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

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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 ΔdksA 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.

IMPACT 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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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 alanine 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 β′ 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 α-helix by a variable hinge region. In most annotated DksA orthologues, this critical interface contains a
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 Zn²⁺ cofactor. The structures shown were previously reported for E. coli DksA (C4; Protein Data Bank [PDB] code ITL) 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 tetrathosphate [(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 (OD₆₀₀) of bacteria grown in LB, EG, or EGCA medium was measured over time. Data are the means ± SEM (n = 3). P < 0.001 when ΔdksA Salmonella grown in EG or EGCA medium were compared to stmdksA-expressing controls.

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 functional complementation 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.

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) parologue (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 *padksA* and *padksA2* (see Fig. S2) under the control of the native stmdksA promoter and cloned these alleles into the chromosome of *ΔdksA Salmonella*. The expression of *padksA* and *padksA2* by *Salmonella* was indistinguishable from that of *stmdksA* 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 hydroxamate (SHX), which elicits stringent control (16), was also equal among *stmdksA*, *padksA*, and *padksA2*-expressing *Salmonella* (Fig. 1C). Moreover, complementation with either *padksA* or *padksA2* relieved amino acid auxotrophy in *ΔdksA 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 stmdksA, padksA, or padksA2. 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 *padksA* and *padksA2*-expressing *Salmonella* to resist oxidative killing by hydrogen peroxide (H2O2) (Fig. 2A) and the bacteriostatic effects of nitric oxide (NO) (Fig. 2B). *Salmonella* expressing stmdksA or padksA 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 padksA2 were hypersusceptible to the antimicrobial effects of H2O2 and the NO donor (Z)-1-[N-(2-aminoethyl)-N-(2-aminominoethyl)amino]diazene-1-ium-1,2-diol (DETA NONOate [dNO]). We previously reported that *ΔdksA Salmonella* exhibit diminished levels of the cellular antioxidant GSH (15). Consistent with this determination, *ΔdksA 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 stmdksA, padksA, or padksA2 displayed comparably reduced redox potentials. This observation indicates that the increased susceptibility of *padksA2*-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 paDksAs are capable of sensing reactive species, we examined thiol reactivity toward the ROS H2O2 and the RNS peroxynitrite, the sum of peroxynitric acid (ONOOH) and peroxynitrite anion (ONOO–). Each of these molecules has been shown previously to modify cysteine thiols in stmdksA (10).

Thiol oxidation with H2O2 was quantified over time for recombiant stmdksA, paDksA, and paDksA2 (Fig. 3A). The pseudo-first-order rate constant (kdso) was plotted against the H2O2 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 ± 2.5 M–1 s–1 [mean ± 95% confidence interval]) to be more readily modified with H2O2 than stmdksA (k = 0.64 ± 0.03 M–1 s–1) and paDksA (k = 1.33 ± 0.05 M–1 s–1). The reactivities of paDksA and paDksA2 toward peroxynitrite were also assessed and showed paDksA (k = 2,420 ± 60 M–1 s–1) and paDksA2 (k = 1,430 ± 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 Zn2+...
Thiol reactivities of stmDksA, paDksA, and paDksA2 toward H$_2$O$_2$ and peroxynitrite. Thiol oxidation in the indicated recombinant DksA proteins (30 μM) 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$_2$O$_2$, 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 μM H$_2$O$_2$ 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 thiol content of zinc-deficient stmDksA were also hypersensitive to oxidation with H$_2$O$_2$ 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 hypersusceptible to the bactericidal effects of H$_2$O$_2$, we examined the transcriptional changes that occur in response to 15 μM H$_2$O$_2$. At this concentration of H$_2$O$_2$, stmDksA-, padksA-, and padksA2-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 padksA2-expressing Salmonella underwent significantly greater down-regulation of livJ, rplU, and rpsM upon exposure to H$_2$O$_2$ than did stmDksA- and padksA-expressing bacteria (Fig. 4A). Salmonella expressing stmDksA or padksA responded similarly to one another upon treatment, and changes in gene expression were consistent with the use of suboptimal levels of H$_2$O$_2$ for triggering responses from zinc-bound C4 DksA orthologues. As anticipated, treatment of stmDksA- and padksA-expressing Salmonella with higher levels of H$_2$O$_2$ (100 μM) resulted in the down-regulation of livJ, rplU, and rpsM expression to levels comparable to those mediated by paDksA2 at 15 μM H$_2$O$_2$ (see Fig. S5C). To determine
whether the disparate transcriptional responses mediated by C4 and C2 DksA orthologues to \( \text{H}_2\text{O}_2 \) reflect direct effects on transcription, we compared the ability of reduced or oxidized paDksA and paDksA2 to regulate in vitro transcription from the \( \text{livJ} \) promoter (\( \text{livJ} - \)). As previously reported (10), RNAP alone was unable to initiate transcription from \( \text{livJ} - \). 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 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), paDksA2 but not paDksA mediated a marked reduction in \( \text{livJ} - \) transcription.

In vivo and in vitro transcriptional analyses were also performed to define RNAs-mediated changes in gene expression. Exposure to 25 \( \mu \text{M} \) of the \( \text{NO} \) donor spermine NONOate (sNO) did not affect the growth of either \( \text{padksA} - \) or \( \text{padksA2} - \) expressing \( \text{Salmonella} \) (see Fig. S6A in the supplemental material). The expression of \( \text{livJ} - \), \( \text{rplU} - \), and \( \text{rpsM} - \) was down-regulated in \( \text{padksA} - \) expressing \( \text{Salmonella} \) upon treatment with sNO (Fig. 5A). Bacteria expressing \( \text{padksA} - \) also exhibited reduced transcription of \( \text{livJ} - \), \( \text{rplU} - \), and \( \text{rpsM} - \); however, these effects were milder than those observed for \( \text{padksA2} - \) organisms. Similar results were observed when dNO was used as the \( \text{NO} \) donor (see Fig. S6B). In vitro transcription from \( \text{livJ} - \) 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 \( \text{Salmonella} \) to survive and replicate in macrophages following phagocytosis is an essential aspect of pathogenesis and requires that \( \text{Salmonella} \) 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 \( \Delta \text{dksA} - \), \( \text{stm} \text{dksA} - \), \( \text{padksA} - \), and \( \text{padksA2} - \) expressing \( \text{Salmonella} \). Importantly, under the experimental conditions examined here, the amount of superoxide (\( \text{O}_2^- \)) 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 \( \text{Salmonella} \) within host cells, independent of oxidative stress. As expected due to their inability to mediate stringent control, \( \Delta \text{dksA} - \) bacteria were incapable of intracellular growth in J774 cells (Fig. 6A). \( \text{Salmonella} \) expressing \( \text{stm} \text{dksA} - \), \( \text{padksA} - \), or \( \text{padksA2} - \) 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 from congenic gp91phox\(^{-/-}\)
animals unable to generate $O_2^{-}$ (see Fig. S7B and C). Survival within immunocompetent macrophages was significantly reduced among ΔdksA and padksA2-expressing Salmonella compared to the survival of stmdksA- and padksA-expressing bacteria (Fig. 6B). In the absence of phagocyte-derived $O_2^{-}$, Salmonella expressing padksA2 were able to survive and replicate. In contrast, while ΔdksA 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 ΔdksA, stmdksA-, padksA-, and padksA2-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 stmdksA-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 $O_2^{-}$ 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 RssA (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 × 10⁻³ s⁻¹) react with H₂O₂ less efficiently than does the principle cellular reductant GSH (0.87 × 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 μM C4 DksA protein reportedly in the cytoplasm of Gram-negative bacteria from steady-state intracellular H₂O₂ concentrations estimated to be ~20 nM (25–27). The 15 to 40 μM of H₂O₂ 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 × 10⁻³ s⁻¹) would be a preferred target for thiol oxidation with H₂O₂ 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 O₂⁻ 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 orientation 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 α-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(lux) by oxidized DksA in vitro is dose dependent in regard to DksA (10).

The reactivity of DksA proteins toward H₂O₂ is several orders of magnitude lower than that of dedicated peroxide sensors like OxyR or thiol-dependent peroxidases, which catalyze H₂O₂ and peroxynitrite reduction with rate constants of ~10⁵ to 10⁷ 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 Piof (−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, pdksA, and pdksA2 was performed by glutathione S-transferase (GST) affinity isolation followed by size exclusion chromatography (10). Protein thiol reactivity toward H2O2 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 pdksA and pdksA2 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 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 pdksA and pdksA2 with peroxynitrite were determined using stopped-flow kinetics (33).

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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