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The 4-Cysteine Zinc-Finger Motif of the RNA Polymerase Regulator DksA serves as a Thiol Switch for Sensing Oxidative and Nitrosative Stress

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Abstract

We show that thiols in the 4-cysteine zinc-finger motif of DksA, an RNA polymerase accessory protein known to regulate the stringent response, sense oxidative and nitrosative stress. Hydrogen peroxide- or nitric oxide (NO)-mediated modifications of thiols in the DksA 4-cysteine zinc-finger motif release the metal cofactor and drive reversible changes in the α-helicity of the protein. Wild-type and *relA spoT* mutant *Salmonella*, but not isogenic *dksA*-deficient bacteria, experience the downregulation of r-protein and amino acid transport expression after NO treatment, suggesting that DksA can regulate gene expression in response to NO congeners independently of the ppGpp alarmone. Oxidative stress enhances the DksA-dependent repression of *rpsM*, while preventing the activation of *livJ* and *hisG* gene transcription that is supported by reduced, zinc-bound DksA. The inhibitory effects of oxidized DksA on transcription are reversible with dithiothreitol. Our investigations indicate that sensing of reactive species by DksA redox active thiols fine-tunes the expression of translational machinery and amino acid assimilation and biosynthesis in accord with the metabolic stress imposed by oxidative and nitrosative stress. Given the conservation of Cys^{114}, and neighboring hydrophobic and charged amino acids in DksA orthologues, phylogenetically diverse microorganisms may use the DksA thiol switch to regulate transcriptional responses to oxidative and nitrosative stress.

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Supplementary Table information:

Supplementary Table S1. Reduced and oxidized DksA spectra report.

INTRODUCTION

The univalent and divalent reduction of oxygen by cytosolic and electron transport chain flavoproteins are important sources of endogenous oxidative stress in aerobic organisms (Messner and Imlay, 1999; Seaver and Imlay, 2004). The NADPH oxidase-mediated respiratory burst of professional phagocytes enhances the degree of oxidative stress experienced by pathogenic microorganisms, such as *Salmonella*, during their associations with host cells (Mastroeni *et al.*, 2000; Vazquez-Torres *et al.*, 2000). During infection, microorganisms also encounter nitrosative stress emanating from the production of nitric oxide (NO) generated by the reduction of nitrite or the inducible NO synthase-mediated oxidation of the guanidino group of L-arginine (Fang, 2004; Mastroeni *et al.*, 2000; Vazquez-Torres *et al.*, 2000). The reactive oxygen and nitrogen species generated by these processes modify and disrupt critical metal cofactors and thiol groups in key enzymes of glycolysis, the tricarboxylic acid cycle, and amino acid biosynthetic pathways, thereby exerting considerable metabolic stress on the bacterial cell (Brandes *et al.*, 2007; Keyer and Imlay, 1997; Kuo *et al.*, 1987). Reactive species also exert direct genotoxicity on DNA molecules (Imlay and Linn, 1988; Moody and Hassan, 1982; Richardson *et al.*, 2009). Given the tremendous demands imposed by reactive species, multiple regulatory proteins have subverted redox active cysteines, heme, or metal cofactors to sense reactive oxygen and nitrogen species, and coordinate the transcription of antioxidant and antinitrosative defenses (Crack *et al.*, 2012; Farhana *et al.*, 2012; Spiro and D'Autreaux, 2012; Vazquez-Torres, 2012). *Salmonella* OxyR was the first sensor of oxidative stress to be identified (Christman *et al.*, 1985). Elegant studies using *E. coli* OxyR established that the oxidation of Cys^{199} and Cys^{208} by hydrogen peroxide (H_2O_2) or nitrogen oxides activates the transcription of genes encoding antioxidant and antinitrosative defenses (Hausladen *et al.*, 1996; Kullik *et al.*, 1995). Since the discovery of OxyR in *Salmonella*, diverse saprophytic and pathogenic microorganisms have been shown to express thiol-based sensors of oxidative or nitrosative stress (Chen *et al.*, 2006; Lee *et al.*, 2007; Paget *et al.*, 2001). We recently discovered that Cys^{203} in the dimerization domain of the SsrB response regulator of *Salmonella enterica* senses reactive nitrogen species and plays a role in *Salmonella* pathogenesis (Husain *et al.*, 2010). It is likely that, in addition to OxyR and SsrB, *Salmonella* expresses other thiol-based sensors to regulate specific transcriptional responses to reactive oxygen and nitrogen species encountered in the multiple niches occupied by this enteropathogen.

In addition to being exposed to oxidative and nitrosative stress, intracellular bacteria endure limitations in nutrients during the course of an infection. Nutritional deprivation in general, and amino acid shortages in particular, trigger an adaptation known as the stringent response (Potrykus and Cashel, 2008). The stringent response in starving organisms is characterized by repressed transcription of tRNA, rRNA, and ribosomal proteins, and the activation of amino acid biosynthesis genes. Exposure of Gram-negative and –positive bacteria to reactive oxygen and nitrogen species similarly results in the down-regulation of translational machinery (Bourret *et al.*, 2008; Chi *et al.*, 2011; Chi *et al.*, 2013; Henard *et al.*, 2010; Henard and Vazquez-Torres, 2012), suggesting that the stringent response must offer similar advantages to the metabolic challenges that arise from the oxidative inactivation of key enzymes of central metabolism. The coordinated and independent actions of the ppGpp

alarmone and DksA regulatory protein on the RNA polymerase regulate the stringent response in starving bacteria (Paul *et al.*, 2004; Perederina *et al.*, 2004; Potrykus and Cashel, 2008). The mechanism by which reactive species trigger a down-regulation of translational machinery, however, has not yet been elucidated. DksA of *E. coli* or *Salmonella* contains 4 cysteines. Structural analysis of the *E. coli* DksA protein has revealed that the 4 cysteines form part of a zinc-finger motif strategically placed in the globular domain between a coiled-coil and an α-helix (Perederina *et al.*, 2004). Because zinc fingers are excellent sensors of oxidative and nitrosative stress, we tested the hypothesis that the sulfhydryls coordinating the 4-cysteine zinc-finger motif of DksA serve as a thiol switch able of repressing gene expression in response to biologically relevant reactive species.

RESULTS

Sensing of nitrosative stress by the DksA 4-cysteine zinc-finger motif

To test whether the DksA 4-cysteine zinc-finger motif is a sensor of reactive nitrogen species, a *Salmonella* strain expressing a 3×FLAG-tagged DksA protein was exposed to acidified nitrite (NO_2^-), a primary source of nitrosative stress in the gastric lumen and macrophages (Bourret *et al.*, 2008; McCollister *et al.*, 2007). S-nitrosylated cytoplasmic proteins were derivatized in a biotin-switch assay that distinguishes S-nitrosothiols from other oxidized or reduced thiol groups. The resulting biotinylated proteins were affinity-purified and the specimens were resolved by SDS-PAGE. Western blots specific for the 3 × FLAG tag revealed the S-nitrosylation of DksA in *Salmonella* cultures exposed to acidified NO_2^-, but not to acidified nitrate (NO_3^-) (Figure 1A). Our investigations demonstrate that thiols coordinating the DksA 4-cysteine zinc-finger motif are susceptible to biologically active nitrogen oxides generated from the acidification of NO_2^-.

A *Salmonella* strain expressing a DksA variant lacking all 4 cysteine residues showed a lack of S-nitrosylation of thiol groups in the zinc-finger motif (Figure 1B). On the other hand, *Salmonella* strains expressing the DksA C117S or C135S variants showed diminished levels of S-nitrosylation of DksA, possibly reflecting the lower concentrations of these DksA variants in the cytoplasm of *Salmonella*. We also tested whether DksA can be S-nitrosylated in bacteria treated with the transnitrosylating agent S-nitrosoglutathione (GSNO). Treatment of *Salmonella* with 500 μM GSNO for 30 min resulted in the S-nitrosylation of DksA (Figure 1C). As expected, untreated or 400 μM H_2O_2-treated controls did not harbor S-nitrosylated DksA. Together, these findings indicate that DksA can be S-nitrosylated *in vivo* in response to various reactive nitrogen species encountered by *Salmonella* during their associations with the host. Given these results, we investigated the ability of biologically relevant reactive nitrogen species to S-nitrosylate affinity-purified, recombinant DksA protein *in vitro*. The recombinant, wild-type DksA protein was found to be S-nitrosylated upon treatment with the NO donor spermine NONOate or GSNO, but not with spermine or glutathione controls (Figure 1D). Because DksA cysteine variants are still S-nitrosylated in *Salmonella* treated with acidified NO_2^- (Figure 1B), we used this *in vitro* system to gain insights into the target cysteine residue of S-nitrosylation. Recombinant DksA proteins bearing mutations in single cysteines in the 4-cysteine zinc-finger motif were exposed to 500 μM GSNO for 30 min before processing in the biotin-switch assay. This analysis revealed

that all DksA variants bearing single cysteine mutations were S-nitrosylated by GSNO (Figure 1E), suggesting that 2 or more of the cysteines in the 4-cysteine zinc-finger motif are reactive. In addition to the 17.2 kDa species corresponding to monomeric DksA, GSNO treatment of DksA cysteine mutants gave rise to S-nitrosylated species of different molecular weights (Figure 1E). The high molecular weight species likely reflects oligomers arising from oxidized cysteine residues in monomeric DksA. The nature of the S-nitrosylated species with a smaller molecular weight than monomeric DksA is currently unknown. Collectively, these findings indicate that thiols in the 4-cysteine zinc-finger motif of DksA can be S-nitrosylated *in vivo* and *in vitro* by a variety of reactive nitrogen species.

Oxidative and nitrosative stress releases the zinc cation from DksA

The zinc-finger motif in the globular domain of DksA is coordinated by the only 4 cysteines in the protein (Perederina *et al.*, 2004), and thus S-nitrosylation of thiols forming the zinc-finger motif would be anticipated to destabilize metal binding. The chelator 4-(2-pyridylazo) resorcinol (PAR) (Hunt *et al.*, 1985) was used to test whether reactive nitrogen species affect the binding of zinc by DksA. PAR was not observed to strip the zinc cation from wild-type DksA. Treatment of 50 μM DksA with 500 μM of the NO donor spermine NONOate resulted in the release of about 25 μM zinc (Figure 2A). The NO donor PROLI NONOate also released zinc from DksA (not shown), lending further support to the notion that the NO-dependent modification of thiols does indeed disassemble the 4-cysteine zinc-finger motif. The strong oxidant peroxynitrite ($ONOO^-$) and the transnitrosating agent GSNO also caused zinc release from DksA (Figure 2A). It appears, however, that not all reactive nitrogen species tested were as efficient at releasing the zinc from DksA. Therefore, we studied the kinetics by which these reactive nitrogen species strip the zinc cation from DksA. Nonlinear regression analysis of the concentration of zinc released over time indicated that $ONOO^-$, GSNO, and spermine NONOate release zinc from DksA with estimated $t_{1/2}$ of 2, 21, and 33 min, respectively (Figure 2B). Moreover, $ONOO^-$ and GSNO released 100% of the zinc, whereas spermine NONOate released about 50%. These data indicate that a variety of reactive nitrogen species cause the release of the metal cation from the DksA 4-cysteine zinc-finger motif, although with distinct kinetics. Taking into account the kinetics presented herein, it appears that DksA is a better sensor of $ONOO^-$ and S-nitrosothiols than NO. The addition of 500 μM H_2O_2 stripped about 50% of the zinc from 50 μM DksA (Figure 2C), suggesting that DksA 4-cysteine zinc-finger motif can also sense reactive oxygen species.

Thiol oxidation alters the secondary structure of DksA

The oxidation of the zinc fingers in the anti-sigma factor RsrA and the heat shock protein Hsp33 drives conformational and functional changes in these metalloproteins (Bae *et al.*, 2004; Graumann *et al.*, 2001). Consequently, the modifications of cysteines in the DksA 4-cysteine zinc-finger motif and the release of the metal cofactor noted in NO-treated specimens could induce conformational changes in this RNA polymerase regulatory protein. We first tested this hypothesis by measuring the migration of control and oxidized DksA in nonreducing, SDS-PAGE gels. $ONOO^-$, and to a lesser extent spermine NONOate and GSNO, altered the migration patterns of DksA in SDS-PAGE gels (Figure 3A). The addition of DTT reversed the oligomeric species formed upon the oxidation of DksA. H_2O_2 also

induced the formation of a DTT-reversible, oligomeric DksA species (Figure 3B). These findings suggest that reactive oxygen and nitrogen species induce disulfide bonds that are accompanied by changes in the secondary structure of DksA. To test this idea further, control and oxidized DksA proteins were studied by circular dichroism spectroscopy. All specimens were processed 1 h after treatment with reactive oxygen and nitrogen species, because the oligomeric DksA species formed upon $ONOO^-$ treatment are stable for at least 1 h and because the amount of zinc released by GSNO, spermine NONOate and H_2O_2 reach the V_{max} by 1 h. $ONOO^-$, GSNO, and H_2O_2 each diminished the α-helical secondary structure of DksA as indicated by the loss of the 220 nm minimum (Figure 3C). Consistent with its superb ability to mediate zinc release and induce the reversible oxidation of DksA (Figure 2 and 3A), $ONOO^-$ triggered the most dramatic loss of α-helical content in DksA. The loss of α-helicity noted in $ONOO^-$-treated DksA was reversed upon the addition of DTT (Figure 3C). Collectively, these investigations suggest that the 4-cysteine zinc-finger motif behaves as a redox active thiol switch that can drive reversible changes in the secondary structure of DksA through disulfide bond formation.

LC-MS/MS peptide mapping of reduced and oxidized DksA

The ability of DTT to reverse the loss of secondary structure in oxidized DksA suggests that the cysteines in DksA can undergo reversible disulfide bonds. To test whether disulfide linkages are associated with these structural changes, reduced and oxidized DksA were examined by mass spectrometry. Reduced and $ONOO^-$-treated DksA were first alkylated with iodoacetamide to selectively label free thiol groups. The protein samples were then reduced with tris(2-carboxyethyl)phosphine (TCEP) to break any existing disulfide bonds. The newly reduced cysteine residues were then alkylated with N-ethyl-malemeide (NEM). Differential modifications on individual cysteine residues were subsequently identified by LC-MS/MS. As shown in Table 1, all cysteine residues in DTT-treated DksA were found to be present as reduced thiols. In contrast, the cysteine residues in the $ONOO^-$-treated DksA samples demonstrated considerable disulfide bonding. The raw spectral counts for the peptides containing the cysteine residues can be seen in Table S1. Bond formation preferentially involved Cys^{114} and Cys^{135}, indicating an important role for these residues in the capacity of DksA to sense and respond to reactive species. Cys^{138} displayed a more modest involvement in disulfide bonding as compared to Cys^{114} and Cys^{135}. Interestingly, Cys^{117} was very limited in its ability to participate in disulfide bond formation and was primarily observed to be terminally oxidized following $ONOO^-$ treatment. Cys^{114} and Cys^{117}, as it is the case for Cys^{135} and Cys^{138}, are contained within a single trypsin-digested DksA protein fragment. The LC-MS/MS analysis did not show any N-ethylmaleimide modifications in both Cys^{114} and Cys^{117} within single fragments, suggesting that these two residues do not form intramolecular disulfide bonds with each other. Similarly, only 12% of the fragments contained disulfide bonds between Cys^{135} and Cys^{138} in $ONOO^-$-treated DksA. Surprisingly, tyrosine nitration, which is a frequent oxidative signature of $ONOO^-$, was absent in $ONOO^-$-treated DksA. However, methionine residues 32, 62, and 149 were oxidized to the corresponding sulfoxides. The raw spectral counts for the peptides containing the methionine residues can be seen in Table S1. Methionine oxidation is unlikely to account for DksA sensory function because reduction of sulfoxide derivatives

requires enzymatic catalysis or harsh chemical treatment that is inconsistent with the ability of DTT to reverse the novel properties of oxidized DksA (Figure 3).

Cys^{114} is conserved in DksA of Gram-negative bacteria

DksA is highly conserved among Gram-negative bacteria, suggesting that the ability of DksA to sense reactive oxygen and nitrogen species in *Salmonella* could be generalizable to other microorganisms. Orthologues of DksA express 1, 2 or 4 conserved cysteines. All known DksA proteins contain at minimum the cysteine at position 114 of the *Salmonella* DksA protein (Figure 4A). Analysis of the DksA consensus sequence shows considerable conservation of various charged and hydrophobic residues (Figure 4B) that are known to be critical for thiol-mediated sensing of reactive species (Vazquez-Torres, 2012). To determine the spatial localization of conserved hydrophobic and charged residues relative to Cys^{114}, we performed an analysis of the crystal structure of *E. coli* DksA (Perederina *et al.*, 2004) using Swiss-PDB Viewer v4.1.0 (Figure 5). This analysis revealed that the basic δ-guanido group of Arg^{125} is located 7.06 Å from the thiol group of Cys^{114}, whereas the negatively charged carboxylic groups of Glu^{115} and Asp^{137} are 7.73 Å and 7.83 Å, respectively (Figure 5A). In addition, the conserved residues Ile^{121}, Leu^{126}, and Leu^{134} form a hydrophobic pocket flanking Cys^{114} (Figure 5B).

Binding of oxidized DksA to the RNA polymerase

Next, we tested the effect that oxidation has on the binding of DksA to the RNA polymerase (Figure 6). Reduced and $ONOO^-$-oxidized DksA immobilized in GSH Sephadex columns were incubated with core RNA polymerase. After 2 h of binding, the ββ' and α subunits of the RNA polymerase were eluted from the GST-DksA with 400 mM NaCl. Densitometry of the ββ' and α subunits of the RNA polymerase visualized by silver staining of specimens separated in SDS PAGE gels indicates that oxidized DksA binds about 60% of the RNA polymerase when compared to the reduced DksA protein. The lower binding of oxidized DksA may reflect the formation of oligomeric species (Figure 3A) unable to associate with the secondary channel of the RNA polymerase.

Oxidative stress enhances the DksA-dependent repression of gene transcription

The considerable loss of α-helicity and the release of zinc seen in oxidized DksA could result in a change in the function of this RNA polymerase regulatory protein. We therefore tested whether the sensing of reactive nitrogen species by DksA has a direct effect on transcription. We measured the transcription of the ribosomal protein gene *rpsM* that is not only a direct target of DksA, but is also repressed in response to oxidative stress in a DksA-dependent manner (Henard *et al.*, 2010). As previously noted for H_2O_2 (Henard *et al.*, 2010), the transcription of the *rpsM* gene was repressed in rapidly growing *Salmonella* exposed to 5 μM NO generated by DETA NONOate (Figure 7A). NO also down-regulated *rpsM* expression in a *relA spoT* mutant, but did not repress the expression of this r-protein in *dksA*-deficient *Salmonella*. The *rpsM* transcript levels in wild-type and the *dksA* mutant were similar, and significantly lower than in the $ppGpp^0$ isogenic control. These findings suggest that the down-regulation of *rpsM* transcription seen herein after treatment of *Salmonella* with low amounts of NO is independent of ppGpp.

An *in vitro* transcription system was used to directly test whether DksA can repress *rpsM* in response to reactive nitrogen species. Unexpectedly, *in vitro* transcription of *rpsM* appeared to be stimulated in response to 0.5 and 1.0 μM DksA. However, as described for *E. coli* (Lemke *et al.*, 2011), 2.5 μM DksA repressed *rpsM in vitro* transcription by ~3-fold (Figure 7B). Because of its fast kinetics and short half-life, $ONOO^-$ was chosen to test the effect that the oxidation of DksA has on *rpsM in vitro* transcription. Taking into account a binding constant of 100 nM (Lennon *et al.*, 2009) and that over 90% of DksA should be bound to the RNA polymerase in the cell, we reasoned that the oxidation of DksA *in vivo* must take place in complex with the RNA polymerase. Therefore, we treated the RNA polymerase-DksA complex with 25 μM authentic $ONOO^-$, a species with a half-life of 1.9 sec (Beckman *et al.*, 1990). Of note, the addition of 25 μM $ONOO^-$ did not affect the transcription of *rpsM* supported by the RNA polymerase alone, indicating that the low concentrations of $ONOO^-$ used in these experiments do not have an appreciable effect on the enzymatic activity of the RNA polymerase. At 0.5 and 1 μM DksA concentrations, $ONOO^-$ did not significantly ($p > 0.05$) affect *rpsM in vitro* transcription (Figure 7B). However, as the concentration of DksA increased to 2.5 μM, $ONOO^-$ potentiated ($p < 0.05$) the inhibitory activity of reduced DksA by about 2-fold. These findings indicate that the oxidized DksA protein is a more potent repressor of *rpsM* than reduced DksA.

Because *rpsM* is repressed by DksA, we also measured the effect of oxidation on the transcription of *livJ* that is known to be directly activated by DksA (Paul *et al.*, 2005). *Salmonella* strains deficient in *dksA* or *relA spoT* expressed lower concentrations of *livJ* than the isogenic wild-type control, which is consistent with the idea that the stringent response activates *livJ* transcription (Magnusson *et al.*, 2007). As noted with *rpsM*, 2.5 mM DETA NONOate repressed *livJ* transcription in wild-type and *relA spoT* mutant *Salmonella*, but did not affect its expression in *dksA* isogenic bacteria (Figure 7C). These findings suggest that oxidized DksA represses or fails to activate *livJ* gene transcription independently of ppGpp. Accordingly, the level of *livJ in vitro* transcription was lower in the DksA/RNA polymerase specimens treated with 25 μM $ONOO^-$ (Figure 7D). The lack of activation of *livJ* noted after $ONOO^-$ treatment does not seem to be explained by the oxidation of the RNA polymerase because, in the absence of DksA, $ONOO^-$ did not repress the enzymatic activity sustained by the RNA polymerase alone (Figure 7D). Moreover, the inhibitory effects of $ONOO^-$ were dependent on the concentration of DksA. This is demonstrated by the fact that 1 and 5 μM of oxidized DksA diminished *livJ* transcription by 20% and 70%, respectively, as compared to the corresponding concentrations of reduced, zinc-bound DksA (Figure 7E). The addition of 2.5 mM DTT reversed the inhibition of *livJ in vitro* transcription observed with the $ONOO^-$-treated DksA/RNA polymerase complex (Figure 7F), suggesting that the inhibitory effects exerted by the oxidized DksA protein are reversible. Oxidized DksA also inhibited the *in vitro* transcription of *hisG*, a gene that is activated by reduced DksA (Paul *et al.*, 2005). In contrast, the internal RNA1 promoter that is directly transcribed by the RNA polymerase was not inhibited by oxidized DksA. Cumulatively, these investigations support the idea that DksA can behave as a novel thiol switch that inhibits target gene transcription upon oxidation of cysteines in the zinc-finger motif.

DISCUSSION

Salmonella undergoing nitrosative or oxidative stress down-regulate the transcription of ribosomal proteins, rRNA, and tRNA (Bourret *et al.*, 2008; Henard *et al.*, 2010). The investigations presented herein are consistent with a model by which sensing of reactive species by thiols in the 4-cysteine zinc-finger motif modulate the regulatory activity of the DksA protein in *Salmonella* experiencing oxidative or nitrosative stress. Analogous to the synergism exerted by ppGpp on the regulatory activity of DksA, our investigations indicate that oxidative and nitrosative stress can reversibly improve the inhibitory activity of the DksA protein. However, in contrast to the classical stringent response, DksA-dependent responses to oxidative stress are associated with the down-regulation of both translational machinery and amino acid metabolic gene transcription. Placing the expression of translational machinery and amino acid biosynthesis under the control of oxidized DksA may help bacteria quickly and reversibly adapt to the metabolic constraints associated with oxidative and nitrosative stress.

Zinc coordinating cysteines in the globular domain of DksA form a structural motif (Perederina *et al.*, 2004) that is required for the DksA-dependent regulation of transcription of stringent response targets in nutritionally deprived bacteria. We found that, in addition to serving as a scaffold where a zinc-finger motif is assembled, the thiols of the only four cysteines of DksA respond to a variety of reactive oxygen and nitrogen species. These data are in keeping with the idea that thiolates coordinating zinc cations often serve as redox active sensors of oxidative and nitrosative stress (Ilbert *et al.*, 2006), as this metal promotes cysteine reactivity by lowering the pK_a of the coordinating thiolate groups. Interestingly, it is also possible that the zinc cation ameliorates the reactivity of DksA thiols. In this case, zinc would play an antioxidant role as previously described for the anti-sigma factor RsrA and PerR (Lee and Helmann, 2006a, b; Li *et al.*, 2003). Hence, zinc coordination may limit the oxidation of DksA cysteines to situations in which oxidative stress imposes metabolic demands on the cell.

Cysteine residues are often used as redox sensors because they can adopt up to ten, often reversible, oxidation states that endow proteins with specific structural and functional properties. Our investigations have discovered that thiols in the DksA 4-cysteine zinc-finger motif can undergo a variety of covalent modifications upon exposure to reactive oxygen and nitrogen species. For example, DksA becomes S-nitrosylated in *Salmonella* exposed to acidified NO_2^- or GSNO. S-nitrosylation of DksA could occur through the direct reaction of sulfenyl ($-S^{\bullet}$) and NO radicals, or through the transnitrosation of the nitrososonium cation (NO^+) from low-molecular weight S-nitrosothiols, such as GSNO, to a thiolate group ($-S^-$) in the zinc-finger motif of DksA (Vazquez-Torres, 2012). S-nitrosothiols can alter protein function by their own right, but are often intermediate species that promote stable disulfide bond formation with neighboring cysteines (Kim *et al.*, 2002). As a matter of fact, the LC-MS/MS analysis of the $ONOO^-$-treated protein demonstrates disulfide bonds are formed primarily among Cys^{114}/Cys^{135} and, to a lesser extent, between Cys^{114}/Cys^{138} and Cys^{135}/Cys^{138}. The oxidation of DksA thiols appears to promote changes in α-helicity and increase the ability of DksA to inhibit gene transcription. The novel regulatory effects of oxidized DksA are readily reversible upon reduction. Taken together, these observations

support our proposed model that the DksA 4-cysteine zinc-finger motif is a thiol multiplex that provides DksA the capacity to rapidly integrate nutritional, oxidative, and nitrosative signals into a coordinated transcriptional response.

The conservation of Cys^{114} in the globular domain of DksA from phylogenetically diverse Gram-negative bacteria raises the intriguing possibility that all DksA variants described to date may sense oxidative and nitrosative stress, even though not all of them are capable of assembling a zinc-finger motif. In addition to Cys^{114}, several nearby charged and hydrophobic residues are also highly conserved among all DksA orthologues. The proximity of conserved charged residues potentially lowers the pK_a of Cys^{114}, thus promoting the formation of a thiolate critical for reactivity with peroxides such as H_2O_2 or $ONOO^-$. In fact, according to PROPKA 3.1 analysis (Rostkowski *et al.*, 2011), the pK_a of *Salmonella* DksA Cys^{114} is 5.45. Conserved hydrophobic residues may promote DksA reactivity by forcing the Cys^{114} thiolate group away from the protein's core, thereby increasing its accessibility to reactive species. Importantly, in DksA proteins containing only Cys^{114}, the cysteine residues at positions 117 and 135 are replaced with threonine or serine residues. The close proximity of these residues to Cys^{114}, each less than 4 Å away, would allow stabilization of the thiolate through hydrogen bonding and further promote the reactivity of Cys^{114}. Taken together, these determinants are markedly similar to those reported to influence thiolate formation in the redox-sensing transcriptional regulators OxyR and OhrR (Vazquez-Torres, 2012), and are consistent with a thiol-based sensory function for all known DksA protein variants. While the reactivity of DksA variants containing a single redox active cysteine residue would be expected to differ from those containing the more common C_4 arrangement, each would be capable of dimer formation through disulfide bonding. In addition, all DksA variants could form mixed disulfides with low-molecular weight thiols such as glutathione, a modification known to regulate protein function (Antelmann and Helmann, 2011). Moreover, the C_4 and C_2 DksA variants could form intramolecular disulfide bonds as demonstrated for the C_2 *Pseudomonas aeruginosa* DksA2 protein (Furman *et al.*, 2013). Because all described DksA orthologues express one, two, or four conserved cysteines in their globular domain, phylogenetically diverse microorganisms could use this thiol switch as a mechanism for fine-tuning transcriptional regulation according to the metabolic restrictions imposed by reactive oxygen and nitrogen species.

Two non-mutually exclusive models may explain the DksA-dependent down-regulation of transcription noted in response to oxidative stress. First, the formation of DksA oligomers in the soluble monomeric pool in response to oxidative stress may limit the availability of this transcriptional regulator. As DksA is constitutively expressed (Paul *et al.*, 2004), changes in the amount of monomeric protein available to bind to the RNA polymerase could be an important mechanism of regulation. Indeed, diminished association between DksA and RNA polymerase could account for the observed decrease in binding of RNA polymerase by oxidized DksA, as well as the apparent inhibition of *livJ* and *hisG* transcription noted in the reactions containing oxidized DksA. This model, however, does not easily explain why *rpsM* transcription, classically repressed by reduced DksA, is further down-regulated in response to increasing levels of oxidized, zinc-free DksA. It is therefore possible that monomeric, oxidized, zinc-free DksA could exert increased repressing activity on the RNA

polymerase. In this second model, the regulatory effects seen upon oxidation of DksA could stem from the actual oxidation of cysteine thiols and/or the formation of a demetallated apoprotein.

The ability of *relA spoT* mutant *Salmonella* to down-regulate *rpsM* and *livJ* transcription in response to nitrosative stress argues that, under the experimental conditions tested here, oxidized DksA represses gene transcription independently of ppGpp. Distinct roles for ppGpp and DksA in regulating gene transcription in response to oxidative stress is also suggested by the observation that ppGpp^0 *dksA* mutant *Salmonella* are even more hypersusceptible to the cytotoxicity of NO than isogenic strains lacking either *relA spoT* or *dksA* (Henard and Vazquez-Torres, 2012). Our findings are consistent with data reported by other investigators who have also suggested that DksA can mediate ppGpp-independent roles in gene regulation (Aberg *et al.*, 2008; Aberg *et al.*, 2009; Magnusson *et al.*, 2007). These observations, however, do not preclude a role for ppGpp in the transcriptional response to oxidative and nitrosative stress. Specifically, reactive nitrogen species oxidize thiol groups and [Fe-S] clusters of IlvD dehydroxy-acid dehydratase, LpdA lipoamide-dependent lipoamide dehydrogenase, and MetE cobalamin-independent methionine synthase (Hondorp and Matthews, 2004; Hyduke *et al.*, 2007; Richardson *et al.*, 2011). The inhibition of amino acid biosynthesis and the expected depletion of charged tRNA could induce a surge in the intracytoplasmic ppGpp pool, thereby contributing to the down-regulation of translational machinery seen in bacterial cells undergoing oxidative and nitrosative stress. Separately, the cytoplasmic pool of the rRNA initiating nucleoside (usually an ATP or GTP) exerts feedback regulation on rRNA synthesis (Schneider *et al.*, 2002). Thus, the diminution of ATP synthesis that follows the nitrosylation of terminal quinol cytochrome oxidases of the electron transport chain could also contribute to the down-regulation of translational machinery in NO-treated *Salmonella* (Bourret *et al.*, 2008).

Our investigations indicate that cysteines holding the DksA zinc-finger motif behave as a thiol multiplex that integrates nutritional, oxidative, and nitrosative signals to repress gene transcription. The DksA-dependent regulation of stringent control in response to oxidative and nitrosative stress provides a rapid and reversible mechanism for fine-tuning the level of translational machinery in accord with nutritional shortages associated with the oxidation of redox active cysteines and metal cofactors of central metabolic enzymes. As all known DksA orthologues contain at least one conserved cysteine residue in the globular domain, phylogenetically diverse microorganisms could use this thiol-based sensor in the regulation of transcription according to the metabolic restrictions imposed by reactive oxygen and nitrogen species.

EXPERIMENTAL PROCEDURES

Bacterial strains

Salmonella enterica serovar Typhimurium strain ATCC 14028s and isogenic strains expressing several *dksA* variants are described in Table 2. To generate *Salmonella* strains carrying a wild-type or mutated *dksA* allele, a template plasmid was constructed by cloning an FRT-flanked chloramphenicol cassette from pKD3 into the BamHI and SacI restriction sites of pBluescript SK$^+$ to generate pSK: :cm. The *dksA* open reading frame was cloned

between EcoRI and BamHI restriction sites of pSK: :cm. *dksA* variants with cysteine to serine substitutions were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) and the mutations were confirmed by DNA sequencing. The plasmids were used as templates to generate amplicons with the *dksA* alleles and an FRT-flanked chloramphenicol resistance cassette. The addition of 60-base long primers containing homology to the *dksA* locus allowed for the recombination of the amplicons into the *Salmonella* chromosome using the λ Red recombinase system (Datsenko and Wanner, 2000). The primers are listed in Table 3.

DksA purification

Genes encoding DksA variants were cloned into the BamHI-EcoRI restriction sites of pGEX6P1 (GE Healthcare Biosciences, Fairfield, CT) using the primers listed in Table 3. N-terminal glutathione S-transferase (GST)-tagged DksA proteins were expressed in *E. coli* BL21 (DE3) at 25°C. Expression of GST-DksA was induced for 3 h by adding 0.2–0.5 mM isopropyl-β-D-thiogalactopyranoside to cultures grown in LB broth to an OD_{600} of 0.5. The cells were harvested by centrifugation, resuspended in 50 mM Tris, pH 7, and lysed by sonication. Soluble DksA proteins were purified as GST-fusions, and the GST tag removed using PreScission protease (GE Healthcare Biosciences). DksA proteins were purified further at 4°C on a Superdex-75 size-exclusion FPLC column equilibrated with 50 mM Tris, pH 7.0. Positive fractions were pooled and concentrated using Centricon filter devices.

Biotin switch assay

Salmonella were exposed to 750 μM NO_2^- or NO_3^- in EG medium (0.2 g/L $MgSO_4$, 2 g/L $C_6H_8O_7$-H_2O, 10 g/L K_2HPO_4, 3.5 g/L $Na(NH_4)HPO_4$-$4H_2O$, and 4 g/L D-glucose), pH 5.5. Some of the *Salmonella* cultures were exposed to 400 μM H_2O_2 or 500 μM GSNO for 30 min. The analysis of S-nitrosothiols in the DksA protein in *Salmonella* was done according to a modified method of the protocol originally described by Jaffrey and Snyder (Husain *et al.*, 2010; Jaffrey and Snyder, 2001). Briefly, free thiols were blocked with four volumes of 250 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine (HEN) buffer containing 20 mM methyl methanethiosulfonate and 2.5% (w/v) SDS at 50°C with occasional mixing. After 20 min, the cytoplasmic proteins were precipitated, and the pellets were washed with ice-cold acetone. The proteins were solubilized in 1% (w/v) SDS HEN buffer, and the nitrosothiols present in the samples were reduced with 1 mM ascorbate. The exposed thiol groups were derivatized with 16.66 mM biotin {N-[6-(Biotinamido)hexyl]-3′-(2’-pyridyldithio)-propionamide} (Thermo Fisher Scientific) for 1 h at room temperature. The proteins were then precipitated and washed with ice-cold acetone. The protein pellets were solubilized in 1% (w/v) SDS HEN buffer and mixed with two volumes of neutralization buffer [1 mM EDTA, 0.1 mM neocuproine, 100 mM NaCl, 0.5% (v/v) Triton X-100, 250 mM Hepes-NaOH, pH 7.7]. NeutrAvidin-agarose resin (Thermo Scientific) was added, and the mixture was incubated at 4°C overnight. The resin was washed with high-salt neutralization buffer (1 mM EDTA, 0.1 mM neocuproine, 600 mM NaCl, 0.5% (v/v) Triton X-100, 250 mM Hepes-NaOH, pH 7.7), and the proteins were eluted after boiling the resin in 2× SDS sample buffer containing β-mercaptoethanol. The specimens were resolved on 12% (v/v) SDS/PAGE gels, transferred onto a nitrocellulose membrane, and probed for the DksA: :3 × FLAG protein with the M2 monoclonal antibody (Sigma-Aldrich, St. Louis,

MO). To determine the S-nitrosylation of DksA *in vitro*, 50 μM of the DksA variants were treated with 500 μM spermine NONOate or 100 μM GSNO at 37°C. The nitrosating agents were removed after 1 h of culture using the Micro bio-spin P6 column (BioRad, Hercules, CA). Free thiols were blocked and S-nitrosothiols were derivatized as described above. S-nitrosylated DksA derivatives were resolved using 12% (v/v) SDS-PAGE gels, transferred electrophoretically to a nitrocellulose membrane, and immunoblotted with an anti-biotin monoclonal antibody (Sigma-Aldrich).

Measurement of zinc release

50 μM recombinant protein was exposed to 500 μM spermine NONOate, 500 μM GSNO, 500 μM H_2O_2, or 500 μM $ONOO^-$. GSNO and $ONOO^-$ were synthesized as previously described (Hart, 1985; Mohr *et al.*, 1994). Free zinc was measured by the addition 150 mM of the metal chelator 4-(2-pyridylazo)resorcinol (PAR) (Sigma-Aldrich). PAR-zinc chelates were measured spectrometrically at A_{500nm}, and zinc concentration was calculated by regression analysis using known $ZnCl_2$ standards.

Non-reducing, SDS-PAGE

50 μM recombinant protein was treated with 500 μM spermine NONOate, 500 μM GSNO, 500 μM H_2O_2, or 500 μM $ONOO^-$ for 1 h at 37°C. The specimens were then mixed with 3× Red loading buffer (New England Biolabs, Ipswich, MA) lacking reducing agents. 10 μL (~10 μg protein) of the samples were loaded into a 12% (v/v) SDS-PAGE gels and electrophoresed at 125V on ice. Proteins were visualized by Coomassie blue staining.

Circular dichroism spectroscopy

Circular dichroism spectroscopy was performed on a Jasco-810 spectrometer with constant nitrogen flushing (Jasco, Easton, MD). Circular optical cells with a path length of 0.1 cm were used to determine the spectra of proteins in 50 mM Tris, pH 7 over a wavelength of 195–250 nm in 1 nm increments. Each spectrum is the average of four scans.

Binding of DksA to the RNA polymerase

The effect of oxidation on the binding of DksA with the RNA polymerase was assessed as described earlier with slight modifications (Paul *et al.*, 2004). Briefly, 30 nmol GST-DksA were treated with 1 mM DTT or 1 mM $ONOO^-$ at 37°C for 1 h. Treatment of GST-DksA with $ONOO^-$ stripped all the zinc from the protein as determined spectrophotometrically by measuring the formation of zinc-PAR chelates at A_{500nm}. DTT and $ONOO^-$ oxidation products were removed from the specimens using a Micro bio-spin P6 column. The samples were incubated at 4°C for 1 h with 1 ml of GSH Sepharose beads that had been prewashed with 40 bed volumes of 50 mM Tris, pH 7.0. DTT- and $ONOO^-$-treated DksA proteins were retained in the GSH Sepharose matrix at similar concentrations. 40 pmol of RNA polymerase core (Epicentre, Madison, WI) were incubated at 4°C with the DksA immobilized in the columns. Two hours after incubation, the column was washed 4 times with 30 mM NaCl, and the RNA polymerase was eluted with 4 additional washes with 400 mM NaCl. Low salt and high salt eluates were combined separately. The proteins were

precipitated with 15% TCA, separated by SDS PAGE, and the α and ββ' subunits of the RNA polymerase eluted were visualized by silver staining.

Mass Spectrometry Analysis

Recombinant *Salmonella* DksA (75μM) was treated in the dark with 2 mM DTT for 60 min at room-temperature. DTT was removed using a Zebra desalt spin 25 column (Thermo Scientific Pierce, Rockford, Illinois). Selected samples of reduced DksA were treated with 10 molar equivalents of ONOO− for 60 min at 37°C. Unreacted ONOO− was removed using a Micro bio-spin P6 column and SDS was added to each sample at a final concentration of 0.05%. All samples were treated in the dark with 20 mM iodoacetamide for 60 min at 37°C. Following alkylation, the sample buffer was exchanged with 0.1 M ammonium bicarbonate. Five micrograms of DksA in the specimens were resolved on 10% Bis-Tris PAGE gels, and were subjected to in gel digestion as described previously (Keene *et al.*, 2009). Briefly, individual bands were excised from gel and treated with 5 mM TCEP for 15 min followed by alkylation with 20 mM NEM for 60 min and trypsin digestion. Peptides were extracted from gel pieces and analyzed on a hybrid Obritrap mass spectrometer (Thermo Electron, San Jose, CA) coupled to an Eksigent 2D LC system (Eksigent Technologies, Framingham, MA). Data was search for the presence of carbamidomethylated cysteine (+57 Da), NEM-alkylated cysteine (+125 Da), and cysteic acid oxidized cysteine (+48 Da).

Real Time RT-PCR

Bacteria grown overnight in LB broth were subcultured in EG medium supplemented with 0.4% glucose, 0.1% casamino acids, 10 μM $FeCl_3$, and 2 μg/mL thiamine and grown in a shaker incubator to log phase (OD_{600} of 0.4). Selected samples were treated with 5 mM DETA NONOate for 30 min. After 30 min of culture, untreated controls had grown to an OD_{600} of ~0.77, whereas the DETA NONOate-treated specimens had grown to an OD_{600} of ~0.49. The cultures were mixed 1:5 (v/v) with ice-cold 5% phenol/95% ethanol and the specimens were placed on ice for 20 min for RNA stabilization. Isolation of bacterial RNA, synthesis of cDNA, and real-time RT-PCR for the *rpsM*-encoded ribosomal protein, the amino acid transporter *livJ*, and the *rpoD* housekeeping gene were performed as previously described (Henard *et al.*, 2010). Briefly, the RNA was purified using the high pure RNA isolation kit (Roche Applied Sciences, Indianapolis, IN), and contaminating DNA was removed by performing treatment with Turbo DNase (Life Technologies, Carlsbad, CA) followed by RNeasy clean-up (Qiagen, Valencia, CA). One microgram of total RNA from DETA NONOate-treated or untreated wild-type or *dksA* mutant cultures was used to generate cDNA in reactions that contained 100 U M-MLV reverse transcriptase (Promega, Madison, WI), 0.45 μM N6 random hexamer primers (Life Technologies), and 20 U RNAsin Plus RNase inhibitor (Promega). Reverse transcription was performed for 1 h at 42°C. The cDNA was purified using the QIAGEN PCR purification kit as suggested by the manufacturer (Qiagen, Valencia, CA). The primers and probes used for the real-time RT-PCR are listed in Table 2. The results are expressed as relative expression over the *rpoD* house-keeping gene. Treatment of *Salmonella* with DETA NONOate did not result in any significant changes ($p > 0.05$) in *rpoD* mRNA expression.

In vitro transcription

Linear PCR products used for *in vitro* transcription spanned from −107 to +221, and −128 to +320 regions of the *rpsM* and *livJ* genes, respectively. The *in vitro* transcription vector pRLG4413 containing the *hisG* (−60/+1) promoter from *E. coli* and an internal RNA1 control was used at 1 nM in 10 μL of reaction buffer. RNA polymerase/DksA complexes were treated with 2.5 mM DTT or 25 μM $ONOO^-$ for 5 min at 37°C in buffer containing 40 mM HEPES, pH 7.4, and 2 mM $MgCl_2$ before they were added to the *in vitro* transcription reactions. The RNA polymerase was used at 5 nM, whereas DksA was used between 0.5 and 5 μM. Where indicated, DksA/RNA polymerase complexes were incubated with 2.5 mM DTT 5 min after $ONOO^-$ treatment. The *in vitro* transcription of the *rpsM* and *livJ* genes were performed at 37°C (final volume 10 μL) with 1 nM linear DNA, 40 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 60 mM potassium glutamate, 0.05% NP-40, 0.8 U/μL RNase inhibitor, 200 μM ATP, 200 μM GTP, 200 μM CTP, 10 μM UTP and 1 μCi α-^{32}P UTP. The *in vitro* transcription of *hisG* and RNA1 were initiated by the addition of reaction buffer containing 40 mM Tris·HCl, pH 7.9, 165 mM NaCl, 5% glycerol, 10 mM $MgCl_2$, 0.1 μg/μl BSA, 0.8 U/mL RNase inhibitor, 500 μM ATP, 200 μM CTP and GTP, 10 mM UTP and 1 mCi α-^{32}P UTP. The reactions were carried out at 37°C for *rpsM* and *livJ*, and 30°C for *hisG*. After 10 min, the reactions were terminated with the addition of RNA loading buffer (95% formamide, 0.025% SDS, 5 mM EDTA, 0.025% bromophenol blue), and heating at 70°C for 10 min. Transcripts were resolved by electrophoresis on 5% Bio-Rad TBE-Urea precasted gels, and their abundance quantified after processing in a phosphorimager.

Statistical Analysis

The data were analyzed using an unpaired *t*-test with Welch's correction. Determination of statistical significance between multiple comparisons was achieved using one-way analysis of variance (ANOVA) followed by a Bonferroni post-test using transformed data. Data were considered statistically significant when $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DTT	dithiothreitol
GSNO	S-nitrosoglutathione
GST	glutathione S-transferase

NO_3^-	nitrate
NO	nitric oxide
NO_2^-	nitrite
NO^+	nitrosonium cation
$ONOO^-$	peroxynitrite
$O_2^{\cdot -}$	superoxide anion
PAR	4-(2-pyridylazo) resorcinol
S^-	thiolate
S·	sulfenyl radical

REFERENCES

Aberg A, Shingler V, Balsalobre C. Regulation of the *fimB* promoter: a case of differential regulation by ppGpp and DksA *in vivo*. Mol Microbiol. 2008; 67:1223–1241. [PubMed: 18284577]

Aberg A, Fernandez-Vazquez J, Cabrer-Panes JD, Sanchez A, Balsalobre C. Similar and divergent effects of ppGpp and DksA deficiencies on transcription in *Escherichia coli*. J Bacteriol. 2009; 191:3226–3236. [PubMed: 19251846]

Antelmann H, Helmann JD. Thiol-based redox switches and gene regulation. Antioxid Redox Signal. 2011; 14:1049–1063. [PubMed: 20626317]

Bae JB, Park JH, Hahn MY, Kim MS, Roe JH. Redox-dependent changes in RsrA, an anti-sigma factor in *Streptomyces coelicolor*: zinc release and disulfide bond formation. J Mol Biol. 2004; 335:425–435. [PubMed: 14672653]

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A. 1990; 87:1620–1624. [PubMed: 2154753]

Bourret TJ, Porwollik S, McClelland M, Zhao R, Greco T, Ischiropoulos H, Vazquez-Torres A. Nitric oxide antagonizes the acid tolerance response that protects *Salmonella* against innate gastric defenses. PLoS One. 2008; 3:e1833. [PubMed: 18350168]

Brandes N, Rinck A, Leichert LI, Jakob U. Nitrosative stress treatment of *E. coli* targets distinct set of thiol-containing proteins. Mol Microbiol. 2007; 66:901–914. [PubMed: 17919278]

Chen PR, Bae T, Williams WA, Duguid EM, Rice PA, Schneewind O, He C. An oxidation-sensing mechanism is used by the global regulator MgrA in *Staphylococcus aureus*. Nat Chem Biol. 2006; 2:591–595. [PubMed: 16980961]

Chi BK, Gronau K, Mader U, Hessling B, Becher D, Antelmann H. S-bacillithiolation protects against hypochlorite stress in *Bacillus subtilis* as revealed by transcriptomics and redox proteomics. Mol Cell Proteomics. 2011; 10 M111 009506.

Chi BK, Roberts AA, Huyen TT, Basell K, Becher D, Albrecht D, Hamilton CJ, Antelmann H. S-bacillithiolation protects conserved and essential proteins against hypochlorite stress in firmicutes bacteria. Antioxid Redox Signal. 2013; 18:1273–1295. [PubMed: 22938038]

Christman MF, Morgan RW, Jacobson FS, Ames BN. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell. 1985; 41:753–762. [PubMed: 2988786]

Corpet F. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 1988; 16:10881–10890. [PubMed: 2849754]

Crack JC, Green J, Hutchings MI, Thomson AJ, Le Brun NE. Bacterial Iron-Sulfur Regulatory Proteins As Biological Sensor-Switches. Antioxid Redox Signal. 2012; 17:1215–1231. [PubMed: 22239203]

Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. 2000; 97:6640–6645. [PubMed: 10829079]

Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol. 2004; 2:820–832. [PubMed: 15378046]

Farhana A, Saini V, Kumar A, Lancaster JR Jr, Steyn AJ. Environmental Heme-Based Sensor Proteins: Implications for Understanding Bacterial Pathogenesis. Antioxid Redox Signal. 2012; 17:1232–1345. [PubMed: 22494151]

Furman R, Biswas T, Danhart EM, Foster MP, Tsodikov OV, Artsimovitch I. DksA2, a zinc-independent structural analog of the transcription factor DksA. FEBS Lett. 2013; 587:614–619. [PubMed: 23416301]

Graumann J, Lilie H, Tang X, Tucker KA, Hoffmann JH, Vijayalakshmi J, Saper M, Bardwell JC, Jakob U. Activation of the redox-regulated molecular chaperone Hsp33--a two-step mechanism. Structure. 2001; 9:377–387. [PubMed: 11377198]

Hart TW. Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. Tetrahedron Letters. 1985; 26:2013–2016.

Hausladen A, Privalle CT, Keng T, DeAngelo J, Stamler JS. Nitrosative stress: activation of the transcription factor OxyR. Cell. 1996; 86:719–729. [PubMed: 8797819]

Henard CA, Bourret TJ, Song M, Vazquez-Torres A. Control of redox balance by the stringent response regulatory protein promotes antioxidant defenses of *Salmonella*. J Biol Chem. 2010; 285:36785–36793. [PubMed: 20851888]

Henard CA, Vazquez-Torres A. DksA-dependent resistance of *Salmonella enterica* serovar Typhimurium against the antimicrobial activity of inducible nitric oxide synthase. Infect Immun. 2012; 80:1373–1380. [PubMed: 22311927]

Hondorp ER, Matthews RG. Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in *Escherichia coli*. PLoS Biol. 2004; 2:e336. [PubMed: 15502870]

Hunt JB, Neece SH, Ginsburg A. The use of 4-(2-pyridylazo)resorcinol in studies of zinc release from *Escherichia coli* aspartate transcarbamoylase. Anal Biochem. 1985; 146:150–157. [PubMed: 3887984]

Husain M, Jones-Carson J, Song M, McCollister BD, Bourret TJ, Vazquez-Torres A. Redox sensor SsrB Cys^{203} enhances *Salmonella* fitness against nitric oxide generated in the host immune response to oral infection. Proc Natl Acad Sci U S A. 2010; 107:14396–14401. [PubMed: 20660761]

Hyduke DR, Jarboe LR, Tran LM, Chou KJ, Liao JC. Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in *Escherichia coli*. Proc Natl Acad Sci U S A. 2007; 104:8484–8489. [PubMed: 17494765]

Ilbert M, Graf PC, Jakob U. Zinc center as redox switch--new function for an old motif. Antioxid Redox Signal. 2006; 8:835–846. [PubMed: 16771674]

Imlay JA, Linn S. DNA damage and oxygen radical toxicity. Science. 1988; 240:1302–1309. [PubMed: 3287616]

Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. Sci STKE. 2001; 2001:pl1. [PubMed: 11752655]

Keene SD, Greco TM, Parastatidis I, Lee SH, Hughes EG, Balice-Gordon RJ, Speicher DW, Ischiropoulos H. Mass spectrometric and computational analysis of cytokine-induced alterations in the astrocyte secretome. Proteomics. 2009; 9:768–782. [PubMed: 19132682]

Keyer K, Imlay JA. Inactivation of dehydratase [4Fe-4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. J Biol Chem. 1997; 272:27652–27659. [PubMed: 9346904]

Kim SO, Merchant K, Nudelman R, Beyer WF Jr, Keng T, DeAngelo J, Hausladen A, Stamler JS. OxyR: a molecular code for redox-related signaling. Cell. 2002; 109:383–396. [PubMed: 12015987]

Kullik I, Stevens J, Toledano MB, Storz G. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. J Bacteriol. 1995; 177:1285–1291. [PubMed: 7868603]

Kuo CF, Mashino T, Fridovich I. α, β-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme. J Biol Chem. 1987; 262:4724–4727. [PubMed: 3031031]

Lee JW, Helmann JD. The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. Nature. 2006a; 440:363–367. [PubMed: 16541078]

Lee JW, Helmann JD. Biochemical characterization of the structural Zn^{2+} site in the *Bacillus subtilis* peroxide sensor PerR. J Biol Chem. 2006b; 281:23567–23578. [PubMed: 16766519]

Lee JW, Soonsanga S, Helmann JD. A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. Proc Natl Acad Sci U S A. 2007; 104:8743–8748. [PubMed: 17502599]

Lemke JJ, Sanchez-Vazquez P, Burgos HL, Hedberg G, Ross W, Gourse RL. Direct regulation of *Escherichia coli* ribosomal protein promoters by the transcription factors ppGpp and DksA. Proc Natl Acad Sci U S A. 2011; 108:5712–5717. [PubMed: 21402902]

Lennon CW, Gaal T, Ross W, Gourse RL. *Escherichia coli* DksA binds to Free RNA polymerase with higher affinity than to RNA polymerase in an open complex. J Bacteriol. 2009; 191:5854–5858. [PubMed: 19617357]

Li W, Bottrill AR, Bibb MJ, Buttner MJ, Paget MS, Kleanthous C. The Role of zinc in the disulphide stress-regulated anti-sigma factor RsrA from *Streptomyces coelicolor*. J Mol Biol. 2003; 333:461–472. [PubMed: 14529630]

Magnusson LU, Gummesson B, Joksimovic P, Farewell A, Nystrom T. Identical, independent, and opposing roles of ppGpp and DksA in *Escherichia coli*. J Bacteriol. 2007; 189:5193–5202. [PubMed: 17496080]

Mastroeni P, Vazquez-Torres A, Fang FC, Xu Y, Khan S, Hormaeche CE, Dougan G. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. J Exp Med. 2000; 192:237–248. [PubMed: 10899910]

McCollister BD, Myers JT, Jones-Carson J, Husain M, Bourret TJ, Vazquez-Torres A. N_2O_3 enhances the nitrosative potential of IFNγ-primed macrophages in response to *Salmonella*. Immunobiology. 2007; 212:759–769. [PubMed: 18086377]

Messner KR, Imlay JA. The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. J Biol Chem. 1999; 274:10119–10128. [PubMed: 10187794]

Mohr S, Stamler JS, Brune B. Mechanism of covalent modification of glyceraldehyde-3-phosphate dehydrogenase at its active site thiol by nitric oxide, peroxynitrite and related nitrosating agents. FEBS Lett. 1994; 348:223–227. [PubMed: 8034046]

Moody CS, Hassan HM. Mutagenicity of oxygen free radicals. Proc Natl Acad Sci U S A. 1982; 79:2855–2859. [PubMed: 7045874]

Paget MS, Bae JB, Hahn MY, Li W, Kleanthous C, Roe JH, Buttner MJ. Mutational analysis of RsrA, a zinc-binding anti-sigma factor with a thiol-disulphide redox switch. Mol Microbiol. 2001; 39:1036–1047. [PubMed: 11251822]

Paul BJ, Barker MM, Ross W, Schneider DA, Webb C, Foster JW, Gourse RL. DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell. 2004; 118:311–322. [PubMed: 15294157]

Paul BJ, Berkmen MB, Gourse RL. DksA potentiates direct activation of amino acid promoters by ppGpp. Proc Natl Acad Sci U S A. 2005; 102:7823–7828. [PubMed: 15899978]

Perederina A, Svetlov V, Vassylyeva MN, Tahirov TH, Yokoyama S, Artsimovitch I, Vassylyev DG. Regulation through the secondary channel--structural framework for ppGpp-DksA synergism during transcription. Cell. 2004; 118:297–309. [PubMed: 15294156]

Potrykus K, Cashel M. (p)ppGpp: still magical? Annu Rev Microbiol. 2008; 62:35–51. [PubMed: 18454629]

Richardson AR, Soliven KC, Castor ME, Barnes PD, Libby SJ, Fang FC. The Base Excision Repair system of *Salmonella enterica* serovar Typhimurium counteracts DNA damage by host nitric oxide. PLoS Pathog. 2009; 5:e1000451. [PubMed: 19478870]

Richardson AR, Payne EC, Younger N, Karlinsey JE, Thomas VC, Becker LA, Navarre WW, Castor ME, Libby SJ, Fang FC. Multiple targets of nitric oxide in the tricarboxylic acid cycle of *Salmonella enterica* serovar Typhimurium. Cell Host Microbe. 2011; 10:33–43. [PubMed: 21767810]

Rostkowski M, Olsson MH, Sondergaard CR, Jensen JH. Graphical analysis of pH-dependent properties of proteins predicted using PROPKA. BMC Struct Biol. 2011; 11:6. [PubMed: 21269479]

Schneider DA, Gaal T, Gourse RL. NTP-sensing by rRNA promoters in *Escherichia coli* is direct. Proc Natl Acad Sci U S A. 2002; 99:8602–8607. [PubMed: 12060720]

Schneider TD, Stephens RM. Sequence logos: a new way to display consensus sequences. Nucleic Acids Res. 1990; 18:6097–6100. [PubMed: 2172928]

Seaver LC, Imlay JA. Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? J Biol Chem. 2004; 279:48742–48750. [PubMed: 15361522]

Spiro S, D'Autreaux B. Non-Heme Iron Sensors of Reactive Oxygen and Nitrogen Species. Antioxid Redox Signal. 2012; 17:1264–1276. [PubMed: 22304730]

Uzzau S, Figueroa-Bossi N, Rubino S, Bossi L. Epitope tagging of chromosomal genes in *Salmonella*. Proc Natl Acad Sci U S A. 2001; 98:15264–15269. [PubMed: 11742086]

Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC. 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. 2000; 192:227–236. [PubMed: 10899909]

Vazquez-Torres A. Redox Active Thiol Sensors of Oxidative and Nitrosative Stress. Antioxid Redox Signal. 2012; 17:1201–1214. [PubMed: 22257022]

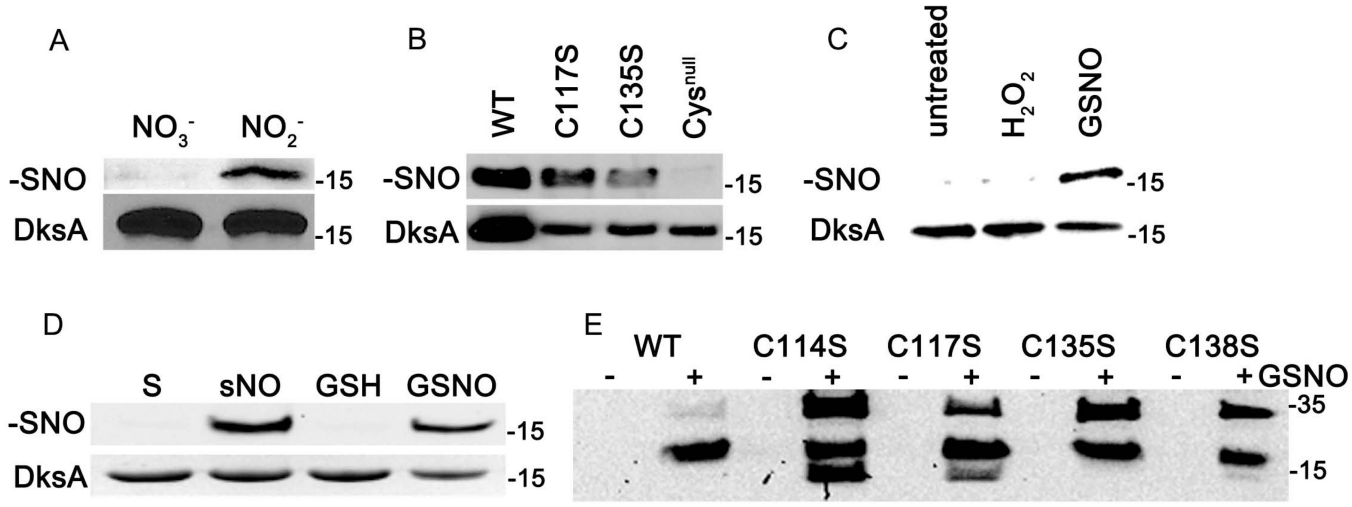

Figure 1. Sensing of reactive nitrogen species by thiols in the 4-cysteine zinc-finger motif of DksA

Salmonella strain AV08016 expressing the *dksA*∷*3 × FLAG* allele was grown for 6 h in EG medium, pH 5.5, in the presence of 750 μM NO_3^- or NO_2^-. S-nitrosothiols (−SNO) in cytoplasmic extracts were derivatized in the biotin switch assay. DksA∷3 × FLAG was detected in affinity-purified, biotinylated fractions (upper panel, A). The effect that reactive nitrogen species had on DksA content was measured in unfractionated bacterial cytoplasmic extracts (lower panel, A). S-nitrosylation of DksA was also studied in *Salmonella* strains expressing 3 *dksA* variants bearing mutations in 1 or all cysteines in the zinc-finger motif (B). The formation of S-nitrosylated DksA was also tested in *Salmonella* treated with 400 μM H_2O_2 or 500 μM S-nitrosoglutathione (GSNO) for 30 min (C). *Salmonella* grown in EG medium, pH 5.5, were used as controls (untreated). (D) 50 μM recombinant DksA protein was treated with 10 equivalents of the NO-donor spermine NONOate (sNO), or 2 equivalents of GSNO for 1 h at 37°C in the dark. Spermine (S) and glutathione (GSH) were used as controls. S-nitrosothiolated DksA derivatives were detected using the biotin switch assay (upper panel) and total recombinant DksA protein was visualized by Coomassie blue staining (lower panel). (E) S-nitrosylation of recombinant DksA variant expressing the wild-type or serine substitutions in the indicated cysteine residues. Where indicated (+), the specimens were treated with GSNO. The molecular weight markers (kDa) are shown on the right side of the immunoblots. The data are representative of 2–3 independent experiments.

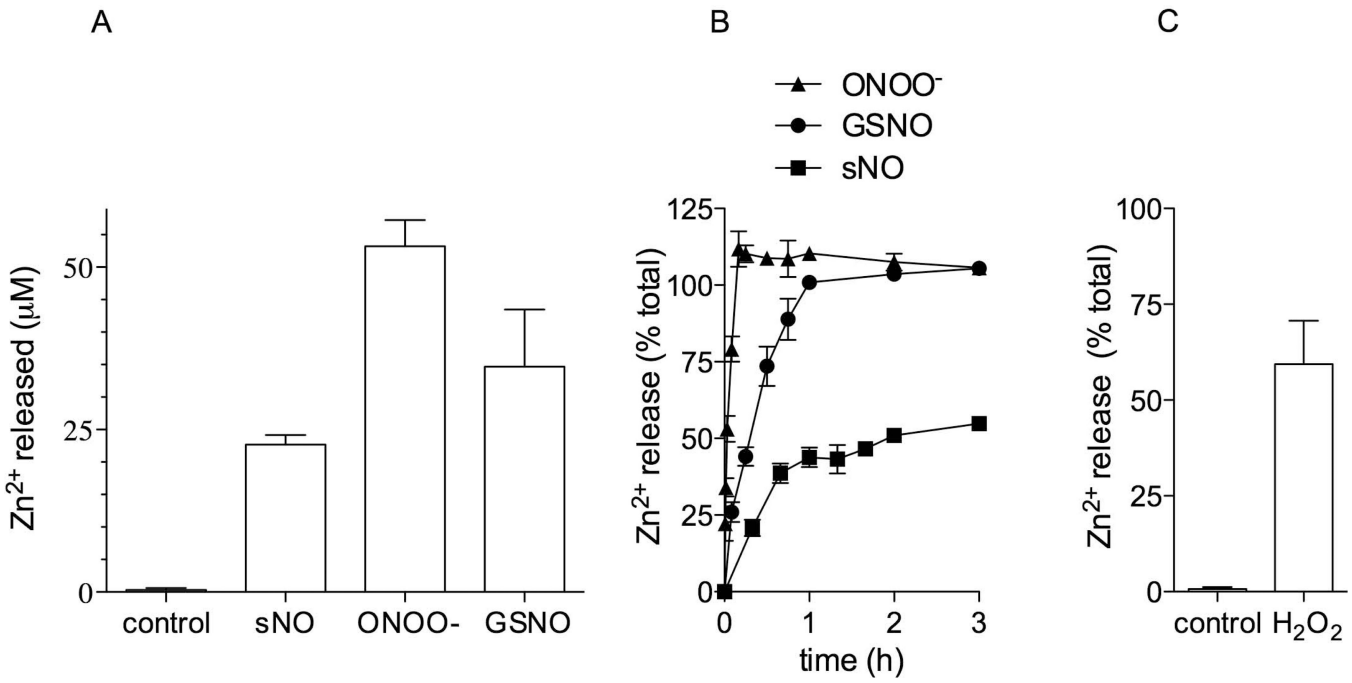

Figure 2. Reactive oxygen and nitrogen species release zinc from DksA

The release of zinc from 50 μM DksA was measured by monitoring the complexation of Zn^{2+} with 150 mM 4-(2-pyridylazo) resorcinol 1 h after the protein was treated with 10 equivalents of spermine NONOate (sNO), S-nitrosoglutathione (GSNO), or peroxynitrite ($ONOO^-$) at 37°C (A). Percent of zinc released from 50 μM DksA over time after treatment with 10 equivalents of the indicated reactive nitrogen species (B) or H_2O_2 (C). The data are the mean ± SEM from at least 2 separate experiments (n=4–6).

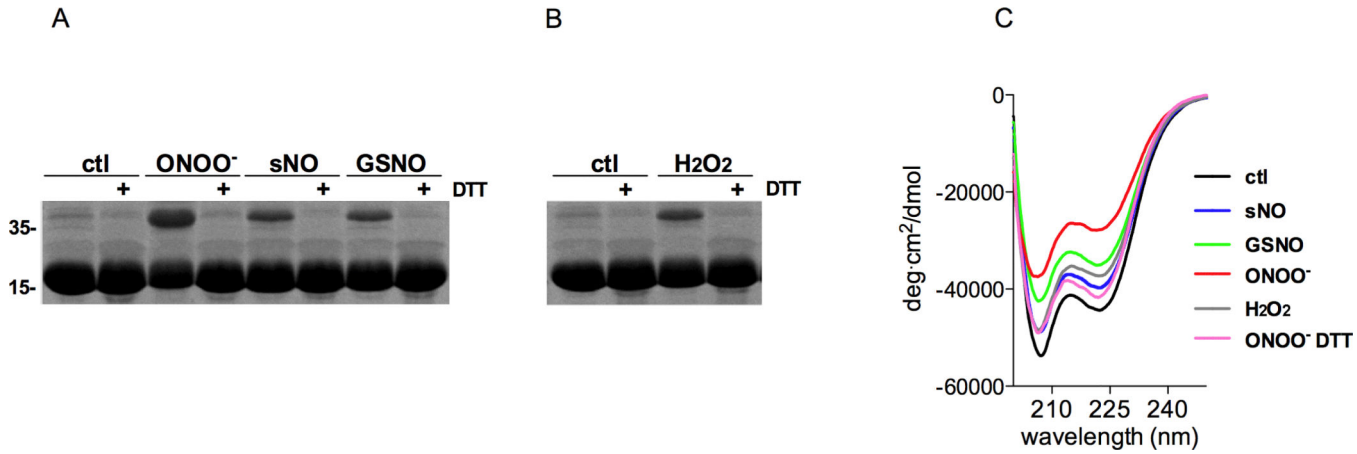

Figure 3. Changes in DksA α-helicity following the reversible oxidation of cysteines in the zinc-finger motif

50 μM DksA was treated for 1 h at 37°C in the dark with 10 equivalents of peroxynitrite ($ONOO^-$), spermine NONOate (sNO), or S-nitrosoglutathione (GSNO) (A), or hydrogen peroxide (H_2O_2) (B). Reduced, zinc-bound DksA was used as control (ctl). Selected samples were co-incubated with 1 mM DTT. DksA was visualized by Coomassie blue staining after the samples were separated in non-reducing, SDS-PAGE gels. (C) CD spectra of untreated or $ONOO^-$-, sNO-, GSNO-, or H_2O_2-treated DksA. Where indicated, 1 mM DTT was added to oxidized DksA 1 h after $ONOO^-$ treatment. Data on A and B are representative of 2–3 independent experiments. Panel C represents the mean of 6 independent scans.

A

C1	*Advenella kashmirensis*	RDYGW CEETGEPIGL RRLLARPTAT LSLEAQERRE
	Aequorivita sublithincola	KTYGV CRVTGKLINK ERLKLVPHAT LSIEAKNLQK
	Agrobacterium tumefaciens	GTYGY CEETGEPIGL KRLDARPIAT LSIEAQERHE
	Alicycliphilus denitrificans	GDYGY CDETGEPIGV GRLLARPTAT LSLEAQQRRE
	Bartonella henselae	GTYGF CEETGEPISI KRLEARPIAV LSLEAQERHE
	Bordetella pertussis	GEYGW CEETGEPIGV PRLLARPTAT LSLEAQERRE
	Brucella melitensis	GTYGF CEETGDPISL KRLDARPIAT LSIEAQERHE
	Burkholderia mallei	GDYGW CEETGEPIGI PRLLARPTAT LSLEAQERRE
	Chlamydia trachomatis	ASYGI CDVSGEEIPL ARLMAIPYAT MTVKSQEKFE
	Kingella kingae	GEYGF CRDTGEPIGL RRLLARPTAT LSVEAQERRE
	Leptospira interrogans	GTYGV CEGTGKKIPI ARLKAIPWTR YTVEYAETLS
	Neisseria gonorrhoeae	GDYGF CADTGEPIGL KRLLARPTAT LSVEAQERRE
	Parabacteroides distasonis	KTYGI CRETGKLIPK ERLRAVPHAT LSIEAKQGGA
	Rhizobium leguminosarum	GTYGY CEETGEPIGL KRLDARPIAT LSIEAQERHE
	Xanthomonas gardneri	GDYGY CVDTGEEIGL DRLEARLTAE RTIDAQERWE
C2	*Alishewanella aestuarii*	KEFGY CLETGEPIGL ARLFIRPVSE YCADVKLLHE
	Alteromonas macleodii	KSFGY CLETGEPIGI PRLLIRPVSE YCADVKMVNE
	Anaplasma phagocytophilum	GLYGY CEETGEKIGL GRLKANPVTL YCIEEQERRE
	Ehrlichia ruminantium	GSYGY CEETGEEIGI ARLKANPITL YCIEEQERRE
	Pseudomonas aeruginosa	GDYGW CQETGEPIGL RRLLLRPTAT LCIEAKERQE
	Rickettsia rickettsii	GEYGY CEETGNPIGI KRLEARPIAA LCIEAQERHE
C4	*Acinetobacter baumannii*	EDYGF CETCGIEIGL RRLEARPTAT LCIDCKTLAE
	Aeromonas hydrophila	DDFGY CEHCGIEIGI RRLEARPTAD LCVDCKTLAE
	Borrelia burgdorferi	NSYGK CLACEREIAR ERLLAIPYAF LCISCQTKKE
	Campylobacter jejuni	GTYGI CESCDDEIDS QRLKIKPHAR YCITCRQIAE
	Citrobacter freundii	EDFGY CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Coxiella burnetii	GDYGF CEDCGAEIGI RRLEARPTAT KCIDCKTFEE
	Edwardsiella tarda	DDFGF CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Enterobacter cloacae	EDFGY CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Escherichia coli	EDFGY CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Haemophilus influenzae	DDFGY CDCCGEEIGI RRLEARPTAD LCIDCKTLAE
	Helicobacter canadensis	GVYGI CEMCDEPIGI QRLRAKPHAR YCIVCREIVE
	Klebsiella pneumoniae	EDFGY CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Legionella pneumophila	EDFGY CEACGIEIGL KRLEARPTAT LCIDCKTLSE
	Moraxella catarrhalis	LDYGY CETCGTEIGL RRLEARPTAT QCIDCKTLSE
	Morganella morganii	DDFGF CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Nitrosococcus oceani	GDYGY CDGCGAEIGI RRLEARPTAT LCIDCKTLDE
	Pasteurella multocida	GDFGY CDSCGIEIGI RRLEARPTAD LCIDCKTLAE
	Photobacterium leiognathi	DDFGF CDSCGIEIGI RRLEARPTAE LCIDCKTLAE
	Propionibacterium acnes	GTWGT CESCGEPIGK ARLQAFPRAT MCVKCKQRQE
	Proteus mirabilis	DDFGF CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Pseudomonas aeruginosa	EEYGW CDSCGVEIGI RRLEARPTAT LCIDCKTLAE
	Ralstonia eutropha	GQYGT CIDCQQPIPF SRLQAYPTAK RCTACQRRHE
	Rhodobacter sphaeroides	DEYGF CVKCGAEIGE ARLDVLPYTP FCRKCAG---
	Salmonella enterica	EDFGY CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Shewanella denitrificans	DDFGF CDSCGVEIGI RRLEARPTAD QCIDCKTLAE
	Shigella flexneri	EDFGY CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Vibrio cholerae	EDFGY CESCGVEIGI RRLEARPTAD LCIDCKTLAE
	Yersinia pestis	DDFGF CESCGVEIGI RRLEARPTAD LCIDCKTLAE

B

Figure 4. Amino acid sequence alignment of the C-terminal region of DksA homologs

Selected annotated protein sequences obtained from the NCBI Protein database were aligned using Multalin (Corpet, 1988). Sequences are grouped according to their cysteine content; cysteine residues are highlighted in yellow. The DksA consensus sequence was determined using 74 protein sequences from NCBI, including those presented in panel A. The graphical representation of the consensus sequence was generated using Sequence Logo (Schneider and Stephens, 1990) and is displayed as amino acid frequency.

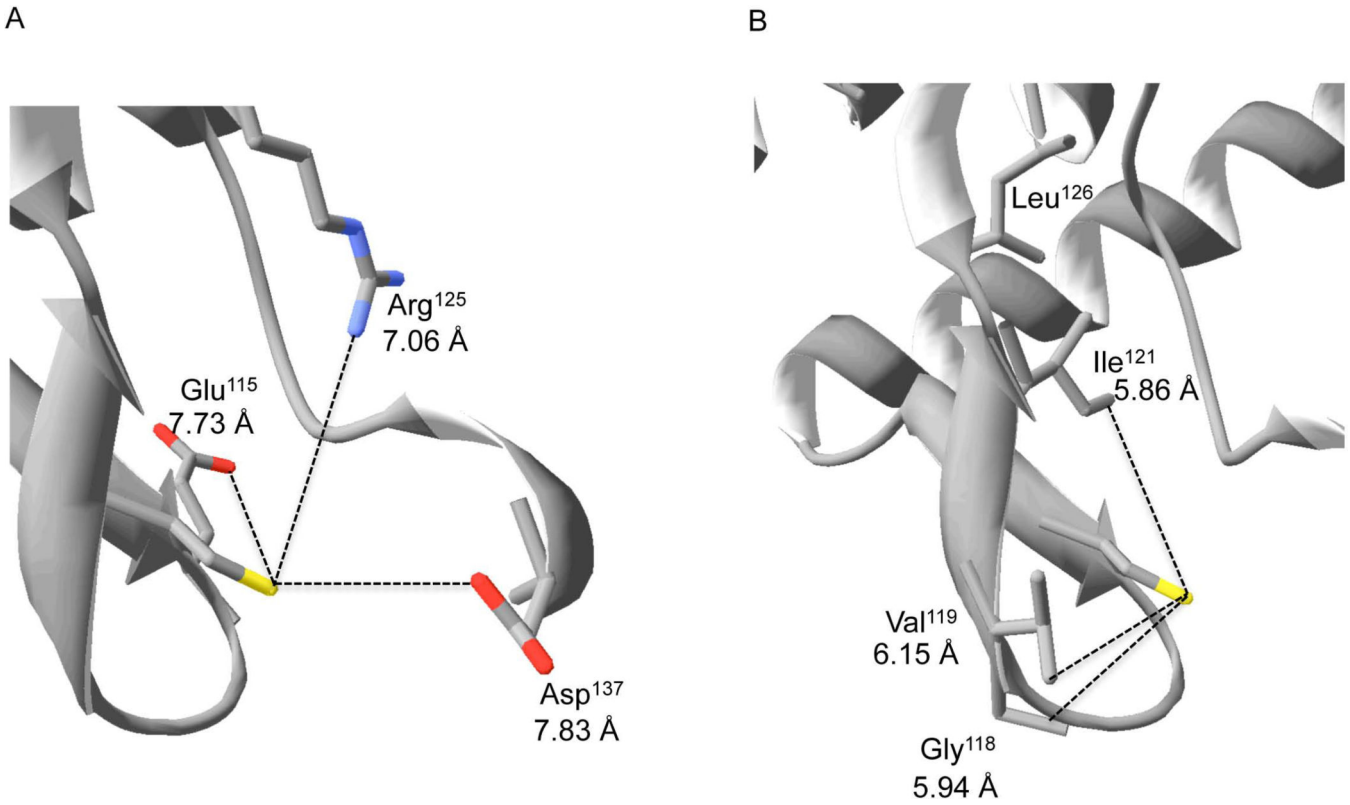

Figure 5. Charged and hydrophobic residues near Cys^{114}

Analysis of the crystal structure of *E. coli* DksA protein (Perederina *et al.*, 2004) reveals the proximity of conserved positively- and negatively-charged (A) and hydrophobic residues (B) to the thiol group of Cys^{114}.

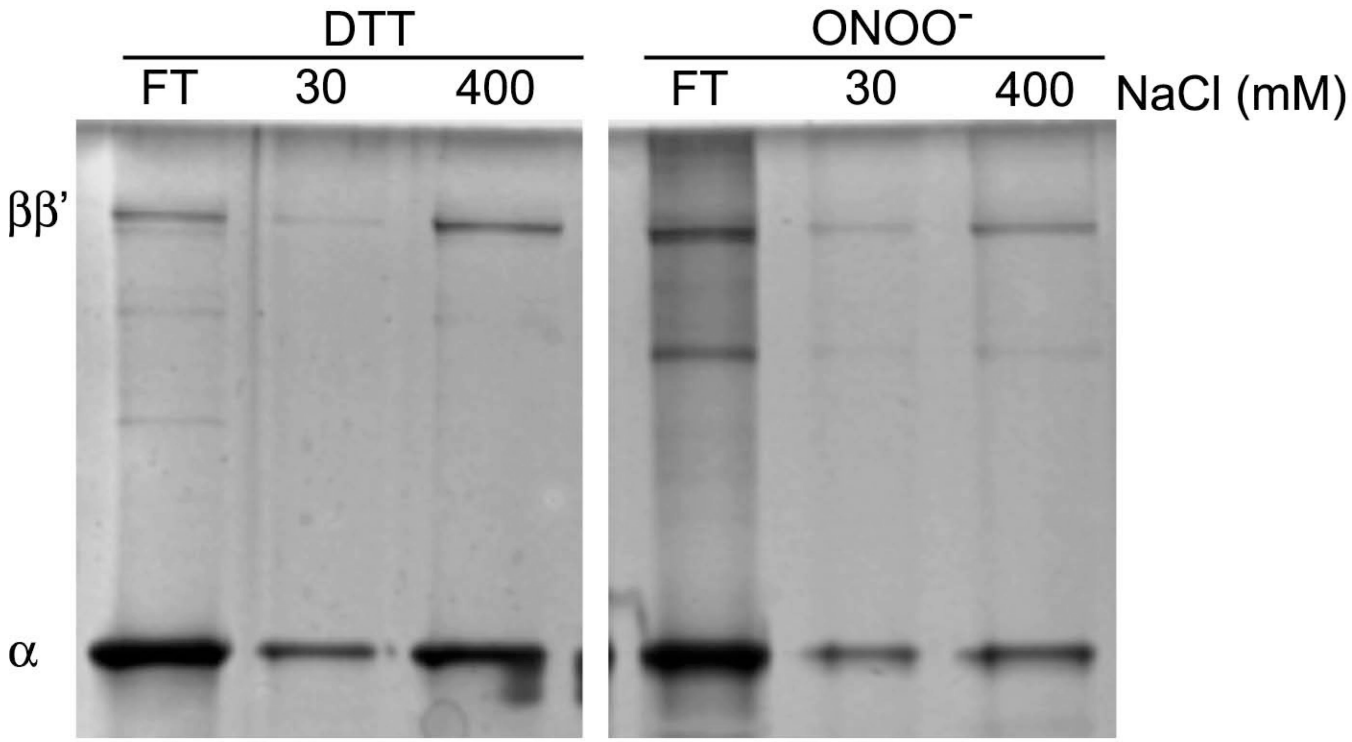

Figure 6. Binding of oxidized DksA to the RNA polymerase

Binding of core RNA polymerase to GST-DksA proteins that had been treated with 1 mM DTT or 1 mM $ONOO^-$ before they were immobilized on a GSH Sepharose matrix. The gels show the α and ββ' subunits of the RNA polymerase in the flow through (FT) or the fractions collected after the addition of NaCl. The proteins were visualized by silver staining of specimens separated in SDS-PAGE gels. The data are representative of 3 independent experiments.

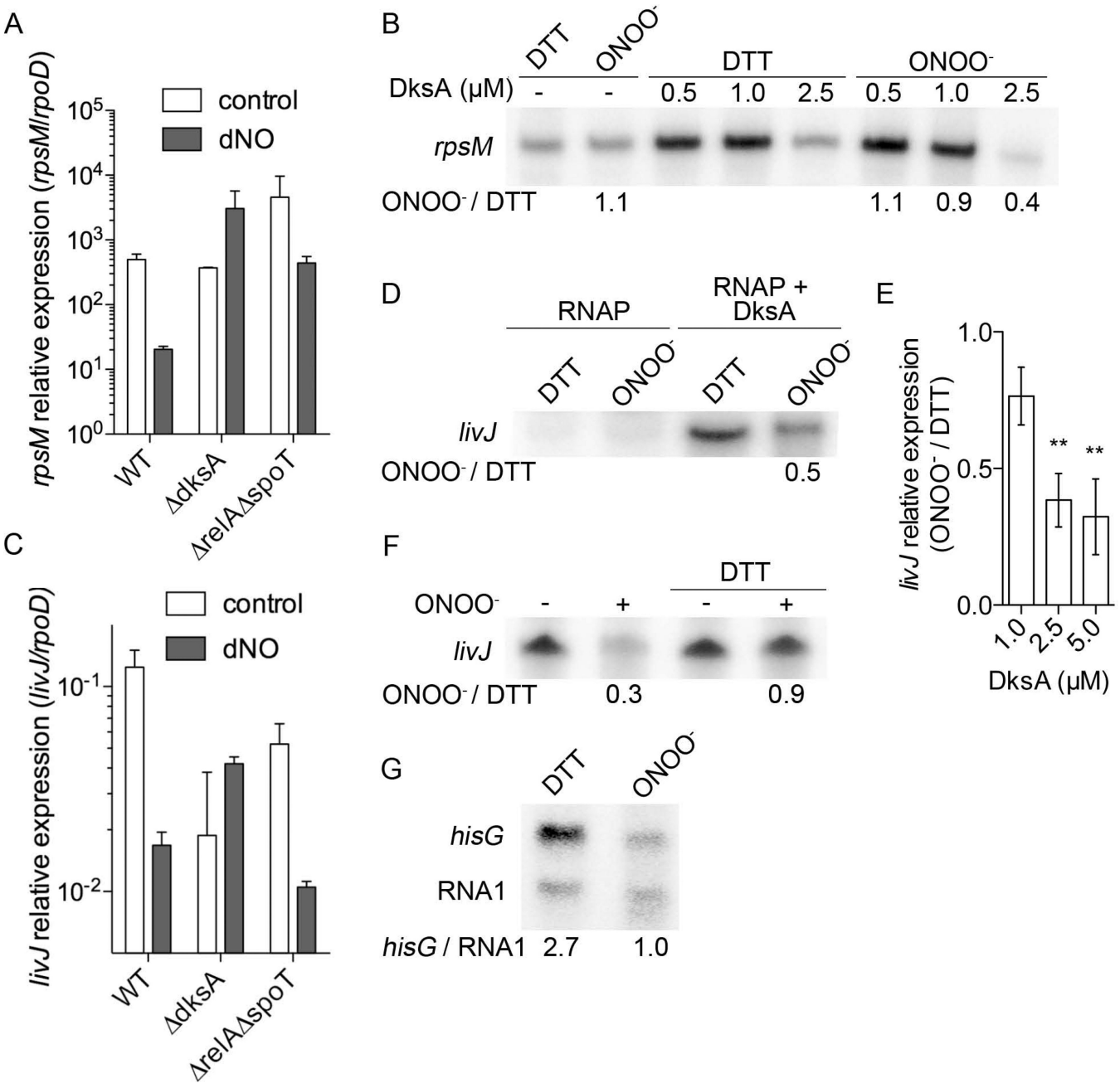

Figure 7. DksA-dependent inhibition of gene transcription in response to reactive nitrogen species

Relative expression of *rpsM* (A) and *livJ* (C) in control and DETA NONOate (dNO)-treated bacteria. Increasing concentrations of DksA complexed with 5 nM RNA polymerase were treated with 25 μM $ONOO^-$ before *rpsM* (B) and *livJ* (D) *in vitro* transcription reactions were initiated. The ratio of *rpsM* and *livJ* transcripts in oxidized over reduced samples ($ONOO^-$/DTT) are shown at the bottom of the autoradiographs. 2.5 μM of DksA were used in the experiments shown in panel D. Increasing concentrations of DksA and 5 nM RNA polymerase were treated with 25 μM $ONOO^-$ before *livJ in vitro* transcription was initiated upon the addition of DNA template and reaction buffer (E). The results in E show the ratio

of *livJ* transcription supported by the oxidized over the corresponding reduced specimens. * $p < 0.01$ when compared to the *in vitro* transcription reactions containing 1 μM DksA. 2.5 mM DTT was added to DksA/RNA polymerase complexes 5 min after treatment with 25 μM $ONOO^-$; the transcription of *livJ* was initiated with the addition of the DNA template and reaction buffer (F). Effect of oxidation on the *in vitro* transcription of *hisG* and the internal standard RNA1 is shown in G. The ratio of *hisG*/RNA1 is shown below the autoradiograph. The results in A, C, and E are the mean ± SEM of 3 independent experiments. The data in B, D, F and G are representative of 2–3 independent experiments.

Table 1

Oxidation status of cysteine residues in reduced and $ONOO^-$-treated DksA

	DTT-treated				$ONOO^-$-treated			
	C114	**C117**	**C135**	**C138**	**C114**	**C117**	**C135**	**C138**
Reduced[1]	100*	100	99	100	20	0	6	18
Disulfide-bonded[2]	0	0	0	0	50	8	52	39
Terminally-oxidized[3]	0	0	1	0	30	92	42	43

*% of cysteine residue modifications in all peptides measured

[1] carbamidomethyl (+57 Da)

[2] N-ethylmaleimide (+125 Da)

[3] cysteic acid (+48 Da)

Table 2

Strains and plasmids

Strain	Genotype	Source
S. Typhimurium ATCC 14028s	Wild-type	ATCC
AV08016	*dksA*: :3×FLAG	(Henard *et al.*, 2010)
AV10305	*dksA* C135S: :3×FLAG	This study(Henard *et al.*, 2010)
AV10310	*dksA* C117S: :3×FLAG	This study
AV10311	*dksA* C114S C117S C135S C138S: :3×FLAG	This study
Plasmids		
pSUB11	3×FLAG FRT *ahp* FRT *bla* R6KoriV	(Uzzau *et al.*, 2001)
pGEX6P1	*bla* pBR322 ori *lacI*q P_{tac} *gst*	GE Healthcare
pGEX6P1: :*dksA*	*bla* pBR322 ori l*acI*q P_{tac} *gst dksA*	This study
pGEX6P1: :*dksA*C114S	*bla* pBR322 ori *lacI*q P_{tac} *gst dksA*C114S	This study
pGEX6P1: :*dksA*C117S	*bla* pBR322 ori *lacI*q P_{tac} *gst dksA*C117S	This study
pGEX6P1: :*dksA*C135S	*bla* pBR322 ori *lacI*q P_{tac} *gst dksA*C135S	This study
pGEX6P1: :*dksA*C138S	*bla* pBR322 ori *lacI*q P_{tac} *gst dksA*C138S	This study
pRLG4413	P_{hisG} (−60/+1) RNA1	(Paul *et al.*, 2005)

Table 3

Primers

Mutation	Primer sequence
*dksA*C114S	F:TGGAAGATGAAGACTTCGGTTATAGCGAGTCCTGCG
	R:CGCAGGACTCGCTATAACCGAAGTCTTCATCTTCCA
*dksA*C117S	F:TATTGCGAGTCCAGCGGGGTGGAGATT
	R:AATCTCCACCCCGCTGGACTCGCAATA
*dksA*C135S	F:ACAGCCGATCTGAGCATCGACTGCAAAACGCTGGCT
	R:AGCCAGCGTTTTGCAGTCGATGCTCAGATCGGCTGT
*dksA*C138S	F:ACAGCCGATCTGTGCATCGACAGCAAAACGCTGGCT
	R:AGCCAGCGTTTTGCTGTCGATGCACAGATCGGCTGT
pGEX6P1: :*dksA*	F:AAGCGCGGATCCATGCAAGAAGGGCAAAACCG
	R:GCCGGAATTCTTAACCCGCCATCTGTTTTTCG
Real time PCR	Primer sequence
rpsM	F:AGTTGCCAAATTTGTCGTTG
	R:TACGAGCGTTGGTCTTGGTA
	PROBE: 6-FAM-TGAAATCAGCATGAGCATCAAGCGCCTGAT-3BHQ-1
livJ	F:CGCAGGGCTGAAAACCCA
	R: CACACGAATGCGCCGCTA
	PROBE: 6-FAM-TCAGCGGAAGGCTTACTGGTCAC-3BHQ-1
In vitro transcription	
livJ	F: GGAATTCCAATACGTTTGCCCGATGG
	R: TGCACTGCAGTGCATATTTCACCGCGACGAGC
rpsM	F: CGGAATTCCTGTCATCCGTGTGATTTGCAG
	R: TGCAGCTAGCTGCATTTTCAGCGATACCCGCT