thiol reactivity (23). Differences in reactivity among C4 and C2 DksA orthologues may also be influenced by other local determinants of thiol reactivity, such as hydrogen bond networks and charged amino acid residues (19). Cumulatively, it appears that distinct variations in the thiol microenvironment of DksA sensory cysteine residues finely tune the threshold at which DksA proteins respond to oxidative and nitrosative stress.

Oxidized C4 and C2 DksA orthologues mediate similar transcriptional responses, indicating that the adaptive changes mediated by DksA in response to oxidative and nitrosative stress are a generalizable survival strategy. However, paDksA2 failed to direct appropriate antioxidative and antinitrosative responses in *Salmonella* and was not well suited for promoting the fitness of this enteropathogen in macrophage and murine models of infection. Our observations indicate that the capacity of DksA to appropriately sense and respond to host-derived ROS and RNS contributes to *Salmonella* pathogenesis. Moreover, the underperformance of the C2 orthologue paDksA2 in *Salmonella* was associated with hypersensitivity toward reactive species, suggesting that the threshold of sensing of oxidative or nitrosative stress by DksA orthologues is important to the promotion of bacterial fitness. Taking into account estimated intrabacterial thiol concentrations and rates of oxidation (see Table S1 in the supplemental material), the C4 orthologues stmDksA and paDksA (~$0.10 \times 10^{-3}$ $s^{-1}$) react with $H_2O_2$ less efficiently than does the principle cellular reductant GSH ($0.87 \times 10^{-3}$ $s^{-1}$) at physiological pH (24). The presence of 1 to 3 mM GSH in the bacterial cell would therefore be expected to protect the 25 $\mu$M of C4 DksA protein reportedly in the cytoplasm of Gram-negative bacteria from steady-state intracellular $H_2O_2$ concentrations estimated to be ~20 nM (25–27). The 15 to 40 $\mu$M of $H_2O_2$ generated in the inflammatory response to *Salmonella* consumes GSH (15, 28). The resulting drop in redox buffering capacity may allow for the oxidation of C4 DksA thiols, thus inducing DksA-mediated antioxidative and antinitrosative defenses critical to reestablishing reducing power and promoting *Salmonella* pathogenesis. In contrast to the zinc-bound C4 orthologues, paDksA2 ($3.26 \times 10^{-3}$ $s^{-1}$) would be a preferred target for thiol oxidation with $H_2O_2$ compared to GSH. Therefore, in the context of *Salmonella*, the increased sensitivity of paDksA2 toward ROS and RNS may undermine pathogenesis by inappropriately triggering transcriptional changes at levels of oxidative and nitrosative stress tolerated by C4 DksA proteins. Along these lines, it is important to note that the respiratory burst of host phagocytes does not end abruptly; rather, the production of $O_2^{\cdot -}$ diminishes over several hours as measured by lucigenin- or luminol-dependent chemiluminescence (21). This observation, taken together with the hyperreactivity of paDksA2, may suggest that the adaptive changes mediated by paDksA2 in response to reactive species inappropriately persist during association with phagocytes, perhaps explaining the attenuation of *Salmonella* expressing paDksA2 in macrophage and murine models of infection.

*P. aeruginosa* is an extremely versatile bacterium, capable of colonizing an extensive range of environmental and host niches. It has been shown that *Pseudomonas* preferentially employs paDksA2 under zinc-limited growth conditions (9), indicating that which DksA paralog *Pseudomonas* utilizes might be dictated by environmental cues. The more sensitive C2 paralog paDksA2 might be advantageous for monitoring redox homeostasis during the colonization of hypoxic environments met by *P. aeruginosa* in soil and cystic fibrosis lesions (29). The notion that DksA proteins might be specialized according to the levels of oxidative stress experienced by bacteria in their environments is similar to the preferential utilization of specific PerR orthologues according to the reactivity of these sensory regulators (30). DksA and PerR exemplify the novel realization that the sensory activity of redox-active regulatory proteins is finely tuned to the levels of reactive species encountered by bacteria.

The unique biochemical features of the amino acid cysteine are utilized in protein structure, redox-based sensing, enzymatic catalysis, and metal coordination. While all known DksA orthologues maintain distinct hinge regions, containing one, two, or four cysteine residues, variations in cysteine content seem to have little effect on DksA structure or regulatory control (8, 9). Mutagenesis of these cysteine residues, however, invariably disrupts the structural integrity of DksA and abolishes transcriptional control (4, 8, 9). Structural analyses of C4 and C2 DksA orthologues have demonstrated DksA to have a small, marginally stable hydrophobic core (5, 8). It has been proposed that cysteine residues in the hinge region of DksA, regardless of zinc coordination, may promote structural integrity, especially in regard to the orientations of the globular and coiled-coil domains (5, 8). Thus, the conservation of cysteine residues in all DksA orthologues may serve interdependent roles in structure and redox-active sensing. The changes in $\alpha$-helical content that occur upon oxidative or nitrosative modification of DksA support this interdependence (10). Posttranslational modification of DksA by ROS or RNS may trigger conformational changes that influence interaction with RNAP and result in altered transcriptional control. This proposed mechanism is generally consistent with a number of prokaryotic and eukaryotic redox-active transcription factors that display reversible ROS- and RNS-dependent structural changes associated with altered regulatory profiles (12, 13, 31). DksA-dependent transcriptional changes mediated in response to ROS and RNS could also result from the functional inactivation of this RNAP regulatory protein. Indeed, the induction of antioxidant programs by several redox-active transcriptional regulators, including RsrA and PerR, stems precisely from functional inactivation by reactive species (12, 31). If this were the case for DksA, however, we would expect to observe derepression among genes encoding ribosomal proteins, as these loci are repressed in a DksA-dependent manner. However, as illustrated by the results for *rpsM* and *rplU*, oxidized DksA further represses the expression of these genes. Additionally, transcriptional down-regulation from P*livJ* by oxidized DksA *in vitro* is dose dependent in regard to DksA (10).

The reactivity of DksA proteins toward $H_2O_2$ is several orders of magnitude lower than that of dedicated peroxide sensors like OxyR or thiol-dependent peroxidases, which catalyze $H_2O_2$ and peroxynitrite reduction with rate constants of ~$10^5$ to $10^7$ $M^{-1}$ $s^{-1}$ (12, 18, 19). Thus, it is possible that DksA thiol groups could be oxidized by bacterial peroxidases *in vivo*, as has been described for eukaryotic transcription factors such as Yap1 (32). The oxidation of DksA by bacterial peroxidases would form a robust redox relay approach by which to affect the integration of nutritional, oxidative, and nitrosative signals by DksA over a range of physiological conditions.

Collectively, our investigations suggest that individual Gram-negative bacterial species maintain DksA proteins that possess uniquely tailored sensory cysteine residues that are finely tuned by variations in the thiol microenvironment to the oxidative and

nitrosative stresses encountered in their particular niches. These findings demonstrate an essential role for the variability observed among the hinge regions of DksA orthologues and raise the intriguing possibility that the specific control of thiol-based sensing is a critical driving force behind the molecular evolution of DksA.

## MATERIALS AND METHODS

Detailed descriptions of the methodologies and statistical analyses used in this study are provided in Text S1 in the supplemental material.

**Bacterial strains.** *Salmonella enterica* serovar Typhimurium strain 14028s and its derivatives used in this study are described in Table S2 in the supplemental material.

**Growth conditions.** Amino acid auxotrophies were examined by growth in EG medium. Unless otherwise noted, bacterial susceptibilities and transcriptional responses to ROS and RNS were examined in EGCA medium.

**Measurement of (p)ppGpp and intrabacterial redox potential.** The production of (p)ppGpp in nucleotide extracts isolated from radiolabeled bacteria treated with or without SHX was measured using formic acid extraction and separation by thin-layer chromatography. Intrabacterial redox potentials were determined by fluorescence measurement as reported for roGFP2 in *Salmonella* (17).

**Transcriptional analysis.** Changes in gene expression were determined by qPCR; the primers and probes used for these analyses are listed in Table S2 in the supplemental material. *In vitro* transcriptional analysis from P*livJ* (−128 to +320) was performed as described previously by our laboratory (10). The resultant transcripts were resolved by electrophoresis and visualized by phosphorimaging.

**Protein purification and measurement of thiol reactivity.** The purification of recombinant stmDksA, paDksA, and paDksA2 was performed by glutathione *S*-transferase (GST) affinity isolation followed by size exclusion chromatography (10). Protein thiol reactivity toward $H_2O_2$ was determined using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) labeling of free thiol groups (24). Zinc-deficient stmDksA was prepared by treatment with MMTS, followed by reduction with DTT. The reactivities of paDksA and paDksA2 with peroxynitrite were determined using stopped-flow kinetics (33).

**Macrophage isolation and animal challenges.** All studies involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado—Denver Anschutz Medical Campus, under protocol number 56413(07)1E. The levels of intracellular survival and replication of *Salmonella* were determined using a standard gentamicin protection assay with a multiplicity of infection of 10. *Salmonella* virulence in a murine model of acute infection was examined by inoculating mice with approximately 300 bacteria by intraperitoneal injection.

**Statistical analysis.** Statistical analysis and graphing were performed using GraphPad Prism 4.0 software; a $P$ value of $<0.05$ was considered to be significant.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02161-15/-/DCSupplemental.

Figure S1, TIF file, 0.9 MB.
Figure S2, TIF file, 2.9 MB.
Figure S3, TIF file, 1.8 MB.
Figure S4, TIF file, 3.8 MB.
Figure S5, TIF file, 6.2 MB.
Figure S6, TIF file, 4.6 MB.
Figure S7, TIF file, 2.4 MB.
Table S1, DOCX file, 0.01 MB.
Table S2, DOCX file, 0.02 MB.
Text S1, DOCX file, 0.05 MB.

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