IAI
Journals.ASM.org

DksA-Dependent Resistance of *Salmonella enterica* Serovar Typhimurium against the Antimicrobial Activity of Inducible Nitric Oxide Synthase

Calvin A. Henard and Andrés Vázquez-Torres

Department of Microbiology, University of Colorado School of Medicine, Aurora, Colorado, USA

In coordination with the ppGpp alarmone, the RNA polymerase regulatory protein DksA controls the stringent response of eubacteria, negatively regulating transcription of translational machinery and directly activating amino acid promoters and *de novo* amino acid biosynthesis. Given the effects of nitric oxide (NO) on amino acid biosynthetic pathways and the intimate relationship of DksA with amino acid synthesis and transport, we tested whether DksA contributes to the resistance of *Salmonella* to reactive nitrogen species (RNS). Our studies show that the zinc finger predicted to position DksA in the secondary channel of the RNA polymerase is essential for the resistance of *Salmonella enterica* serovar Typhimurium to RNS in a murine model of systemic salmonellosis. Despite exhibiting auxotrophies for various amino acids, Δ*dksA* mutant *Salmonella* strains regain virulence in mice lacking inducible NO synthase (iNOS). DksA is also important for growth of this intracellular pathogen in the presence of NO congeners generated by iNOS during the innate response of murine macrophages. Accordingly, *dksA* mutant *Salmonella* strains are hypersusceptible to chemically generated NO, a phenotype that can be prevented by adding amino acids. The DksA-dependent antinitrosative defenses do not rely on the Hmp flavohemoprotein that detoxifies NO to NO_3^- and appear to operate independently of the ppGpp alarmone. Our investigations are consistent with a model by which NO produced in the innate response to *Salmonella* exerts considerable pressure on amino acid biosynthesis. The cytotoxicity of NO against *Salmonella* amino acid biosynthetic pathways is antagonized in great part by the DksA-dependent regulation of amino acid biosynthesis and transport.

Intracellular pathogens are exposed to a plethora of defense mechanisms within host cells. *Salmonella enterica* cells residing in macrophages encounter a burst of reactive oxygen and nitrogen species generated through the enzymatic activities of NADPH phagocyte oxidase and inducible nitric oxide (NO) synthase (iNOS) hemoproteins. Macrophages express iNOS in response to lipid A, fimbriae, and porins decorating the *Salmonella* cell envelope (6, 42, 43). The proinflammatory cytokine gamma interferon (IFN-γ) further enhances the nitrosative potential of *Salmonella*-infected mononuclear phagocytic cells (28, 41). NO or reactive nitrogen species (RNS) engendered through the reaction of this diatomic radical with molecular oxygen (O_2), low-molecular-weight thiols, iron, or superoxide (O_2^-) modify several bacterial biomolecules, including terminal cytochromes of the electron transport chain, DNA, and DNA binding metalloproteins, as well as [Fe-S] cluster- and thiol-containing enzymes of intermediary metabolism (20, 35). The RNS-mediated inhibition of respiration, central metabolism, and DNA replication is a likely cause of the growth arrest experienced by *Salmonella* exposed to nitrogen oxides (14). The enzymatic activity of iNOS plays an active role in the anti-*Salmonella* arsenal of rodent and human mononuclear phagocytes (38, 41) and in host defense against systemic and oral salmonellosis (1, 17, 26).

Salmonella expresses multiple defenses against RNS encountered in the host. The *Salmonella* pathogenicity island 2 type III secretion-dependent avoidance of iNOS-containing vesicles and DNA damage repair and scavenger systems all increase the antinitrosative defenses of *Salmonella* (2, 7, 36). In addition, the flavohemoprotein Hmp, which dinitrosylates NO to nitrate, serves as a primary defense against nitrosative stress generated by iNOS (2). Hmp protects the respiratory activity of terminal cytochromes of the electron transport chain (37), increases the resistance of *Salmonella* against NO congeners produced by human and murine macrophages, and reduces *S*-nitrosoglutathione formation (9, 38), thereby fostering *Salmonella* virulence (2). Due to the selective pressure imposed by products of iNOS against *Salmonella*, this intracellular pathogen must count on a rich regulatory network to fine-tune the expression of antinitrosative defenses. Multiple *Salmonella* regulators respond to RNS. For example, NsrR and NorR are dedicated sensors of NO, and the transcriptional regulators OxyR, SoxR, fumarate-nitrate reduction (FNR), and Fur, which primarily function as sensors of O_2, reactive oxygen species (ROS), and iron, also respond to RNS (10, 12, 15, 18). To sense nitrogen oxides, these regulatory proteins use cysteine residues, non-heme iron centers, and [Fe-S] clusters (10, 12, 15, 18). Nitrosylation of cysteines or iron cofactors in OxyR, SoxR, and NorR activates transcription, whereas the iron-nitrosyl complexes in NsrR and Fur derepress gene expression (12, 13, 15, 18, 39).

Recent evidence indicates that the addition of amino acids to growth media prevents the bacteriostatic actions of NO against *Salmonella*, suggesting that inhibition of amino acid biosynthesis

Received 12 December 2011 Returned for modification 31 December 2011
Accepted 28 January 2012
Published ahead of print 6 February 2012
Editor: A. J. Bäumler
Address correspondence to Andrés Vázquez-Torres, andres.vazquez-torres@ucdenver.edu.

doi:10.1128/IAI.06316-11

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
S. Typhimurium ATCC 14028s	Wild type	ATCC
AV03005	Δ*hmp*::*lacZY*	27
AV04068	Δ*hmp*::FRT	27
AV08140	Δ*relA*::FRT Δ*spoT*::FRT	19
AV08174	Δ*dksA*::FRT Δ*hmp*::*lacZY*	This study
AV08261	Δ*dksA*::cm Δ*relA*::FRT Δ*spoT*::FRT	This study
AV09091	Δ*dksA*::cm Δ*hmp*::FRT	This study
AV09294	Δ*dksA*::FRT	19
AV10359	*put*::*dksA*::cm Δ*dksA*::FRT	This study
AV10360	*put*::*dksA*C114S::cm Δ*dksA*::FRT	This study
AV10361	*put*::*dksA*C117S::cm Δ*dksA*::FRT	This study
AV10362	*put*::*dksA*C135S::cm Δ*dksA*::FRT	This study
AV10363	*put*::*dksA*C138S::cm Δ*dksA*::FRT	This study
Plasmids		
pKD3	*bla* FRT *cat* FRT *oriR6K*	11
pKD13	*bla* FRT *ahp* FRT *oriR6K*	11
pCP20	*bla cat* cI857 *flp* pSC101	8
pSK	*bla* pUC *ori* f1 *lacZα*	Stratagene
pSK::cm	*bla cat* pUC *ori* f1 *lacZα*	This study
pSK::*dksA*::cm	*bla cat* pUC *ori* f1 *lacZα dksA*	This study
pSK::*dksA*C114S::cm	*bla cat* pUC *ori* f1 *lacZα dksA*C114S	This study
pSK::*dksA*C117S::cm	*bla cat* pUC *ori* f1 *lacZα dksA*C117S	This study
pSK::*dksA*C135S::cm	*bla cat* pUC *ori* f1 *lacZα dksA*C135S	This study
pSK::*dksA*C138S::cm	*bla cat* pUC *ori* f1 *lacZα dksA*C138S	This study
pCE36	*ahp* FRT *lacZY*$^{+}$ t_{his} *oriR6K*	16

TABLE 2 Primers used for the construction of strains used in this study

Mutation	Primer sequence[a] (5′–3′)
*dksA*C114S	F: TGGAAGATGAAGACTTCGGTTATAGCGAGTCCTGCG
	R: CGCAGGACTCGCTATAACCGAAGTCTTCATCTTCCA
*dksA*C117S	F: TATTGCGAGTCCAGCGGGGTGGAGATT
	R: AATCTCCACCCCGCTGGACTCGCAATA
*dksA*C135S	F: ACAGCCGATCTGAGCATCGACTGCAAAACGCTGGCT
	R: AGCCAGCGTTTTGCAGTCGATGCTCAGATCGGCTGT
*dksA*C138S	F: ACAGCCGATCTGTGCATCGACAGCAAAACGCTGGCT
	R: AGCCAGCGTTTTGCTGTCGATGCACAGATCGGCTGT
pSK::cm	F: ACGCGGATCCATGGGAATTAGCCATGGTCC
	R: CTGCAGGAGCTCGTGTAGGCTGGAGCTGCTTC
pSK::*dksA*::cm	F: ATCGTAGAATTCCGTTGTAGTGGAATAACAGC
	R: CCGCGGATCCTTAACCCGCCATCTGTTTTT
put::*dksA*	F: TAGCGATGGGAGAGAGGACACGTTAATTATTCCATT TTAA
	TAGTGGAATAACAGCCTGATTATTA
	R: TACTGCGGGTATTAATGCTGAAAACATCCATAACCC ATTGGTGT
	AGGCTGGAGCTGCTTC

[a] Underlined sequences denote SacI, BamHI, or EcoRI restriction sites.

may be a key target of the anti-*Salmonella* activity of RNS (35). Work from our laboratory indicates that *Salmonella* cells undergoing nitrosative stress repress transcription of rRNA, tRNA, and other components of translational machinery, all of which are hallmarks of the stringent response often associated with amino acid deprivation (3). The [Fe-S] cluster in the dihydroxy acid dehydratase of the branch chain amino acid biosynthetic pathway is a prime example of a molecular target of RNS (21), as is the lipoamide-dependent lipoamide dehydrogenase (35). A sudden decrease in the availability of amino acids likely triggers ppGpp synthesis and a stringent response. In addition, RNS could elicit a stringent response by targeting the DksA protein that activates amino acid biosynthetic gene transcription (31). In support of this hypothesis, exposure of *Salmonella* to acidified nitrite derepresses DksA-inhibited flagellar gene transcription (3, 22). The investigations presented herein tested whether the RNA polymerase binding protein DksA contributes to the antinitrosative defenses of the enteric pathogen *S. enterica* serovar Typhimurium.

MATERIALS AND METHODS

Bacterial strains. *S. enterica* serovar Typhimurium strain ATCC 14028s was used as the wild type and as a background in the construction of mutants according to the method previously described by Datsenko and Wanner (11) (Table 1). PCR amplicons containing kanamycin or chloramphenicol resistance cassettes flanked by the Flp recognition target (FRT) were generated from a pKD13 or pKD3 template, respectively, using AccuPrime *Taq* high-fidelity polymerase (Invitrogen, Carlsbad, CA) and 60-base-long primers with homology to target genes (Table 2). PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and were electroporated into *S.* Typhimurium strain TT22236 harboring the plasmid pTP223, which expresses an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible λ Red recombinase system (34). 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 (8). In-frame deletions were verified by PCR analysis. To create complementing strains carrying a wild-type or mutated *dksA* allele, we constructed a template plasmid by cloning an FRT-flanked chloramphenicol cassette from pKD3 into the BamHI and SacI restriction sites of pBluescript SK(+) to generate pSK::cm. A DNA region containing the native promoter and the *dksA* open reading frame was cloned between EcoRI and BamHI restriction sites of pSK::cm to generate pSK::*dksA*::cm. *dksA* variants with a serine substitution at position 114, 117, 135, or 138 were generated by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by DNA sequencing. The Δ*dksA* mutant strain AV09294 was complemented ectopically by inserting a P_{dksA}-driven, wild-type *dksA* gene or *dksA* C114S, C117S, C135S, or C138S variant into the intergenic site downstream of the chromosomal *putP* locus using the λ Red recombinase system and the pSK::*dksA*::cm plasmid templates described above (19). The *hmp*::*lacZY* transcriptional fusion was transduced from strain AV03005 (27) into the Δ*dksA* mutant, generating strain AV08174.

Quantification of NO by polarography. Wild-type *Salmonella* cells grown overnight in LB broth were diluted 1:250 in LB broth and treated with 5 mM the NO donor diethylenetriamine (DETA) NONOate, an NO donor with an estimated half-life of 20 h at 37°C, pH 7.4 (Cayman Chemical, Ann Arbor, MI). The amount of NO released into the culture was measured polarographically using an NO-specific probe (World Precision Instruments, Sarasota, FL). The NO concentration was calculated by regression analysis using known standards generated with the NO donor Proli NONOate, which has a half-life of 1.8 s at 37°C and pH 7.4 (Cayman Chemical). Data are represented as μM NO over time.

Susceptibility of *Salmonella* to NO. The cytotoxicity of the NO donor DETA NONOate or the polyamine DETA against wild-type and *dksA*-deficient *Salmonella* strains was monitored spectrometrically by monitoring bacterial growth on a Bioscreen C microbiology microtiter plate (Growth Curves USA, Piscataway, NJ). *Salmonella* cultures grown overnight in LB broth were diluted 1:500 in LB broth and treated with 5 mM the NO donor DETA NONOate or the polyamine DETA control. Some cultures were treated with 1 mM DETA NONOate (see Fig. 4). The half-life of DETA NONOate at neutral pH is about 20 h. Bacterial growth was recorded at an optical density at 600 nm (OD_{600}) every 15 min while cultures were shaken at 37°C.

β-Galactosidase assay. LacZ transcriptional activity was quantified spectrometrically from bacterial cultures treated with 250 μM the NO donor spermine NONOate as β-galactosidase activity by monitoring

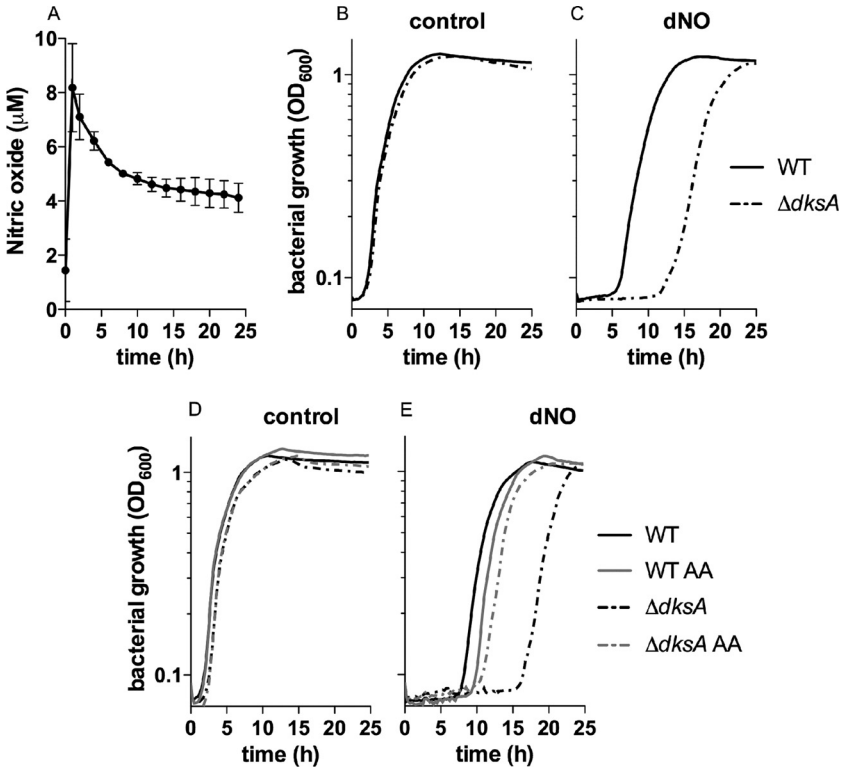

FIG 1 *dksA* mutant *Salmonella* strains are hypersusceptible to the bacteriostatic activity of NO. (A) The concentrations of NO released by 5 mM DETA NONOate (dNO) in LB broth incubated with 2×10^7 CFU/ml of *Salmonella* Typhimurium strain 14028s were measured polarographically over time at 37°C in a shaker incubator. Data from two independent observations are expressed as means ± standard deviations (SD). (B and C) Wild-type (WT) and the isogenic Δ*dksA Salmonella* strain AV09294 grown overnight in LB broth were diluted to 2×10^7 CFU/ml in fresh LB broth and treated with 5 mM polyamine DETA (control) (B) or dNO (C). (D and E) Selected control (D) and dNO-treated (E) cultures were supplemented with 0.1% Casamino Acids (AA). Bacterial growth is expressed as the OD_{600} over time, and the data are represented as the means of 10 independent observations from 2 separate experiments. The *P* value for the difference between the Δ*dksA* and wild-type strains was <0.0001 as determined by one-way ANOVA of all data points in the curve followed by Bonferroni's posttest.

the conversion of *o*-nitrophenyl-β-D-galactopyranoside to ortho-nitrophenyl compared to that of untreated controls. β-Galactosidase activity is expressed as Miller units calculated according to the equation $1{,}000 \times \{[OD_{420} - (1.75 \times OD_{550})]/[(T\ min) \times (V\ ml) \times OD_{600}]\}$. Under the experimental conditions used in our assays, the background β-galactosidase activity is ~1 Miller unit.

Macrophage-killing assays. The anti-*Salmonella* activity of macrophages was evaluated as previously described (27). Briefly, peritoneal exudate cells were harvested from C57BL/6 or iNOS-deficient (24) mice 4 days after intraperitoneal (i.p.) inoculation of 1 mg/ml of sodium periodate. The cells were cultured for 36 to 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, St. Louis, MO), 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Cellgro) ($RPMI^+$). 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 $RPMI^+$ medium containing 25 μg/ml of gentamicin. Macrophages were lysed with 0.25% deoxycholic acid at the indicated time points after infection, and the surviving intracellular bacteria were 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.

Mouse infections. Six- to 8-week-old C57BL/6 or congenic iNOS-deficient (24) 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 followed by cervical dislocation.

Statistical analysis. Determination of the statistical significances of multiple comparisons was achieved using one-way analysis of variance (ANOVA) followed by Bonferroni's posttest. Data were considered statistically significant when *P* was <0.05.

RESULTS

DksA mutant *Salmonella* strains are hypersusceptible to the bacteriostatic effects of NO. The expression of DksA enhances the antioxidant defenses of *Salmonella* (19). Due to overlaps in cellular toxicity between ROS and RNS, many defense mechanisms serve a dual role in defending against oxidative and nitrosative stresses (5, 29). Therefore, we evaluated whether DksA contributes to the antinitrosative defenses of *Salmonella*. The sensitivity of *dksA*-deficient *Salmonella* to RNS was tested by assessing bacterial growth in the presence of the NO donor DETA NONOate (Fig. 1). Defects in *dksA* result in auxotrophies for isoleucine, leucine, valine, glycine, phenylalanine, and threonine, and therefore Δ*dksA* strains do not grow in minimal media (4). Consequently, the effect of the slow NO releaser DETA NONOate on the growth of *dksA* mutant *Salmonella* strains was evaluated in bacteria cultured in LB broth. DETA NONOate has a half-life of

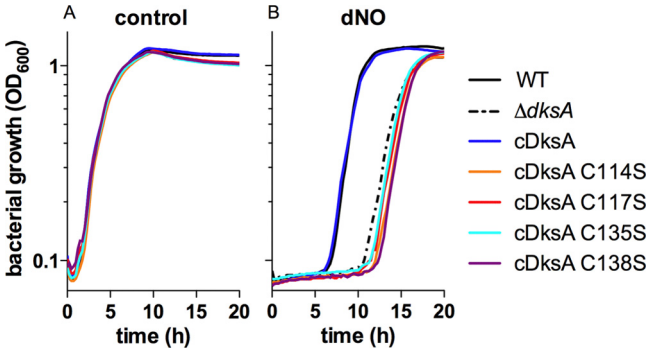

FIG 2 Cysteine residues in the zinc finger motif are critical for DksA-dependent antinitrosative defense. Wild-type (WT), Δ*dksA*, or isogenic Δ*dksA Salmonella* strains complemented with wild-type DksA (cDksA) or a DksA C114S, C117S, C135S, or C138S variant were treated with 5 mM DETA (control) (A) or DETA NONOate (dNO) (B) as described in the legend for Fig. 1. Bacterial growth is expressed as the OD_{600} over time, and the data are represented as the means of 10 independent observations from 2 separate experiments. The *P* value for the difference between the Δ*dksA* and isogenic Δ*dksA Salmonella* strains complemented with DksA C114S, C117S, C135S, or C138S and the wild-type strain was <0.0001 as determined by one-way ANOVA of all data in the curve followed by Bonferroni's posttest.

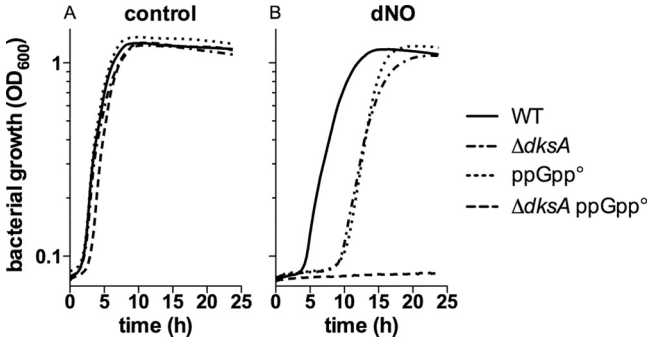

FIG 3 DksA and ppGpp independently contribute to the antinitrosative defense of *Salmonella*. The wild type (WT), the Δ*dksA* strain, and the Δ*relA* Δ*spoT*-deficient *Salmonella* strain lacking the ppGpp synthases (ppGpp°) and a triple Δ*dksA* ppGpp° strain were diluted to 2×10^7 CFU/ml in fresh LB broth from overnight cultures. The cells were treated with 5 mM DETA (control) (A) or DETA NONOate (dNO) (B). Bacterial growth is expressed as the OD_{600} over time, and the data are represented as the means of 10 independent observations from 2 separate experiments. The *P* value for the differences between Δ*dksA* and Δ*dksA* ppGpp° *Salmonella* strains was <0.0001 as determined by one-way ANOVA of all data points in the curve followed by Bonferroni's posttest.

20 h at pH 7. Polarographic determination of the NO generated with 5 mM DETA NONOate under the culture conditions used in our assays indicated an 8 μM NO peak shortly after exposure, followed by a steady 5 μM NO for the duration of the experiment (Fig. 1A). Wild-type and isogenic Δ*dksA Salmonella* strains exhibited similar growth in LB broth supplemented with 5 mM DETA (Fig. 1B). The Δ*dksA* mutant *Salmonella* strain AV09294 was hypersusceptible to the bacterostatic effects of 5 mM DETA NONOate, as shown by its 12-h lag compared to 5 h for the wild-type control (Fig. 1C). Given the inhibitory effects of NO on amino acid biosynthetic pathways and the DksA-dependent regulation of the transcription of amino acid synthesis and transport, we tested whether the addition of amino acids had any effect on the marked susceptibility of Δ*dksA* mutant *Salmonella* strains to chemically generated NO. The addition of 0.1% Casamino Acids did not seem to affect the growth of wild-type or Δ*dksA* mutant bacteria in LB broth (Fig. 1D). However, supplementation of LB broth with 0.1% Casamino Acids abrogated most of the hypersusceptibility of *dksA*-deficient *Salmonella* strains to NO (Fig. 1E). These data suggest that DksA contributes to antinitrosative defenses by *Salmonella*, possibly by fine-tuning the expression of amino acid biosynthetic pathways and transport.

The cysteine residues in the DksA zinc finger are crucial for antinitrosative function. The DksA protein has a zinc finger in its C terminus coordinated by four cysteine residues (32). Because no function has been ascribed to the zinc finger of DksA and because zinc fingers are notorious for their reactivity with reactive oxygen and nitrogen species, we tested whether the zinc finger is necessary for the antinitrosative defenses associated with DksA. *Salmonella* strains expressing DksA variants with a single serine substitution at position 114, 117, 135, or 138 were evaluated for susceptibility to NO (Fig. 2B). All four coordinating cysteine residues were found to be critical for DksA-mediated defense against the bacteriostatic effects of NO. All strains showed similar growth in LB broth (Fig. 2A). These data suggest that the integrity of the zinc finger motif is needed for the contribution of DksA to the antinitrosative defenses of *Salmonella*.

DksA and ppGpp independently increase *Salmonella* resistance to nitrosative stress. DksA is thought to regulate RNA polymerase function in conjunction with the alarmone ppGpp (30, 32). The ppGpp synthases RelA and SpoT produce ppGpp in response to nutrient deprivation (33). We examined the effect that NO has on the growth of *Salmonella* strains lacking both RelA and SpoT ppGpp synthases (ppGpp°) and/or *dksA*. All strains grew with apparently similar kinetics in LB broth (Fig. 3A). A ppGpp° *Salmonella* strain and a *dksA*-deficient *Salmonella* strain displayed the same increased lag phase after treatment with NO as the wild-type isogenic controls. However, the triple *dksA relA spoT* mutant did not resume growth even after 24 h of NO treatment (Fig. 3B). Strain AV08261 lacking *relA*, *spoT*, and *dksA* did not lose viability under these experimental conditions (data not shown), indicating that the hypersusceptibility of this triple mutant to NO cannot be rationalized by direct killing by RNS. The marked susceptibility of strain AV08261 to NO could be interpreted as a sign that DksA and ppGpp contribute independently to the antinitrosative defenses of *Salmonella*.

Independent contributions of DksA and Hmp to antinitrosative defenses of *Salmonella*. Δ*dksA* mutant bacteria grown 24 h in the presence of 5 mM DETA NONOate became resistant to a subsequent challenge with NO (data not shown). The increased resistance of a Δ*dksA* mutant *Salmonella* strain preexposed to NO likely reflects an adaptive response rather than the selection of suppressor mutants, as indicated by the failure of these bacteria to grow in E salts medium (1.66 mM $MgSO_4$, 9.5 mM citric acid monohydrate, 57 mM K_2HPO_4, 16.7 mM $NaHH_3PO_4$) supplemented with 0.4% glucose. Moreover, NO-treated Δ*dksA* mutant *Salmonella* strains became hypersusceptible to 5 mM DETA NONOate after overnight growth to stationary phase in fresh LB broth. By detoxifying NO to NO_3^-, the Hmp flavohemoprotein serves a critical role in the antinitrosative defense of *Salmonella* (2). Because DksA indirectly regulates gene transcription by interacting with the RNA polymerase, we tested whether the Hmp flavohemoprotein may have played a role in the adaptive response of Δ*dksA* mutant *Salmonella* strains pretreated with NO. Toward this

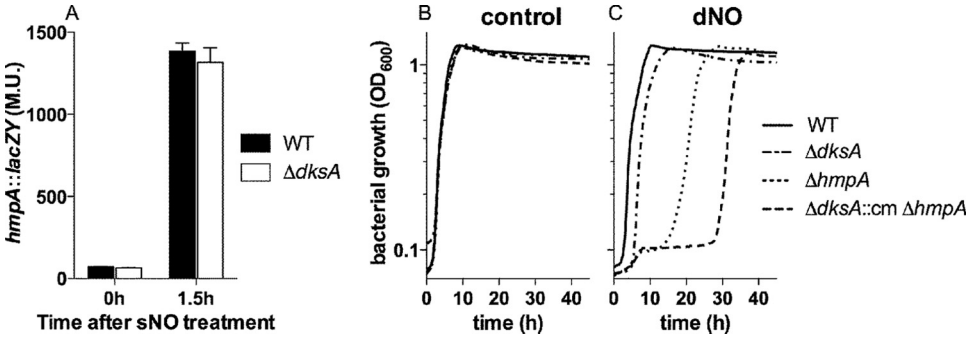

FIG 4 The antinitrosative role of DksA is independent of Hmp. (A) Transcription of the *hmp*::*lacZY* transcriptional fusion 1.5 h after wild-type (WT) and isogenic Δ*dksA* mutant *Salmonella* strains were treated with 250 μM spermine NONOate (sNO). The data are presented as Miller units (M.U.) ± standard errors of the means (SEM) of four observations collected in two independent experiments. The addition of 5 mM DETA NONOate (dNO) completely inhibited the growth of *hmp*-deficient *Salmonella* for 48 h. Consequently, 1 mM DETA (control) (B) or dNO (C) was instead tested in these experiments. At this concentration of dNO, Δ*dksA* mutant *Salmonella* strains were slightly more susceptible than the WT controls. Data represent the means of six independent observations obtained from two separate experiments. The *P* value for the difference between the Δ*hmpA* and Δ*dksA*::*cm* Δ*hmpA* mutant strains was <0.0001 as revealed by one-way ANOVA of all data points in the curve followed by Bonferroni's posttest.

end, an *hmp*::*lacZY* chromosomal fusion was transduced into the Δ*dksA* mutant *Salmonella* strain AV09294. Transcription of the *hmp* gene was induced ~20-fold in both wild-type and *dksA*-deficient *Salmonella* strains 1.5 h after treatment with 250 μM the NO-donor spermine NONOate (Fig. 4A), suggesting that DksA is not involved in the regulation of *hmp* transcription. The wild-type, Δ*dksA*, Δ*hmp*, and Δ*dksA*::*cm* Δ*hmp* strains showed similar growth in LB broth (Fig. 4B). Treatment of *hmp*-deficient *Salmonella* with 5 mM DETA NONOate resulted in a lag phase of about 48 h (data not shown), making this concentration of DETA NONOate impractical to compare the relative contributions of Hmp and DksA to the resistance of *Salmonella* to nitrosative stress. Consequently, the concentration of NO was decreased to levels that make the differences between wild-type and Δ*dksA* mutant *Salmonella* practically unnoticeable but could still elucidate DksA and Hmp synergism. *Salmonella* strain AV04064 lacking the flavohemoprotein-encoding *hmp* gene remained hypersusceptible to 1 mM DETA NONOate, whereas the *dksA*-deficient strain was slightly more susceptible than its parent, the wild-type control (Fig. 4C). Interestingly, the double Δ*dksA*::*cm* Δ*hmp* mutant strain AV09091 showed increased susceptibility to NO compared to single isogenic mutant controls, further supporting the idea that Hmp and DksA independently contribute to the antinitrosative defenses of *Salmonella*.

DksA fosters intracellular survival of *Salmonella* within NO-producing macrophages. RNS generated by the iNOS hemoprotein plays a role in the innate response of macrophages against *Salmonella* (41). Therefore, the contribution of DksA to the antinitrosative defenses of *Salmonella* was evaluated further in primary macrophages expressing a functional iNOS. Compared to the wild-type parent strain, the *dksA*-deficient *Salmonella* strain was hypersusceptible to the early phases of the anti-*Salmonella* activity of macrophages from C57BL/6 mice (Fig. 5A). These findings are consistent with the known hypersusceptibility of *dksA* mutant *Salmonella* strains to oxidative products generated by the NADPH phagocyte oxidase-dependent respiratory burst of macrophages (19). Interestingly, later during the course of infection, the viability of the Δ*dksA* mutant *Salmonella* strain continued to decline, while the wild-type strain increased in number (Fig. 5A). Together, these findings suggest that in addition to being susceptible to ROS generated by the NADPH phagocyte oxidase, *dksA* mutant *Salmonella* strains are hypersusceptible to nitrogen oxides generated by iNOS expressed at later times in infection. To test whether DksA-dependent gene expression antagonizes the anti-*Salmonella* activity of iNOS-expressing macrophages, we studied the intracellular survival of wild-type and *dksA*-deficient *Salmonella* strains in macrophages lacking iNOS (Fig. 5B). iNOS-deficient macrophages showed an even greater difference in the numbers of Δ*dksA* and wild-type *Salmonella* cells recovered at early times of infection (Fig. 5B), likely reflecting the higher concentrations of ROS produced by macrophages lacking the iNOS hemoprotein (41). Later during the course of infection, the Δ*dksA* mutant strain displayed signs of growth in iNOS-deficient macrophages, suggesting that in the absence of NO, *dksA*-deficient *Salmonella* can grow intracellularly (Fig. 5B). Collectively,

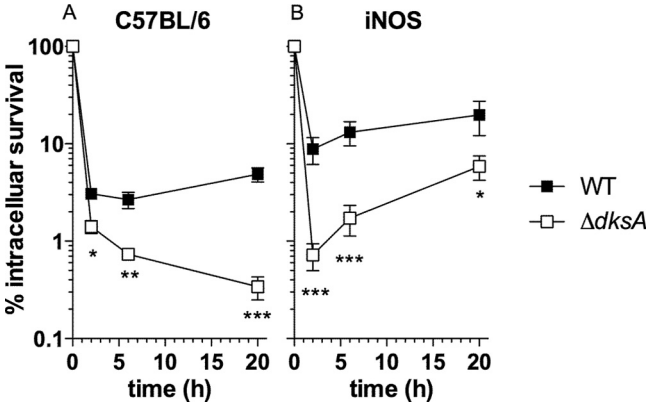

FIG 5 Role of DksA in the intracellular survival of *Salmonella* in iNOS-expressing macrophages. The antimicrobial activities of periodate-elicited peritoneal macrophages isolated from C57BL/6 mice (A) or congenic iNOS-deficient controls (B) were evaluated over time after mice were challenged with the wild-type (WT) or the Δ*dksA* mutant *Salmonella* strain. The number of surviving intracellular bacteria was estimated after culture on LB agar plates as described in Materials and Methods. The data represent the mean percent survival ± the SEM of four to eight independent observations from at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to results for WT controls.

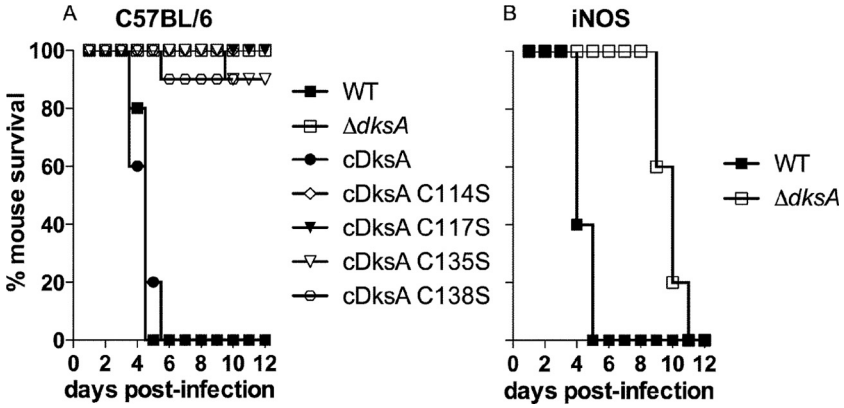

FIG 6 DksA protects *Salmonella* from nitrogen oxides produced by a functional iNOS hemoprotein in a murine model of salmonellosis. (A) C57BL/6 mice were inoculated intraperitoneally with ~200 to 400 CFU of either the wild-type (WT), Δ*dksA*, or Δ*dksA* mutant *Salmonella* strain complemented with a wild-type *dksA* allele (cDksA) or with a *dksA* C114S-, C117S-, C135S-, or C138S-expressing variant. (B) Congenic iNOS-deficient mice were inoculated intraperitoneally with ~400 CFU of either the WT or Δ*dksA* mutant *Salmonella* strain. The fractions of mice surviving the experimental *Salmonella* infections were evaluated over time. Data represent 10 mice per group and were obtained from two separate experiments.

these findings underscore the importance of DksA-mediated antioxidant and antinitrosative defenses for the intracellular fitness of *Salmonella*.

DksA antagonizes the cytotoxicity of iNOS in mice. Several groups of investigators have shown that DksA is crucial for *Salmonella* virulence in mice (19, 40, 44). In agreement with these observations, a Δ*dksA* mutant *Salmonella* strain was attenuated in a C57BL/6 murine model of acute systemic infection (Fig. 6A). The Δ*dksA* mutation could be complemented with a P_{dksA}-*dksA* wild-type allele expressed ectopically downstream of the *putP* chromosomal locus but not by P_{dksA}-*dksA* cysteine variants, suggesting that the integrity of the zinc finger in DksA is critical for *Salmonella* pathogenesis. Given the hypersusceptibility of the Δ*dksA* mutant *Salmonella* strain to the cytotoxic effects of NO and its attenuation in iNOS-sufficient macrophages, we tested whether antinitrosative defenses associated with the expression of a functional DksA promote *Salmonella* virulence in an acute model of infection. To test this hypothesis, iNOS-deficient mice were challenged intraperitoneally with ~400 CFU of the wild-type or isogenic Δ*dksA* mutant *Salmonella* strain. The *dksA*-deficient strain regained virulence in iNOS-deficient mice; strain AV09294 killed iNOS-immunodeficient mice 6 days later than the isogenic wild-type controls (Fig. 6B). Since NADPH oxidase contributes to early anti-*Salmonella* activity, it is possible that the marked susceptibility of Δ*dksA* mutant bacteria to oxyradicals produced by NADPH phagocyte oxidase may have contributed to the delayed virulence of this strain in iNOS-deficient mice. All *Salmonella* cells recovered from the spleens of moribund iNOS-deficient mice that had been inoculated i.p. 8 days earlier with Δ*dksA* mutant bacteria remained auxotrophic for amino acids, as determined by their inability to grow in E salts minimum medium supplemented with 0.4% glucose. Moreover, the bacteria recovered from iNOS-deficient mice contained the Δ*dksA* mutant allele, as determined by PCR analysis. Together, these findings indicate that in the absence of the amino acid biosynthetic restrictions imposed by nitrosative stress, the phagosomal lumen harbors sufficient amino acids to satisfy the auxotrophies of the Δ*dksA* mutant *Salmonella* strain.

DISCUSSION

We previously demonstrated that both oxyradicals and nitrogen oxides produced in the innate response of macrophages activate a stringent DksA-dependent response (3, 19). Furthermore, the RNA polymerase regulatory protein DksA plays a crucial role in the expression of antioxidant defenses of *Salmonella* (19). Therefore, we evaluated the role of the DksA protein in the antinitrosative defenses of *Salmonella*. The investigations presented herein indicate that DksA is critical for the ability of *Salmonella* to resist NO-dependent antimicrobial defenses. DksA increases *Salmonella* resistance to the bacteriostatic effects of NO, and DksA-mediated antinitrosative defenses support intracellular growth of *Salmonella* strains within iNOS-sufficient macrophages while fostering virulence in an acute model of infection dependent on the presence of a functional iNOS hemoprotein. The phagosomal lumen is a nutrient-limiting environment. By inhibiting amino acid biosynthetic pathways, NO produced in the innate response of macrophages to *Salmonella* appears to further restrict the availability of nutrients. According to this model, the regulatory effects of DksA on amino acid biosynthetic synthesis and transport greatly reduce the NO-dependent antimicrobial activity seen in the innate immune response of professional phagocytes. However, in the absence of the iNOS hemoprotein and the consequent burst in nitrosative stress, amino acids must not be a limiting factor in the *Salmonella* phagosome, since auxotrophic Δ*dksA* mutant *Salmonella* strains do in fact replicate in iNOS-deficient macrophages and kill iNOS-deficient mice.

The DksA metalloprotein contains a C-terminal zinc finger coordinated by four cysteine residues (30, 44). Our studies indicate that the DksA zinc finger is critical for the antinitrosative function of the protein. According to a model of the RNA polymerase, ppGpp, and the DksA ternary complex, the globular domain containing the DksA zinc finger is needed for proper positioning of the protein within the secondary channel of the RNA polymerase (32). The zinc finger is likely necessary for the interaction of the acidic residues at the tip of the coiled-coil domain of DksA with the Mg^{2+} ion bound to the distal diphosphate of the ppGpp molecule inside RNA polymerase (30). Mutations in any

of the DksA zinc finger cysteine residues abolish its ability to coordinate antinitrosative defenses, likely due to improper positioning of DksA within the RNA polymerase secondary channel. In accordance with this hypothesis, a mutation in a single cysteine within the *Escherichia coli* DksA zinc finger results in a relaxed phenotype similar to that of *dksA*-deficient bacteria (30).

ppGpp° and *dksA* mutant *Salmonella* strains exhibit similar susceptibility to NO, suggesting that both stringent-response mediators contribute to the antinitrosative defenses of *Salmonella*. However, the hypersusceptibility of the triple mutant indicate that ppGpp and DksA may have independent roles in NO defense. In fact, ppGpp and DksA regulate a variety of cellular processes, including motility, adhesion, autoaggregation, and filamentation, independently (22, 25). Moreover, DksA independently activates the P_{livJ}, P_{his}, and P_{thrABC} promoters critical for amino acid synthesis and transport (25). The fact that DksA can independently increase amino acid biosynthesis genes suggests that this regulatory protein can modulate RNA polymerase activity without ppGpp. Our data could be alternatively interpreted in light of a model by which ppGpp and DksA may be overexpressed in the Δ*dksA* and ppGpp° strains, respectively. Overexpression of *dksA* in ppGpp° *E. coli* can overcome many of the amino acid auxotrophies associated with the Δ*relA* Δ*spoT* mutant (25). The mechanism by which both ppGpp and DksA add to the antinitrosative defenses of *Salmonella* awaits further investigation.

The flavohemoprotein Hmp detoxifies NO and protects *Salmonella* from the iNOS-mediated antimicrobial activity of macrophages (2, 38). Our investigations indicate that DksA does not appear to contribute to *hmp* transcription, and *dksA*-deficient and *hmp*-deficient *Salmonella* strains display distinct hypersusceptibilities to NO. These findings indicate that DksA protects *Salmonella* from iNOS-mediated antimicrobial activity independently of the flavohemoprotein-mediated detoxification of NO. Given the NO-mediated targeting of amino acid biosynthetic pathways, it is likely that the DksA-mediated transcriptional regulation of amino acid biosynthesis is crucial for *Salmonella* to overcome the pressure exerted by RNS on intermediary metabolism.

The addition of amino acids partially abrogated the increased antimicrobial activity exhibited by NO against *dksA*-deficient *Salmonella*. DksA-dependent regulation of amino acid biosynthesis and transport may contribute to both the antioxidant and antinitrosative defenses of *Salmonella*. However, amino acids did not completely reverse the hypersusceptibility of the Δ*dksA* mutant *Salmonella* strain to NO. The positive effects of DksA on glutathione biosynthesis and redox homeostasis may also contribute to antioxidant and antinitrosative defenses. *Salmonella* lacking *dksA* is auxotrophic for several amino acids, including isoleucine, leucine, valine, threonine, phenylalanine, and glycine. Glycine serves as a substrate in the formation of the tripeptide glutathione, and consequently, *dksA*-deficient *Salmonella* contain reduced cytoplasmic glutathione concentrations (19). DksA also maintains proper levels of NAD(P)H-reducing equivalents, which are vital for defense against both oxidative and nitrosative stress (19, 23). The fact that glutathione and the NADPH-dependent glutathione reductase play a critical role in the defense against both ROS and RNS by repairing oxidized and/or nitrosylated thiols raises the interesting possibility that DksA-dependent regulation of low-molecular-weight thiol metabolism could contribute to the antioxidant and antinitrosative defenses of *Salmonella*.

The ability of *Salmonella* to survive inside professional phagocytes is a critical aspect in the pathogenesis of this enteric pathogen. Collectively, our investigations demonstrate that DksA helps *Salmonella* survive the antimicrobial activity of iNOS expressed in the innate immune response of macrophages.

ACKNOWLEDGMENTS

We thank Jessica Jones-Carson, Lin Liu, and James Laughlin for supplying the mice used in the course of these investigations.

This project was supported by National Institutes of Health project AI54959, the Burroughs Wellcome Fund, and Institutional Training Grant T32 AI052066.

REFERENCES

1. **Alam MS, et al.** 2008. Nitric oxide produced in Peyer's patches exhibits antiapoptotic activity contributing to an antimicrobial effect in murine salmonellosis. Microbiol. Immunol. **52**:197–208.
2. **Bang IS, et al.** 2006. Maintenance of nitric oxide and redox homeostasis by the *Salmonella* flavohemoglobin hmp. J. Biol. Chem. **281**:28039–28047.
3. **Bourret TJ, et al.** 2008. Nitric oxide antagonizes the acid tolerance response that protects *Salmonella* against innate gastric defenses. PLoS One **3**:e1833.
4. **Brown L, Gentry D, Elliott T, Cashel M.** 2002. DksA affects ppGpp induction of RpoS at a translational level. J. Bacteriol. **184**:4455–4465.
5. **Bryk R, Griffin P, Nathan C.** 2000. Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature **407**:211–215.
6. **Buzzo CL, et al.** 2010. A novel pathway for inducible nitric-oxide synthase activation through inflammasomes. J. Biol. Chem. **285**:32087–32095.
7. **Chakravortty D, Hansen-Wester I, Hensel M.** 2002. *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. J. Exp. Med. **195**:1155–1166.
8. **Cherepanov PP, Wackernagel W.** 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158**:9–14.
9. **Crawford MJ, Goldberg DE.** 1998. Regulation of the *Salmonella typhimurium* flavohemoglobin gene. A new pathway for bacterial gene expression in response to nitric oxide. J. Biol. Chem. **273**:34028–34032.
10. **Cruz-Ramos H, et al.** 2002. NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. EMBO J. **21**:3235–3244.
11. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97**:6640–6645.
12. **D'Autreaux B, Touati D, Bersch B, Latour JM, Michaud-Soret I.** 2002. Direct inhibition by nitric oxide of the transcriptional ferric uptake regulation protein via nitrosylation of the iron. Proc. Natl. Acad. Sci. U. S. A. **99**:16619–16624.
13. **D'Autréaux B, Tucker NP, Dixon R, Spiro S.** 2005. A non-haem iron centre in the transcription factor NorR senses nitric oxide. Nature **437**:769–772.
14. **De Groote MA, et al.** 1995. Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. Proc. Natl. Acad. Sci. U. S. A. **92**:6399–6403.
15. **Ding H, Demple B.** 2000. Direct nitric oxide signal transduction via nitrosylation of iron-sulfur centers in the SoxR transcription activator. Proc. Natl. Acad. Sci. U. S. A. **97**:5146–5150.
16. **Ellermeier CD, Janakiraman A, Slauch JM.** 2002. Construction of targeted single copy *lac* fusions using λ Red and FLP-mediated site-specific recombination in bacteria. Gene **290**:153–161.
17. **Giacomodonato MN, et al.** 2003. Involvement of intestinal inducible nitric oxide synthase (iNOS) in the early stages of murine salmonellosis. FEMS Microbiol. Lett. **223**:231–238.
18. **Hausladen A, Privalle CT, Keng T, DeAngelo J, Stamler JS.** 1996. Nitrosative stress: activation of the transcription factor OxyR. Cell **86**:719–729.
19. **Henard CA, Bourret TJ, Song M, Vazquez-Torres A.** 2010. Control of redox balance by the stringent response regulatory protein promotes antioxidant defenses of *Salmonella.* J. Biol. Chem. **285**:36785–36793.
20. **Henard CA, Vazquez-Torres A.** 2011. Nitric oxide and *Salmonella* pathogenesis. Front. Microbiol. **2**:84.
21. **Hyduke DR, Jarboe LR, Tran LM, Chou KJ, Liao JC.** 2007. Integrated

network analysis identifies nitric oxide response networks and dihydroxy-acid dehydratase as a crucial target in *Escherichia coli.* Proc. Natl. Acad. Sci. U. S. A. **104**:8484–8489.
22. **Lemke JJ, Durfee T, Gourse RL.** 2009. DksA and ppGpp directly regulate transcription of the *Escherichia coli* flagellar cascade. Mol. Microbiol. **74**: 1368–1379.
23. **Lundberg BE, Wolf RE, Jr, Dinauer MC, Xu Y, Fang FC.** 1999. Glucose 6-phosphate dehydrogenase is required for *Salmonella typhimurium* virulence and resistance to reactive oxygen and nitrogen intermediates. Infect. Immun. **67**:436–438.
24. **MacMicking JD, et al.** 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell **81**: 641–650.
25. **Magnusson LU, Gummesson B, Joksimovic P, Farewell A, Nystrom T.** 2007. Identical, independent, and opposing roles of ppGpp and DksA in *Escherichia coli.* J. Bacteriol. **189**:5193–5202.
26. **Mastroeni P, et al.** 2000. 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. **192**:237–248.
27. **McCollister BD, Bourret TJ, Gill R, Jones-Carson J, Vazquez-Torres A.** 2005. Repression of SPI2 transcription by nitric oxide-producing, IFNγ-activated macrophages promotes maturation of *Salmonella* phagosomes. J. Exp. Med. **202**:625–635.
28. **McCollister BD, et al.** 2007. N_2O_3 enhances the nitrosative potential of IFNγ-primed macrophages in response to *Salmonella.* Immunobiology **212**:759–769.
29. **McLean S, Bowman LA, Poole RK.** 2010. Peroxynitrite stress is exacerbated by flavohaemoglobin-derived oxidative stress in *Salmonella* Typhimurium and relieved by nitric oxide. Microbiology **156**:3556–3565.
30. **Paul BJ, et al.** 2004. DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell **118**:311–322.
31. **Paul BJ, Berkmen MB, Gourse RL.** 2005. DksA potentiates direct activation of amino acid promoters by ppGpp. Proc. Natl. Acad. Sci. U. S. A. **102**:7823–7828.
32. **Perederina A, et al.** 2004. Regulation through the secondary channel—structural framework for ppGpp-DksA synergism during transcription. Cell **118**:297–309.
33. **Potrykus K, Cashel M.** 2008. (p)ppGpp: still magical? Annu. Rev. Microbiol. **62**:35–51.
34. **Price-Carter M, Tingey J, Bobik TA, Roth JR.** 2001. The alternative electron acceptor tetrathionate supports B12-dependent anaerobic growth of *Salmonella enterica* serovar Typhimurium on ethanolamine or 1,2-propanediol. J. Bacteriol. **183**:2463–2475.
35. **Richardson AR, et al.** 2011. Multiple targets of nitric oxide in the tricarboxylic acid cycle of *Salmonella enterica* serovar Typhimurium. Cell Host Microbe **10**:33–43.
36. **Richardson AR, et al.** 2009. The base excision repair system of *Salmonella enterica* serovar Typhimurium counteracts DNA damage by host nitric oxide. PLoS Pathog. **5**:e1000451.
37. **Stevanin TM, et al.** 2000. Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes *bo'* or *bd*, from nitric oxide. J. Biol. Chem. **275**:35868–35875.
38. **Stevanin TM, Poole RK, Demoncheaux EA, Read RC.** 2002. Flavohemoglobin Hmp protects *Salmonella enterica* serovar Typhimurium from nitric oxide-related killing by human macrophages. Infect. Immun. **70**: 4399–4405.
39. **Tucker NP, et al.** 2008. The transcriptional repressor protein NsrR senses nitric oxide directly via a [2Fe-2S] cluster. PLoS One **3**:e3623.
40. **Turner AK, Lovell MA, Hulme SD, Zhang-Barber L, Barrow PA.** 1998. Identification of *Salmonella typhimurium* genes required for colonization of the chicken alimentary tract and for virulence in newly hatched chicks. Infect. Immun. **66**:2099–2106.
41. **Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC.** 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro.* J. Exp. Med. **192**:227–236.
42. **Vazquez-Torres A, et al.** 2004. Toll-like receptor 4 dependence of innate and adaptive immunity to *Salmonella*: importance of the Kupffer cell network. J. Immunol. **172**:6202–6208.
43. **Vitiello M, et al.** 2008. Role of mitogen-activated protein kinases in the iNOS production and cytokine secretion by *Salmonella enterica* serovar Typhimurium porins. Cytokine **41**:279–285.
44. **Webb C, Moreno M, Wilmes-Riesenberg M, Curtiss R III, Foster JW.** 1999. Effects of DksA and ClpP protease on sigma S production and virulence in *Salmonella typhimurium.* Mol. Microbiol. **34**:112–123.