THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 47, pp. 36785–36793, November 19, 2010

Control of Redox Balance by the Stringent Response Regulatory Protein Promotes Antioxidant Defenses of *Salmonella*[*][S]

Received for publication, July 2, 2010, and in revised form, September 15, 2010 Published, JBC Papers in Press, September 17, 2010, DOI 10.1074/jbc.M110.160960

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We report herein a critical role for the stringent response regulatory DnaK suppressor protein (DksA) in the coordination of antioxidant defenses. DksA helps fine-tune the expression of glutathione biosynthetic genes and discrete steps in the pentose phosphate pathway and tricarboxylic acid cycle that are associated with the generation of reducing power. Control of NAD(P)H/NAD(P)$^+$ redox balance by DksA fuels downstream antioxidant enzymatic systems in nutritionally starving *Salmonella*. Conditional expression of the glucose-6-phosphate dehydrogenase-encoding gene *zwf*, shown here to be under DksA control, increases both the NADPH pool and antioxidant defenses of *dksA* mutant *Salmonella*. The DksA-mediated coordination of redox balance boosts the antioxidant defenses of stationary phase bacteria. Not only does DksA increase resistance of *Salmonella* against hydrogen peroxide (H_2O_2), but it also promotes fitness of this intracellular pathogen when exposed to oxyradicals produced by the NADPH phagocyte oxidase in an acute model of infection. Given the role of DksA in the adjustment of gene expression in most bacteria undergoing nutritional deprivation, our findings raise the possibility that the control of central metabolic pathways by this regulatory protein maintains redox homeostasis essential for antioxidant defenses in phylogenetically diverse bacterial species.

Bacterial pathogens have the remarkable ability to survive harsh conditions encountered during the course of infection. For instance, Gram-negative bacteria of the genus *Salmonella* must endure the extreme acidity of the stomach, osmotic stress in the intestinal lumen, as well as oxygen-dependent and -independent cytotoxicity in the intracellular environment of macrophages. Of all of these stresses, reactive oxygen species are arguably the most extensively studied host defenses. *Salmonella* infection triggers the assembly of an NADPH phagocyte oxidase respiratory complex (1) for the production of superoxide, a radical that rapidly dismutates to give rise to hydrogen peroxide (H_2O_2). Membrane-permeable H_2O_2 causes extensive damage to DNA bases and [4Fe-4S] prosthetics groups of dehydratases via Fenton-like chemistries that are dependent on the ferrous iron-catalyzed univalent or divalent reduction of H_2O_2 (2, 3). H_2O_2 is also thought to mediate cytotoxicity through the oxidation of thiol groups in redox active cysteines (4). The importance of reactive oxygen species in host defense against *Salmonella*, as well as an assortment of other fungal and bacterial pathogens, is underscored by the increased incidence of recurrent infections in chronic granulomatous disease patients who lack a functional NADPH phagocyte oxidase (5). A murine model deficient in the gp91*phox* membrane-bound component of the NADPH oxidase accurately recapitulates the clinical importance of this enzymatic complex in the innate response of humans during *Salmonella* infections (6).

Bacteria, including *Salmonella*, have a diverse arsenal of antioxidant defenses. Superoxide dismutases, catalases, alkylhydroperoxidases, glutathione, and thioredoxins all directly or indirectly detoxify reactive oxygen species (7, 8). Because of the membrane-remodeling activity of effectors secreted into the host cell by the type III secretion system encoded within *Salmonella* pathogenicity island 2, *Salmonella* can avoid NADPH phagocyte oxidase-containing vesicles (9–11). Recent investigations on the electron transport chain highlight the importance of reduced nucleotides for the resistance of *Salmonella* against oxidative stress (12). Maintaining cellular reductive power may provide the fuel needed for detoxification of oxyradicals because many of the antioxidant enzymatic systems consume reduced pyridine nucleotides. It is still uncertain whether and to what extent central metabolic pathways contribute reducing power for classical antioxidant defenses. In the present work, we have elucidated a novel role for DksA[3] in the control of discrete steps of central metabolism that fuel antioxidant defenses.

Previous studies in *Escherichia coli* have revealed DksA to have pleiotropic regulatory roles in gene expression, cell division, amino acid biosynthesis, quorum sensing, stress resistance, and virulence (13–19). Some of the global regulatory effects of DksA are dependent on alarmone guanine tetraphosphate (ppGpp) and the stringent response. The stringent response was originally identified as a global adaptation of bacteria to nutritional stress. Starvation for amino acids, glucose, phosphate, or fatty acids leads to the accumulation of ppGpp (20, 21). This alarmone helps the cell adapt to starving conditions by down-regulating transcription of translational machinery, thereby balancing protein production with nutrient

* This work was supported by the National Institute of Health Grant AI54959. This work was also supported by the Burroughs Welcome Fund and the Institutional Training Grant T32 AI052066.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2 and additional references.

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[3] The abbreviations used are: DksA, DnaK suppressor protein; FRT, flippase (Flp) recognition target; ppGpp, guanine tetraphosphate.

availability. Some of the regulatory actions of ppGpp on the activity of RNA polymerase are achieved in conjunction with DksA, a protein with structural homology to Gre transcription factors (22). However, independent and opposing roles for DksA have also been observed, supporting unique functions for DksA (23, 24). DksA has been shown to play a role in the virulence of several pathogens including *Salmonella*; however, it is still unclear how adjustments in gene expression by DksA contribute to *Salmonella* pathogenesis (25). We report herein that DksA exerts transcriptional control of discrete steps in central metabolic pathways of the pentose phosphate pathway and tricarboxylic acid cycle during the stationary phase of *Salmonella*. Our investigations indicate that DksA helps maintain a redox balance critical for *Salmonella* virulence in response to reactive oxygen species generated intracellularly by the NADPH phagocyte oxidase.

EXPERIMENTAL PROCEDURES

Bacterial Strains—Salmonella enterica sv. *typhimurium* strain ATCC 14028s was used as wild-type and as a background in the construction of mutants following the method previously described by Datsenko and Wanner (26) (supplemental Table 1). PCR amplicons containing kanamycin or chloramphenicol resistance cassettes, or a 3×FLAG tag, flanked by the flippase (Flp) recognition target (FRT) were generated from pKD13, pKD3, and pSUB11 templates using LA *Taq* high fidelity polymerase (TaKaRa, Madison, WI) and 60-base-long primers containing homology to the target gene (supplemental Table 2). Purified PCR products were electroporated into *S. typhimurium* strain TT22236 harboring the plasmid pTP223 that expresses an isopropyl 1-thio-β-D-galactopyranoside-inducible λ Red recombinase system. Mutations were moved into *S. typhimurium* strain 14028s via P22-mediated transduction, and pseudolysogens were eliminated by streaking on Evans blue uranine agar. The antibiotic cassette was removed by recombining the flanking FRT sites with the Flp recombinase expressed from the temperature-sensitive pCP20 plasmid (27). In-frame deletions were verified by PCR analysis. Strain AV09294 lacking *dksA* was complemented with the low copy vector pWSK29 encoding a wild-type *dksA* allele expressed from its native promoter. Transcriptional fusions were constructed by pCP20-mediated integration of pCE36 encoding a promoterless *lacZY* into FRT scars of selected genes (28).

Conditional Expression of zwf—Construction of a *zwf* conditional mutant was achieved by placing the *zwf* open reading frame under the control of the tetracycline promoter/operator. The tetracycline promoter/operator containing plasmid pASK75 was modified for the vector to be inserted into the *Salmonella* chromosome. Briefly, the origin of replication in pASK75 was replaced with the R6K origin amplified from plasmid pKD13. The amplicon containing the R6K origin and pASK75 were ligated after SpeI and PflMI digestion, generating a *pir*-dependent suicide vector. The FRT site from pCE36 was directionally cloned into the MscI unique site of the modified pASK75. The ampicillin resistance cassette of the modified pASK75 plasmid was disrupted by directionally cloning a gentamicin cassette from pPS856 into ScaI and PvuI unique sites to generate pTX (see Fig. 6*A*). A *zwf* conditional mutant was constructed by placing the gene with its native ribosomal binding site into EcoRI and BamHI restriction sites placed downstream of the tet promoter. The final construct was confirmed by sequence analysis. The suicide vector was recombined by Flp expressed from pCP20 into a unique FRT site engineered downstream of *putP* in the chromosome of *Salmonella* strain AV09422. The P_{tet}-controlled *zwf* was transduced into wild-type and *dksA* mutant *Salmonella* strain AV09294. Expression of the allele was initiated with 0.2 μg/ml anhydrotetracycline (Sigma-Aldrich).

Real Time PCR—Bacterial cultures grown in Luria Bertani (LB) broth for 20 h were diluted to 10^8 cfu/ml in PBS and treated with 1 mM H_2O_2 for 30 min at 37 °C with shaking. Even though the concentration of H_2O_2 used in these experiments is 10 times higher than that used for the killing assays described below, the high bacterial densities used for the isolation of RNA prevented the loss of bacterial viability. Total bacterial RNA was isolated in a bead-beater (Biospec Products, Inc.) as described previously (29) using TRIzol reagent (Invitrogen) containing silicon beads. RNA was extracted with chloroform, precipitated with isopropyl alcohol, washed with ethanol, and dried in a speed vacuum. Pellets were resuspended in RNase-free water and treated with RNase-free DNase (Ambion, Austin, TX). Complimentary cDNA was synthesized at 42 °C using Moloney murine leukemia virus reverse transcriptase (Promega), RNasin (Promega), dNTPs, and random hexamers. cDNA was used as a template for real time and reverse transcription PCR. Transcripts of *rpsM* and *rplN* were normalized for the housekeeping gene *rpoD*.

β-Galactosidase Assays—Strains carrying chromosomal *lacZY* fusions to genes encoding enzymes of pentose phosphate pathway or tricarboxylic acid cycle known to generate reducing power were constructed as described previously (28). To test whether these metabolic pathways are under the control of DksA, the *lacZY* fusions were transduced into the *dksA* mutant strain AV09294. LacZ enzymatic activity was quantified spectrophotometrically in bacterial cultures grown overnight in LB broth as β-galactosidase activity by following the conversion of *o*-nitrophenyl-β-D-galactopyranoside to *o*-nitrophenyl. β-Galactosidase activity is expressed as Miller units calculated according to the equation: $1000 \times ((A_{420\ nm} - 1.75 \times A_{550\ nm})/(T(\text{min}) \times V(\text{ml}) \times A_{600\ nm}))$. Under the experimental conditions used in our assays, the background β-galactosidase activity was ~1 Miller unit.

Western Blotting—An overnight culture of *dksA::*3×FLAG grown in LB broth was diluted to 10^8 cfu/ml and treated for 1 h with 1 mM H_2O_2 in PBS. Samples were taken every 15 min, pelleted, resuspended in lysis buffer, and disrupted by sonication. Samples normalized to 100 ng were resolved using 12% (v/v) SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane, and immunoblotted with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich).

In Vitro Susceptibility to Reactive Oxygen Species—Wild-type and *dksA* mutant bacteria grown overnight in LB broth were diluted in PBS to a final concentration of 10^5 cfu/well. Where indicated, the expression of *zwf* was induced by adding 0.2 μg/ml anhydrotetracycline (Sigma-Aldrich) to bacteria diluted in PBS. Bacteria were challenged with 100 or 250 μM H_2O_2 at

37 °C for 2 h. The cultures were serially diluted in PBS, and the bacteria capable of forming a colony were estimated after overnight culture on LB agar plates. Data are represented as percent survival compared with untreated controls.

H_2O_2 Consumption—Strains grown overnight in LB broth were diluted in prewarmed LB medium to an $A_{600\,nm}$ of 1. H_2O_2 was added to a final concentration of 250 μM. H_2O_2 consumption was measured polarographically using a H_2O_2-specific probe (World Precision Instruments, Sarasota, FL). Data are represented as μM H_2O_2 over time.

Pyridine Nucleotide Quantification—Pyridine nucleotides were extracted and quantified using a modified cycling method described by Husain *et al.* (12). Briefly, oxidized and reduced nucleotides were extracted in 0.2 M HCl or 0.2 M NaOH, respectively, from bacterial cultures grown for 20–22 h in LB medium or diluted in PBS with or without 0.4% glucose (w/v) or 0.1% casamino acids (w/v). Quantification of oxidized and reduced forms of pyridine nucleotides was achieved utilizing a modified thiazolyl tetrazolium blue cycling assay (12) using NADH/NAD^+-specific alcohol dehydrogenase or NADPH/$NADP^+$ glucose-6-phosphate dehydrogenase (30–34). Nucleotide concentrations calculated by regression analysis of known standards were normalized for bacterial density as measured spectrophotometrically at $A_{600\,nm}$. Intracellular nucleotide concentrations were calculated taking into account a bacterial cell volume of 10^{-15} liters (35).

Glutathione Quantification—Total (GSH-GSSG) or oxidized glutathione (GSSG) was extracted and measured using a glutathione recycling method described by Baker *et al.* (36). Samples were prepared by pelleting 250 μl of cultures grown 20–22 h in LB broth. For experiments aimed at measuring the recovery of GSH after oxidative stress, bacterial cultures grown overnight in LB broth were pelleted, resuspended in PBS, and treated with 1 mM H_2O_2. 200 units of catalase were added after 5 min to eliminate the remaining H_2O_2. 250-μl samples collected 5 and 15 min after the addition of catalase were resuspended in an equal volume of 20 mM EDTA. Samples used for the determination of GSSG were resuspended in 20 mM EDTA containing 2 mM *N*-ethylmaleimide. Bacteria were lysed by sonication using a 40-s pulse. One volume of 10% $HClO_4$ was added to the lysates to precipitate proteins. Lysates were neutralized with 93.5 μl of KOH, freeze/thawed, and centrifuged to remove $KClO_4$ and obtain cleared lysates. *N*-Ethylmaleimide was removed by ether extraction. A reaction mixture was freshly prepared by adding 2.8 ml of 1 mM 5,5′-dithiobis (2-nitrobenzoic acid), 3.75 ml of 1 mM NADPH, 5.85 ml of 100 mM NaH_2PO_4-5 mM EDTA phosphate-EDTA buffer, and 20 units of glutathione reductase (Sigma-Aldrich). 100 μl of reaction mixture was immediately added to 50 μl of sample in a 96-well microtiter plate and read at $A_{412\,nm}$ for 5 min. Concentrations were obtained by regression analysis of known standards. Intracellular GSH-GSSG concentrations were calculated taking into account the number of colony-forming units and a bacterial cell volume of 10^{-15} liters.

Mouse Infections—Six- to 8-week old C57BL/6 or congenic gp91*phox*-deficient (37) mice bred in our animal facility according to Institutional Animal Care and Use Committee guidelines were used to assess the role of DksA in *Salmonella* virulence. Briefly, individual animals were inoculated intraperitoneally with ~400 cfu of *Salmonella* grown overnight to stationary phase in LB broth. Mouse survival was monitored over time. Mice manifesting signs of distress (*i.e.* low spontaneous activity and ruffled coat) were humanely euthanized by CO_2 inhalation.

Macrophage Killing Assays—The anti-*Salmonella* activity of macrophages was evaluated as described previously (29). Briefly, peritoneal exudate cells were harvested from C57BL/6 or gp91*phox*-deficient mice 4 days after intraperitoneal inoculation of 1 mg/ml sodium periodate. The cells were cultured for 36–48 h in $RPMI^+$ 1640 medium (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD), 15 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich), and 100 units/ml penicillin and 100 mg/ml streptomycin (Cellgro). Macrophages washed with media lacking antibiotics were challenged at a multiplicity of infection of 2 with *Salmonella* that had been opsonized with 10% normal mouse serum for 20 min. After 25 min of infection, the medium was exchanged with prewarmed $RPMI^+$ 1640 medium containing 25 μg/ml gentamicin. Macrophages were lysed with 0.25% deoxycholic acid (w/v) at the indicated time points after infection and the surviving intracellular bacteria enumerated on LB agar plates. Killing is expressed as the fraction of bacteria recovered at the indicated time relative to the bacterial burden isolated after 25 min of internalization.

Statistical Analysis—The data were analyzed using Student's paired *t* test. Determination of statistical significance between multiple comparisons was achieved using analysis of variance (ANOVA) followed by a Bonferroni post test using transformed data. Data were considered statistically significant when $p < 0.05$.

RESULTS

Hydrogen Peroxide Induces a Stringent Response—Salmonella, as is true for many bacteria, have several defenses with overlapping roles in the detoxification of reactive oxygen and nitrogen species. Published data indicate that the stringent response is induced in *Salmonella* exposed to reactive nitrogen species (38). In this work, we investigated whether H_2O_2, another reactive species produced in the innate response to *Salmonella*, may induce the stringent response. The stringent response is characterized by a down-regulation of translational machinery, including ribosomal protein gene expression. Consequently, transcription of the 30 S ribosomal protein gene *rpsM* was quantified by real time PCR as a readout of stringent regulation. The expression of *rpsM* was decreased ($p < 0.01$) 10-fold in wild-type *Salmonella* exposed to 1 mM H_2O_2, whereas its transcription remained relaxed in a strain lacking the stringent response regulatory protein DksA (Fig. 1*A*). The transcription of the 50 S ribosomal protein *rplN* gene also remained relaxed in H_2O_2-treated *dksA* mutant bacteria (Fig. 1*B*). These findings indicate that H_2O_2 induces DksA-dependent stringent regulation. H_2O_2 did not, however, alter the amount of DksA protein in the cell (Fig. 1*C*). Moreover, the differences in ribosomal protein transcription between wild-type bacteria and the *dksA* mutant do not appear to reflect

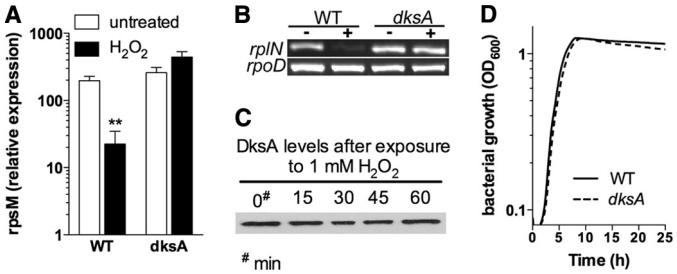

FIGURE 1. **H_2O_2 induces stringent regulation.** *A* and *B*, transcription of the stringently controlled ribosomal protein genes *rpsM* (*A*) and *rplN* (*B*) was evaluated using quantitative real time and reverse transcription PCR, respectively, in wild-type (WT) and *dksA* mutant bacteria. Transcript levels were normalized to the housekeeping σ factor *rpoD*. Bacterial cultures grown overnight in LB medium were subcultured 1:10 for 30 min in PBS with or without 1 mM H_2O_2. The data in *A* represent the mean ± S.E. (*error bars*) from three independent experiments. **, $p < 0.01$. *C*, DksA protein levels were estimated at various times after the bacteria were challenged with 1 mM H_2O_2 in PBS. *D*, bacterial growth in LB medium is shown.

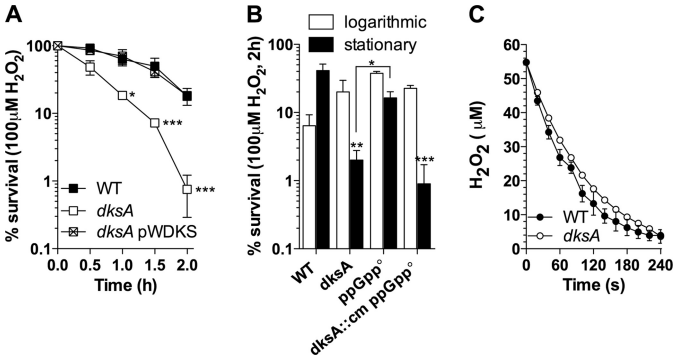

FIGURE 2. ***Salmonella* lacking *dksA* are hypersusceptible to H_2O_2.** *A*, survival of wild type (WT), *dksA* mutant, and *dksA* mutant complemented with a *dksA* gene expressed from the low copy vector pWDKS at various times after exposure to 100 μM H_2O_2. The initial counts of WT and *dksA* mutant at time 0 were ~10^5/well. *B*, killing activity of H_2O_2 against WT *Salmonella* compared with *dksA*, *relA spoT* (ppGpp°), and *dksA* ppGpp° mutants. The bacteria were grown for 20 h (stationary) or to an $A_{600\,nm}$ ~0.5 (logarithmic). *C*, H_2O_2-consuming ability of *Salmonella* grown to stationary phase measured polarographically. The cultures were normalized to bacterial density as determined spectrophotometrically at $A_{600\,nm}$. The data represent the mean percent survival ± S.E. (*error bars*) of 4–12 independent observations from at least two separate experiments. H_2O_2 consumption represents five independent observations from two separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

differences in growth rates as determined in a Bioscreen C growth analyzer (Oy Growth Curves AB Ltd., Helsinki, Finland) (Fig. 1*D*).

DksA Increases the Resistance of Salmonella to Oxidative Stress—Because the *dksA* mutant failed to repress transcription of *rpsM* and *rplN* upon exposure to H_2O_2, we tested whether a lack of *dksA* predisposes this bacterium to reactive oxygen species. H_2O_2, which is generated during the respiratory burst of macrophages in response to *Salmonella* (1), was chosen in these studies. Cytotoxicity assays were initially conducted using 400 μM H_2O_2. However, this concentration killed all *dksA* mutant bacteria. Consequently, lower concentrations of H_2O_2 were tested. The *dksA* mutant exhibited a time-dependent hypersusceptibility to 100 μM H_2O_2 (Fig. 2*A*). Thirty times fewer *dksA*-deficient bacteria survived 2-h exposure to H_2O_2 than did wild-type controls. A wild-type *dksA* allele expressed from the low copy plasmid pWDKS restored the survival of *dksA*-deficient *Salmonella* to levels seen in H_2O_2-treated wild-type bacteria.

TABLE 1

Effect of the *dksA* stringent response locus on growth phase levels of pyridine nucleotide pools

Data are from 8–20 observations recorded in at least three independent experiments. ***, $p < 0.001$ compared with wild-type (WT) bacteria.

Nucleotide pool	Logarithmic		Stationary	
	WT	*dksA*	WT	*dksA*
NADH	257 ± 68[a]	357 ± 92	131 ± 24	87 ± 15***
NAD^+	1632 ± 232	1532 ± 355	731 ± 64	1202 ± 161***
$NADH/NAD^+$	0.158	0.233	0.179	0.073
NADPH	1535 ± 149	1418 ± 240	394 ± 108	280 ± 39***
$NADP^+$	1391 ± 98	1247 ± 173	915 ± 141	1238 ± 230***
$NADPH/NADP^+$	1.103	1.137	0.431	0.227

[a] μM/cell.

At the low concentrations of bacteria used in these studies (*i.e.* 10^5 cfu/well), the decay of H_2O_2 was insignificant over 2 h. The susceptibility of *dksA* mutant bacteria to H_2O_2-mediated killing is growth-dependent. Logarithmically grown *dksA* mutant bacteria were remarkably resistant to H_2O_2, whereas they became highly susceptible upon reaching stationary phase (Fig. 2*B*). The contribution of DksA to the antioxidant defenses of *Salmonella* appears to be independent of the stringent response alarmone ppGpp because a *relA spoT* (ppGpp°) mutant was significantly ($p < 0.05$) less susceptible to H_2O_2 than the isogenic *dksA*-deficient strain. Moreover, a triple mutant lacking *dksA relA spoT* was as susceptible to H_2O_2 as the single *dksA*-deficient *Salmonella* ($p = 0.170$). The hypersusceptibility of *dksA* mutant bacteria to oxidative stress does not appear to be due to a defect in the consumption of H_2O_2 ($p = 0.637$ compared with wild-type bacteria) (Fig. 2*C*).

DksA Controls the Redox Balance of Stationary Phase Salmonella—Recently, it has been suggested that NADH plays a role in resistance of *Salmonella* to reactive oxygen species (12). Consequently, the pools of oxidized and reduced pyridine nucleotides were quantified in *dksA* mutant bacteria. Logarithmically grown wild-type and *dksA*-deficient *Salmonella* harbored similar levels of pyridine nucleotides (Table 1). The reduced NADH and NADPH pyridine pools were nonetheless significantly ($p < 0.001$) lower in stationary phase *dksA* mutant bacteria than in wild-type controls. Conversely, the oxidized pyridine conjugates were increased in the former (Table 1). The dramatic decrease in reducing power seen in stationary phase *dksA*-deficient bacteria raises the possibility that DksA controls central metabolic pathways that supply most of the reducing power in the cell. The majority of the NAD(P)H-reducing equivalents are generated in discrete enzymatic reactions of the pentose phosphate pathway and tricarboxylic acid cycle. To test whether steps associated with the production of reducing equivalents may be under control of DksA, *lacZY* transcriptional fusions were engineered to selected genes. Genes encoding glucose-6-phosphate dehydrogenase (*zwf*), isocitrate dehydrogenase (*icdA*), and malic enzyme (*maeB*) responsible for the generation of NADPH, and the *sucAB*-encoded subunits of α-oxaloacetate dehydrogenase associated with generation of NADH all showed DksA-dependent regulation (Fig. 3*A*, *bold red font*). Several other genes of the pentose phosphate pathway, glycolysis, and tricarboxylic acid cycle such as *gnd*, *aceEF*, and *mdh*, which are also associated with production of reductive power, do not appear to be regulated by DksA.

Mutations were constructed in genes whose expression was decreased 2-fold or more in a *dksA* background. Strains deficient in *sucAB* or *zwf* showed decreases in reductive power compared with wild-type bacteria (Fig. 3, *B* and C). The *sucAB* and *zwf* mutants contained significantly ($p < 0.05$) lower NADPH intracytoplasmic concentrations than wild-type bacteria. These two strains were also hypersusceptible to 100 μM H_2O_2 (Fig. 3*D*). On the other hand, strain AV08257 lacking *icdA* harbored slight increases in NADH/NAD^+ and NADPH/$NADP^+$ ratios and was hyperresistant to H_2O_2 (Fig. 3, *B–D*), whereas the *maeB*-deficient strain was hypersusceptible to H_2O_2. Reduced cycling of metabolites from the tricarboxylic acid cycle into glycolysis may explain the hypersusceptibility of the *maeB* mutant to H_2O_2 because pyruvate is known to be an efficient scavenger of reactive oxygen species (39). Similar to overnight cultures grown in LB medium, the *dksA* mutant strain cultured in PBS for 1 h harbored lower NAD(P)H/$NAD(P)^+$ ratios than wild-type controls (Table 2). The addition of glucose or casamino acids to PBS not only raised the pools of reduced nucleotides but also increased the resistance of *dksA* mutant *Salmonella* to H_2O_2 (Table 2 and Fig. 3*E*). Together, these findings are interpreted as evidence in favor of a model in which carbon fluxes through central metabolic pathways increase the pool of reduced nucleotides that can be used for antioxidant defenses. Nonetheless, excessive carbon fluxing through these metabolic pathways appears to predispose against oxidative stress as shown by the fact that glucose and casamino acids enhanced the susceptibility of wild-type *Salmonella* to H_2O_2 (Fig. 3*E*).

Redox Balance Associated with a Functional DksA Supports the Proper Function of Antioxidant Defenses—Intracellular redox potentials can be calculated with the Nernst equation ($E = E^{\circ}\text{-}\ RT/nF \times \ln(\text{reduced/oxidized})$), where E° is the potential of the redox couple, R is the gas constant (8.3145 $J \cdot K^{-1} \cdot mol^{-1}$), n is the number of electrons transferred in the reaction, and F is Faraday's constant (9.6485×10^4 $C \cdot mol^{-1}$) (40). Using the intracytoplasmic NADPH/$NADP^+$ concentrations found in our studies, we calculated that wild-type and *dksA* mutant bacteria have an intracellular redox environment of −305 and −287 mV, respectively (Fig. 4*A*). Although noticeably lower than wild-type bacteria, the values in the *dksA* mutant should still be thermodynamically favorable for fueling glutathione (GSH) or thioredoxin redox couples with E values of −240 and −270 mV, respectively (41, 42). Given this potential paradox, we investigated whether the decreased reductive power seen in *dksA* mutant *Salmonella* may be of consequence to the redox state of GSH. Compared with wild-type controls grown to stationary phase in LB broth, the *dksA* mutant had significantly ($p < 0.001$) lower concentrations of reduced GSH, al-

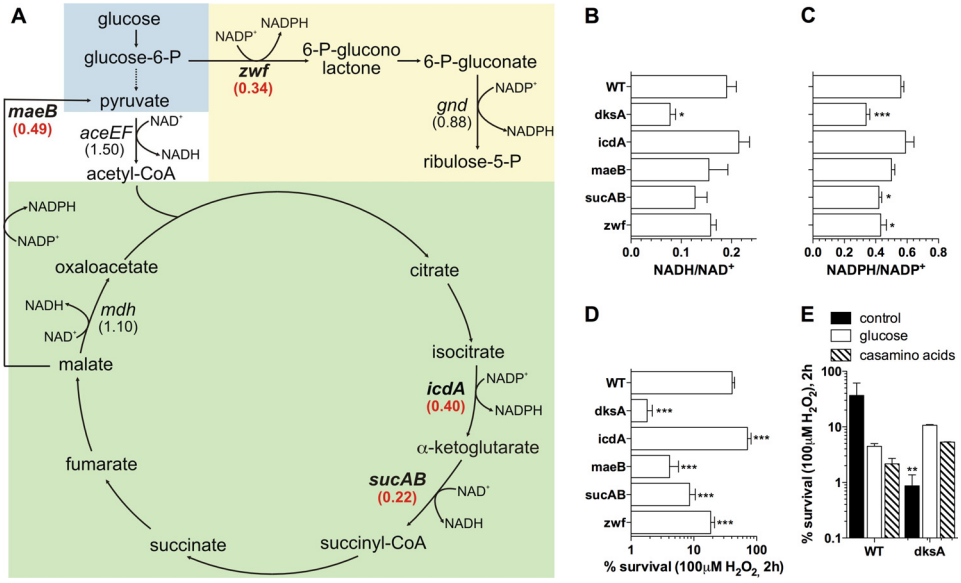

FIGURE 3. **DksA-mediated control of central metabolism boosts cellular reductive power and resistance to H_2O_2.** *A*, expression of key dehydrogenases associated with generation of reduced pyridine nucleotides in the pentose phosphate pathway (*yellow*), glycolysis (*blue*), or the tricarboxylic acid cycle (*green*) was evaluated by following *lacZY* transcriptional activity of the indicated genes. Transcriptional activity was measured in wild-type (WT) *Salmonella* or *dksA* mutant bacteria grown for 20 h in LB broth. The data represent the ratio of β-galactosidase activity supported by the mutant bacteria over WT. Genes expressed 2-fold or lower in the absence of *dksA* are shown in *bold*. The data are the mean of four to nine independent observations from at least two separate experiments. *B* and *C*, NAD(P)$^+$ and NAD(P)H pyridine nucleotides were measured in strains bearing mutations in genes shown to be differentially regulated by DksA. The data are presented as the mean ratio ± S.D. (*error bars*) of reduced to oxidized nucleotide levels from at least 2 separate experiments. *D*, survival of the indicated mutants 2 h after H_2O_2 challenge is shown. *E*, survival of WT and *dksA*-deficient *Salmonella* 2 h after challenge with 100 μM H_2O_2 is shown. Where indicated, PBS was supplemented with 0.4% glucose or 0.1% casamino acids. The data are the mean ± S.E. (*error bars*) from 4–12 independent observations from at least two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with WT bacteria.

TABLE 2

Effect of glucose or amino acids on pyridine nucleotide levels in *dksA* mutant *Salmonella*

Data are from 12–16 independent observations recorded in at least four separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The p values in the control group are comparisons between wild-type (WT) and *dksA* mutant. The p values in the glucose and casamino acids groups are comparisons with the corresponding PBS control.

Nucleotide pool	Control		Glucose		Casamino acids	
	WT	*dksA*	WT	*dksA*	WT	*dksA*
NADH	90 ± 21[a]	49 ± 8*	228 ± 8***	243 ± 35***	234 ± 46**	250 ± 92**
NAD^+	690 ± 195	1089 ± 218**	942 ± 121	971 ± 122	710 ± 79	1111 ± 155
NADH/NAD^+	0.130	0.045	0.242	0.250	0.330	0.225
NADPH	410 ± 89	310 ± 80**	446 ± 114	815 ± 228**	620 ± 43**	707 ± 103**
$NADP^+$	1026 ± 87	1390 ± 254*	485 ± 87	570 ± 148**	456 ± 97***	567 ± 90***
NADPH/$NADP^+$	0.399	0.223	0.922	1.430	1.361	1.248

[a] μM/cell.

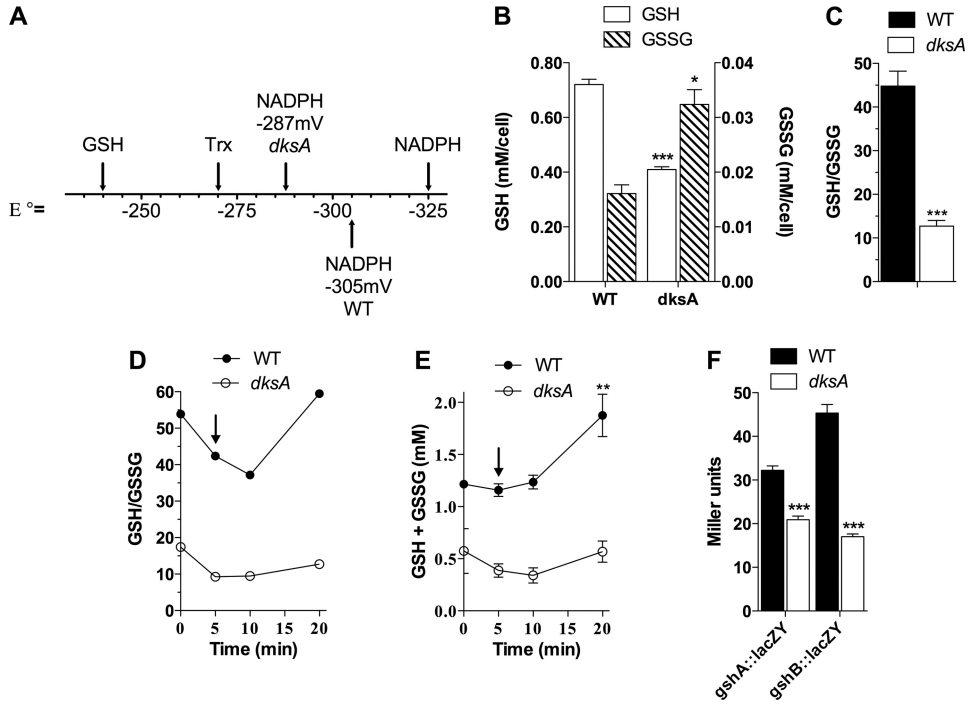

FIGURE 4. ***dksA*-deficient *Salmonella* exhibit a slow turnover of oxidized glutathione.** *A*, redox potentials in wild-type (WT) and *dksA* mutant *Salmonella* were calculated according to the Nernst equation factoring NADPH/$NADP^+$ intracellular concentrations of stationary phase bacterial cultures. Redox potentials of NADPH, glutathione (GSH), and thioredoxin (Trx) are shown for reference. *B*, concentrations of reduced GSH and oxidized GSH (GSSG) were quantified in bacterial cultures grown for 18–20 h in LB broth. *C*, GSH/GSSG ratio is represented. The data are the mean ± S.D. (*error bars*) of six independent observations from three separate experiments. *D* and *E*, recovery of GSH was studied after the bacteria had been exposed to 1 mM H_2O_2 in PBS. 200 units/ml catalase (*arrows*) were added after 5 min of exposure to H_2O_2. The data are shown as mean GSH/GSSG ratios (*D*) or total (GSH+GSSG) glutathione (*E*) from six independent observations collected in three separate experiments. Differences in GSH+GSSG reported in *B* and *E* likely reflect growth of the bacteria in LB medium or PBS. *F*, expression of genes involved in glutathione synthesis was measured as β-galactosidase activity of the indicated transcriptional fusions after the bacteria were grown for 20 h in LB broth. The data are the mean ± S.E. (*error bars*) of four independent observations from two separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

though it contained higher ($p < 0.05$) pools of oxidized GSH (GSSG) (Fig. 4*B*). Overall, the *dksA* mutant had a 3.5-fold lower GSH/GSSG ratio than wild-type bacteria (Fig. 4*C*). Next, we studied the effect of H_2O_2 on glutathione levels in wild-type and *dksA*-deficient *Salmonella*. These studies were performed with 1 mM H_2O_2, which at the high bacterial densities used to biochemically analyze GSH/GSSG ratios, did not affect bacterial viability. The GSH/GSSG ratio decreased in both wild-type and *dksA*-deficient *Salmonella* upon challenge with H_2O_2 in PBS (Fig. 4*D*), demonstrating that GSH is oxidized in response to H_2O_2. To test the turnover of GSSG, 200 units/ml catalase were added to the bacteria 5 min after 1 mM H_2O_2 treatment. The redox GSH/GSSG balance quickly recovered to resting levels in wild-type *Salmonella* (Fig. 4*D*). This recovery was accompanied by a 2-fold increase in the total glutathione pool (GSH+GSSG) (Fig. 4*E*). In contrast, the GSH/GSSG ratio only recovered partially in *dksA*-deficient *Salmonella*, which exhibited slower kinetics compared with wild-type bacteria. Moreover, its already smaller GSH+GSSG pool dropped by half in the *dksA* mutant 10 min after challenge with 1 mM H_2O_2, rebounding to resting levels 10 min later (Fig. 4*E*). The pool of GSH+GSSG did not decrease appreciably in wild-type bacteria 10 min after exposure to H_2O_2, whereas it increased ($p < 0.01$) by close to 2-fold 10 min after the addition of catalase.

The lower GSH+GSSG pool seen in *dksA* mutant bacteria suggests that *de novo* GSH biosynthesis may also be regulated by DksA. To test this hypothesis, *lacZY* fusions were constructed to the genes *gshA* and *gshB* encoding glutamate-cysteine ligase and glutathione synthetase, respectively. Fig. 4*F* shows that the transcription of both steps in the biosynthesis of GSH, and especially the second step catalyzed by GshB, are under control of DksA. Collectively, these data indicate that DksA does not only help maintain a proper reduced nucleotide pool needed in the recycling of GSSG, but also coordinates the expression of glutathione biosynthetic genes.

Conditional Expression of the Glucose-6-phosphate Dehydrogenase Increases the Resistance of the dksA Mutant to H_2O_2—The majority of NADPH-reducing equivalents are generated in the pentose phosphate pathway by Zwf glucose-6-phosphate and Gnd 6-phosphogluconate dehydrogenases. The step catalyzed by Zwf rate limits the flux of carbon through the pentose phosphate pathway. Consequently, it is not surprising that a mutation in the *zwf* locus lowers cellular reductive power needed for resistance to H_2O_2. We therefore selected *zwf* as a candidate for the conditional complementation of the *dksA* mutation. The *zwf* gene containing its native ribosomal binding site was put under the control of the heterologous tetracycline promoter/operator, generating plasmid pTZWF (Fig. 5*A*). The pTZWF and pTX suicide plasmids containing a unique FRT site were integrated into the *Salmonella* chromosome in an intergenic region downstream of the *putP* gene (43). The constructs were transduced into the *dksA* mutant strain AV09294, generating AV10080 and AV10158, respectively. The induction of pTZWF with 0.2 μg/ml anhydrotetracycline resulted in the production of large amounts of glucose-6-phosphate dehydrogenase as indicated by Coomassie Blue staining of cytoplasmic soluble proteins (data not shown). Conditional expression of *zwf* increased the survival of the *dksA* mutant expressing *put*::pTZWF by 10-fold when treated with 250 μM H_2O_2 (Fig. 5*B*). The increased resistance of the *dksA* mutant expressing *zwf* under the *tet* promoter coincided with lower ($p = 0.0027$) $NADP^+$ (*dksA put*::pTX *versus dksA put*::pTZWF; 1388 ± 7 μM *versus* 1255 ± 26 μM) and higher ($p = 0.0306$) NADPH (*dksA put*::pTX *versus dksA put*::pTZWF; 153 ± 4 μM *versus* 186 ± 12 μM) levels at 30 and 60 min after induction. Consequently, the NADPH/$NADP^+$ ratios increased upon induction with anhydrotetracycline (Fig. 5*C*).

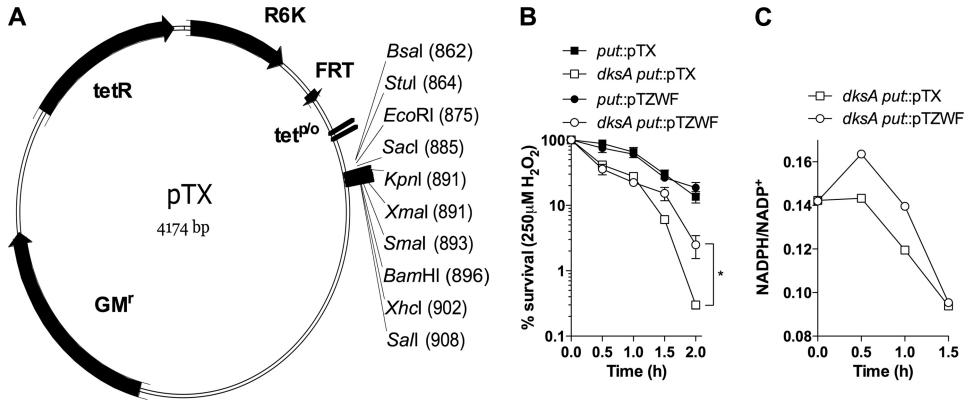

FIGURE 5. **Conditional expression of the glucose-6-phosphate dehydrogenase *zwf* gene boosts reductive power and increases resistance to H_2O_2.** *A*, diagram of the suicide expression vector pTX containing the tetracycline promoter/operator is shown. The position of R6K origin, gentamicin resistance cassette (GM^r), tetracycline repressor (*tetR*), FRT, tetracycline promoter elements (*tet*$^{p/o}$), and multiple cloning site are shown. *B*, pTX empty vector and pTZWF integrated into a neutral site downstream of *putP* were transduced into wild-type and *dksA* mutant bacteria. The bacteria were treated with 0.2 μg/ml anhydrotetracycline at the time of challenge with H_2O_2 in PBS. The data represent the mean percent survival ± S.E. (*error bars*) of three independent observations. *C*, $NADP^+$ and NADPH pyridine nucleotides were measured at the indicated times after the addition of anhydrotetracycline to bacteria cultured in PBS. The data are the mean NADPH/$NADP^+$ ratio of four independent observations collected in 3 separate days. *, $p < 0.05$.

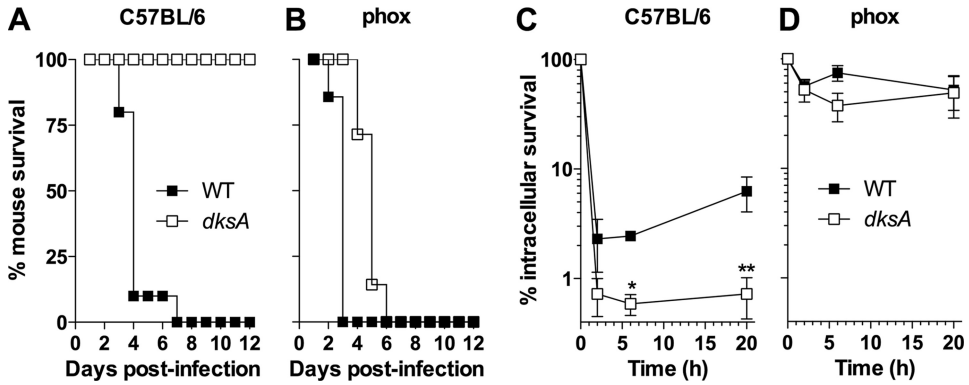

FIGURE 6. **DksA increases fitness of *Salmonella* exposed to the NADPH phagocyte oxidase.** *A* and *B*, C57BL/6 (*A*) and congenic NADPH phagocyte oxidase-deficient (*phox*) (*B*) mice were inoculated intraperitoneally with ~400 cfu of either wild-type (WT) or *dksA*-deficient *Salmonella*. The percent of mice surviving the infection was evaluated over time. The data represent 10 mice/group from two separate experiments. *C* and *D*, the anti-*Salmonella* activity of periodate-elicited peritoneal macrophages isolated from C57BL/6 mice (*C*) or their congenic *phox*-deficient mice (*D*) was evaluated over time. The number of surviving intracellular bacteria was estimated at the indicated times after culture on LB agar plates as described under "Experimental Procedures." The data represent the mean percent survival ± S.E. (*error bars*) of 6–12 independent observations from at least two independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with WT controls.

DksA Contributes to Salmonella Virulence in a Murine Model of Acute Salmonellosis—The induction of stringent regulation by H_2O_2 and the high susceptibility of *dksA* mutant bacteria to H_2O_2 raise the possibility that DksA may play a role in resistance against reactive species generated by NADPH oxidase. To evaluate the role of DksA during acute salmonellosis, C57BL/6 and congenic gp91*phox*-deficient mice were challenged intraperitoneally with wild-type or its isogenic *dksA* mutant *Salmonella* strain AV09294. Bacteria lacking *dksA* were highly attenuated in C57BL/6 mice (Fig. 6*A*). About 8,000 cfu of the *dksA*-deficient strain could be recovered from spleens of infected mice 14 days after inoculation. The isolated bacteria did not grow in E salts minimal medium supplemented with glucose, indicating that suppressor mutations had not developed during the course of the infection. In addition, PCR analysis indicated that the bacteria recovered from viscera of infected mice carried a mutated *dksA* allele. Although wild-type bacteria killed gp91*phox*-deficient mice 2 days earlier than the isogenic *dksA*-deficient *Salmonella*, the latter regained virulence in gp91*phox*-deficient mice (Fig. 6*B*). Together, these data indicate that DksA helps *Salmonella* withstand the innate host response associated with a functional NADPH oxidase.

DksA Mediates Salmonella Fitness inside Professional Phagocytes—The early innate response of macrophages against *Salmonella* is dominated by reactive oxygen species generated through the enzymatic activity of the NADPH phagocyte oxidase. Given the dramatic attenuation of a *dksA* mutant in mice bearing a functional NADPH oxidase, the intracellular survival of wild-type and *dksA* mutant *Salmonella* was compared in primary macrophages. The number of *Salmonella* lacking *dksA* that were isolated from macrophages 2 h after infection was 3-fold lower than that of wild-type bacteria. Differences in bacterial burden of wild-type and *dksA* mutant *Salmonella* augmented at later times in the course of the infection, when wild-type bacteria increased whereas the number of *dksA*-deficient bacteria remained unchanged (Fig. 6*C*). The early disadvantage of *dksA*-deficient *Salmonella* in the intracellular environment of macrophages suggests that DksA helps antagonize the antimicrobial activity emanating from NADPH oxidase. To test this hypothesis, the intracellular survival of wild-type and *dksA* mutant bacteria was studied in gp91*phox*-deficient macrophages. Similar numbers of wild-type and *dksA* mutant bacteria were recovered from gp91*phox*-deficient macrophages (Fig. 6*D*), supporting a role for DksA in defense against products of the respiratory burst.

DISCUSSION

Repression of genes encoding ribosomal proteins, rRNA, and tRNA is a hallmark of the stringent response of bacteria undergoing nutritional deprivation. Findings reported herein indicate that oxidative stress also represses transcription of the *rpsM*- and *rplN*-encoded 30 S and 50 S ribosomal proteins. In analogy to NO (38), the apparent induction of stringent regulation by H_2O_2 may be related to nutritional stress. By modifying redox active centers, NO and H_2O_2 affect a variety of cellular processes. In particular, the [4Fe-4S] prosthetic group of the IlvD dihydroxyacid dehydratase has been shown to react with

both reactive oxygen and nitrogen species, whereas the redox active thiol group of the MetE cobalamin-independent methionine synthase is a primary target of oxidative stress (2, 44). Oxidation of redox active centers in these two proteins is expected to decrease the biosynthesis of branch-chain amino acids and methionine, thereby providing a mechanism for the H_2O_2-dependent induction of the stringent response.

Investigations presented herein indicate that DksA improves the resistance of *Salmonella* to the antimicrobial activity of the NADPH phagocyte oxidase. The antioxidant defenses mediated by DksA in *Salmonella* are functional within macrophages and in an acute model of infection. By exerting transcriptional regulation of central metabolic pathways, DksA controls the production of reducing power that helps detoxify oxyradicals and repair biomolecules damaged by reactive oxygen species. Our investigations indicate that the antioxidant defenses associated with DksA are mostly independent of the traditional stringent response alarmone ppGpp. This is in keeping with the idea that both DksA and ppGpp can have different modes of action and can compensate for amino acid auxotrophies, cell-cell aggregation, motility, filamentation, and stimulation of RpoS accumulation when the other is absent (24). As is the case for the latter examples, further studies are needed to delineate the mechanisms by which DksA regulates redox homeostasis independently of ppGpp.

Bacteria lacking DksA exhibit a normal rate of consumption of H_2O_2 at concentrations of the reactive species frequently seen in inflammatory processes. Thus, DksA does not appear to have a major effect on high rates of H_2O_2 detoxification. In contrast, DksA helps regulate the production of reducing power in stationary phase bacteria. Transcriptional analysis has revealed that DksA enhances the expression of certain steps of the pentose phosphate pathway and tricarboxylic acid cycle that generate NAD(P)H. DksA was found to fine-tune the transcription of *zwf*, *icdA*, *sucAB*, and *maeB*. Conditional expression of *zwf* encoding the glucose-6-phosphate dehydrogenase boosted the redox potential and antioxidant defenses of *dksA* mutant bacteria. Given the fact that DksA plays a global regulatory role and is involved in the transcription of many steps of central metabolism, we found it remarkable that the conditional expression of *zwf* has such a profound effect on the resistance to H_2O_2 in the *dksA* mutant. The resistance to H_2O_2 associated with the heterologous expression of *zwf* was nonetheless delayed 30 min after NADPH accumulated in the bacteria, suggesting that NADPH fuels downstream enzymes. Together, the work presented herein provides evidence in favor of a model in which the generation of reductive power through the DksA-mediated control of central metabolic pathways boosts antioxidant defenses.

Application of the Nernst equation to the calculated intracellular concentrations of the NADPH/$NADP^+$ redox couple indicates that the intracellular environment of *dksA*-deficient bacteria is −287 mV compared with −305 mV of wild type. The diminished redox power of the *dksA* mutant should still be thermodynamically favorable for fueling glutathione and thioredoxin reductases. Nonetheless, the cytoplasmic environment of *dksA*-deficient *Salmonella* is poor in reduced GSH, whereas it harbors high concentrations of GSSG. This observation suggests that the reduced nucleotide pool in *dksA* mutant bacteria is indeed detrimental to downstream antioxidant defenses, including the glutathione oxidoreductase. Further supporting this idea, the *dksA* mutant exhibits lower rates in the reduction of GSSG than wild-type bacteria. The pool of ~200 μM NADPH in *dksA* mutant *Salmonella* is still above the estimated K_m of 20 μM that glutathione reductase has for NADPH (45). According to Michaelis-Menten kinetics, the velocity of an enzymatic reaction is dependent on substrate level ($V = V_{max}[S]/K_m + [S]$, where V is velocity, V_{max} is maximal velocity, and S is substrate concentration). Considering V_{max} and K_m constant and taking into account the large pool of GSSG in the *dksA* mutant, it follows that limited NADPH availability likely drives the slow reduction of GSSG seen in this mutant. Collectively, these findings indicate that low NAD(P)H/$NAD(P)^+$ ratios negatively affect the antioxidant defenses of *dksA*-deficient bacteria.

In addition to low pools of NADPH and NADH, the *dksA* mutant contains low levels of reduced GSH. This tripeptide is synthesized from cysteine, glutamate, and glycine. Auxotrophies for several amino acids, including glycine, have been described for *dksA* mutant bacteria (46). Therefore, the reduced transcription of glutathione synthase reported herein and limited glycine availability likely underlie the low GSH pool in the *dksA* mutant. The defect in GSH biosynthesis might shift the prime targets of oxidative stress from the tripeptide to more essential biomolecules. This idea is supported by the observed drop in measurable GSH+GSSG (possibly through the formation of mixed disulfides) when *dksA* mutant bacteria were challenged with H_2O_2. A defect in GSH biosynthesis might aggravate susceptibility to oxidative stress of nutritionally deprived bacteria because they appear to synthesize GSH *de novo* upon treatment with H_2O_2. Because of the global role for DksA in the regulation of gene transcription, it is still possible that DksA controls the expression of other antioxidant defenses as well.

Control of antioxidant defenses by DksA is growth-dependent. Rapidly growing, *dksA*-deficient *Salmonella* are even more resistant to H_2O_2 than wild-type bacteria. In contrast, stationary phase *dksA* mutant bacteria suffer a considerable loss of viability when exposed to H_2O_2. These findings are consistent with the idea that DksA exerts transcriptional control in stationary phase when nutrients become limited. Accordingly, the addition of carbon in the form of glucose or amino acids enhances both the reductive power and the resistance of *dksA*-deficient *Salmonella* to H_2O_2. The pool of reduced pyridine nucleotides must nonetheless be tightly controlled to minimize collateral damage. On one hand, NADH and NADPH reductive power fuels the antioxidant activities of alkyhydroperoxidase, and glutathione and thioredoxin reductases. On the other, NADH can act as a pro-oxidant that feeds reducing equivalents to flavoproteins, which can adventitiously generate the Fenton catalyst ferrous iron (47). Accordingly, experiments presented herein show that glucose and amino acids increase the cytotoxicity of H_2O_2 against stationary phase wild-type *Salmonella*. Therefore, the fine-tuning of central metabolism by DksA during periods of nutritional starvation helps maintain a delicate balance of reduced nucleotides for use in antioxidant defenses

while limiting excessive buildup of reducing power that could generate undesired damage.

In conclusion, our investigations have elucidated a novel role for DksA in the antioxidant defenses of *Salmonella*. The regulation of central metabolic pathways by DksA coordinates the supply of reducing power that fuels antioxidant defenses crucial for resistance of *Salmonella* to reactive oxygen species produced during the innate host response.

Acknowledgments—We thank Dr. Maroof Husain and Chad Austin for technical assistance. We gratefully acknowledge receiving the pASK75 plasmid from Dr. Arne Skerra, and we thank Dr. Jessica Jones-Carson for supplying the mice used in the course of these investigations and for comments in the preparation of this manuscript.

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