Post-translational modifications and functional studies of DksA in Escherichia coli

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Post-translational modifications and functional studies of DksA in *Escherichia coli*

by

Andrew Charles Isidoridy

A Dissertation

Submitted to the University at Albany, State University of New York

in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

College of Arts & Sciences

Department of Biological Sciences

2019
The majority of my thanks for my doctoral work is reserved for my advisor and mentor Dr. Robert Osuna. From the beginning Robert proved to be hands on and accessible as a PI, personally training me on countless lab techniques and instruments. I owe a particular debt of gratitude for the many late nights (in addition to the long days at the lab) spent on the phone with Robert troubleshooting experiments, reading problematic or confusing results, putting together posters or presentations, and also for his assistance in writing this dissertation. Robert helped me to think more scientifically, showed me how to design experiments to maximize efficiency, and how to make my work failure-proof (resistant) through built-in redundancies. I truly cannot thank you enough for your guidance.

I would also like to acknowledge my committee: Dr. Marlene Belfort, Dr. Richard Cunningham, and Dr. Cara Pager, and thank them for their inputs on the direction and scope of my research, and in the preparation of this dissertation. Their willingness to work with me, along with their patience, is appreciated.

I am grateful to Dr. Ke Xia and Dr. Wilfredo Colón (Department of Chemistry at RPI) for their interest and advice in this work and their contribution with mass spectrometry of DksA, which was a valuable piece in the work described in chapter 2. I also wish to thank Jessica Cheung, whose numerous β-galactosidase assays made fundamental contributions to the work described in chapter 3.
Thank you to my fellow lab mates, Dustin Trufanoff and Dan Woods. You both were there with me from the beginning and it was great having you to joke alongside for the entire grad school process. In addition to your helpful conversations and collaborations, I can’t count how many times you helped pull strains from the freezer, stopped a gel, or spun a sample down for me. Dustin in particular was a great help in piloting a number of interesting experimental threads.

Thank you to Betsy MacCarthy; you’ll always be a second mother to me, and your help with editing was invaluable.

In addition to those who have helped me professionally in this work, I owe tremendous thanks to my friends and family for their emotional (and often food/material) support throughout my time in grad school. A special thanks is owed to my future wife, Laura Wendorf. I would never have been able to face those ultra-late nights in the lab without you and Lily to come home to. You helped me with everything from labeling hundreds of test tubes through driving me into Manhattan for emergency eye surgery. I couldn’t have done it without you.

Finally, I would like to thank my wonderful parents, Ann and George Isidoridy, as well my sisters, Kara and Alyssa, for always encouraging me to achieve my best.
Abstract

DksA is a bacterial gene regulator that functions synergistically with the stress alarmone ppGpp to mediate the stringent response. DksA also functions independently of ppGpp to regulate transcription of a number of genes. DksA function is dependent on its binding affinity to RNA polymerase and requires specific interactions between RNAP and catalytic amino acids located on the coiled coil tip, D74 and A76. While much of the previous work on DksA has focused on understanding the mechanisms of action and the numerous gene targets for transcriptional regulation, little is known about the mechanisms by which DksA expression and function may be regulated at the transcriptional and post-transcriptional levels. In this work we provide evidence from a variety of independent, experimental techniques that DksA is phosphorylated in vivo, and that an N-terminal phosphorylation, likely at amino acid K26, is required for the efficient stimulation of transcription from the uspA promoter. We were unable to identify a functional role associated with the additional phosphorylation site(s) disrupted by the Δ1-31 K101A, T102A, K104A, K105A mutant. In addition to phosphorylation, we probed for other effects of amino acid substitutions on a library of DksA mutants, including some affecting key residues known to be involved in ppGpp binding. We assayed for the ability to repress transcription of the fis promoter (fisp) or to stimulate transcription of uspA promoter (uspAp) in vivo. Our results show that DksA tolerates a large number of mutations with minimal effects on its ability to repress transcription in vivo, suggesting that the integrity and stability of the DksA structure may involve extensive intramolecular interactions not easily disturbed by point mutations. However, we also found several DksA mutations that strongly affect the ability to
stimulate transcription but have little or no effect on its ability to repress transcription, demonstrating that certain functions of DksA are unique to its transcription stimulatory role. Some of these residues (R91, K94, K98, K139) are seen to be involved in ppGpp binding at the ppGpp site 2 of RNA polymerase, according to recent crystallographic evidence. Other residues not interacting with ppGpp are also required for transcription stimulation. Some interact with RNA polymerase in a different conformational state and others form 2 clusters in a solvent-exposed surface of DksA in the ternary complex, suggesting that interactions with other factors besides ppGpp are required for efficient transcription stimulation.
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1. Introduction

Transcriptional regulation in *E. coli*

The ability to respond rapidly and efficiently to changing environmental conditions is greatly important for a bacteria’s survival. Expression levels of a gene can change several-fold over the course of growth and survival. This control is achieved at the transcriptional level via a combination of sigma factors and transcription factors, which act to regulate RNA polymerase enzyme (RNAP). Unlike eukaryotes, a single RNAP is fully responsible for all gene transcription occurring in prokaryotes. Promoter recognition in *E. coli* relies on hexameric DNA regions known as the -10 and -35 promoter sequences. Recognition of a specific promoter by RNAP is dependent on the associated σ factor (7 total in *E. coli*), which provides a mechanism for rapidly responding to environmental conditions with specific changes in transcription. For example, in *E. coli*, σ\(^{70}\) is responsible for the majority of transcription under normal growth conditions while σ\(^{38}\) (σ\(^{5}\)) accumulates intracellularly during stationary phase and other general conditions of stress and starvation, to direct the transcription of genes involved in the response to stress. In addition to regulation via promoter recognition by various σ factors, additional transcriptional regulation is provided by numerous DNA-binding proteins serving as transcription factors. When bound to DNA, transcription factors can repress transcription by blocking promoter access by RNAP or other activator proteins, or activate transcription by recruiting RNAP to the promoter or by facilitating 1 or more steps in the process of forming transcription initiation complexes (Browning and Busby 2004; Browning and Busby 2016). Many of these transcription factors are able to change their intracellular levels or their active state in response to specific environmental
conditions so that their transcription regulatory functions are associated with certain environmental cues.

**Classical transcription factors vs. secondary channel binding factors**

Unlike classical transcription factors, DksA is in a unique category of RNAP secondary channel binding factors (SCBFs) along with the transcription elongation factors GreA, GreB, and the TraR protein. SCBFs are capable of regulating transcription in a promoter-specific manner by binding RNAP directly rather than by binding DNA. DksA, in particular, functions by binding RNAP via the secondary channel, thereby inserting its coiled-coil (CC) tip and positioning the specific, conserved, catalytic amino acids located on the tip (D71xxD74xA76) near the RNAP active site. When these catalytic amino acids are mutated, a loss in DksA’s ability to repress transcription of rRNA promoters (e.g., \( \text{rrnB P1} \)) was observed, while also affecting DksA’s ability to stimulate transcription of amino acid biosynthesis genes such as \( \text{livJ} \) or \( \text{uspA} \).

Additionally, mutations that increase the affinity of DksA for the RNAP secondary channel (N88I, L15F) also correspondingly increase DksA function, suggesting that the transcription regulatory function of DksA is limited in part by its ability to associate stably with RNAP. While structurally similar to GreA and GreB, DksA functions in a largely distinct manner from these other SCBFs; notably, DksA function is affected by the alarmone ppGpp (Paul et al. 2004; Perederina et al. 2004; Molodtsov et al. 2018; Lee et al. 2012; Blankschien et al. 2009).

**DksA function in stringent response and the role of ppGpp**

It has been hypothesized that DksA functions as both a transcriptional stimulator and transcriptional repressor by influencing the kinetics of open complex formation. In this model for
DksA function, RNAP-bound DksA and the small molecule ppGpp lower the free energy (ΔG) of an intermediate or transition state in the process of forming an open complex (or another intermediate complex) from a closed complex. Promoters that are stimulated by DksA are characterized by a high AT sequence content between the -10 regions and the start of transcription and tend to have a relatively low binding affinity for RNAP and are slow to transition to an open complex. However, once formed, these open complexes remain relatively stable because of their lower ΔG relative to the closed complex. For these promoters, lowering a transition-state free energy barrier from a closed to a stable complex would facilitate the formation of open complexes, which would productively initiate transcription faster than they would dissociate to closed complexes. In contrast, promoters that are repressed by DksA are characterized by a high GC sequence content between the -10 region and the start of transcription and tend to exhibit good binding affinities for RNAP but form relatively unstable open complexes because of their relatively higher ΔG compared to the closed complexes. For these promoters, lowering a transition-state free energy barrier between the closed and open complexes would favor the more rapid dissociation of open complexes formed (Paul, Berkmen, and Gourse 2005; Haugen, Ross, and Gourse 2008; Gummesson, Lovmar, and Nyström 2013).

DksA function is often associated with the small ligand alarmone (p)ppGpp, most notably during the stress reaction known as the stringent response. During conditions of amino acid deficiency, the translating ribosomes’ demand for aminoacyl-tRNA is not met, resulting in synthesis of (p)ppGpp by RelA and synthesis/hydrolysis of (p)ppGpp into ppGpp by SpoT (Potrykus and Cashel 2008). It has been shown that ppGpp binding to RNAP results in the suppression of transcription of genes responsible for synthesis of ribosomal proteins (Lemke et al. 2011) while simultaneously up-regulating genes, directly or indirectly, such as σS as well as
those required for amino acid biosynthesis (Barker et al. 2001). In addition to the role of ppGpp, DksA also regulates critical gene transcription during the stringent response. DksA expression is required for transcriptional control of a number of genes during the stringent response, including transcription of the global transcription factor Fis (factor for inversion stimulation) (Mallik et al. 2006) as well as the transcription of ribosomal RNA (rRNA) genes (Paul et al. 2004). These effects, in addition to the repression of ribosomal protein synthesis genes by ppGpp alone, are potentiated when DksA binds RNA polymerase in conjunction with ppGpp (Paul, Berkmen, and Gourse 2005; Lemke et al. 2011).

Understanding the role of binding of ppGpp to RNAP and its relationship to DksA has been complicated by the presence of multiple ppGpp binding sites on RNAP. RNA polymerase crystals generated from *Thermus thermophiles* indicated the presence of a ppGpp binding site relatively far from the location of DksA bound in the secondary channel (Artsimovitch et al. 2004). This ppGpp binding site (site 1) has been subsequently confirmed by a co-crystal structure of the *E. coli* RNA polymerase σ^70^ holoenzyme and (p)ppGpp complexes (Mechold et al. 2013; Zuo, Wang, and Steitz 2013). The effects of disrupting binding site 1 with substitution mutations are not sufficient enough in degree to account for a loss in ppGpp function, suggesting possible alternative ppGpp binding sites (Ross et al. 2013).

More recent work has identified a second ppGpp binding site (site 2) 60 Å from the previously characterized site 1, at the interface of DksA bound to the secondary channel (Ross et al. 2016; Molodtsov et al. 2018). When bound to site 2, the ppGpp 3′ and 5′ phosphate groups are predicted to make multiple interactions with basic residues in DksA.
Example of DksA’s role in a stress response in *E. coli* in LEE expression

Enterohemorrhagic *E. coli* (EHEC) are a group of human pathogens (shigatoxigenic *E. coli*, STEC; and verotoxigenic *E. coli*, VTEC) responsible for bloody diarrhea and hemolytic uremic syndrome. EHEC strains have acquired a pathogenicity island known as the locus of enterocyte effacement (LEE), a chromosomal region of DNA that encodes a number of genes responsible for production of a type III secretion system, the virulence factor intimin, as well as a number of secreted proteins associated with attaching and effacing lesion formation in the human intestine. Conditions of nutrient stress or direct induction of RelA resulting in ppGpp production were able to activate the LEE pathogenicity island transcription in vivo. However, this response is only observed if DksA is also present, demonstrating a role for DksA in pathogenicity in at least some bacteria (Nakanishi et al. 2006).

Regulation of DksA via transcriptional and translational control

DksA appears to be subject to tight regulatory control at the levels of transcription and translation. Transcriptional control of DksA results from variable expression of at least 3 temporally controlled promoters as well as from the binding of a number of global transcriptional regulators. The *dksA* P1 promoter is responsible for the majority of *dksA* transcription during exponential growth and is subject to negative autoregulation as well as growth phase-dependent regulation (Chandrangsu, Lemke, and Gourse 2011; Woods and Osuna unpublished). As *E. coli* enters the stationary phase, DksA, RpoS (σS), and ppGpp play a role in switching transcription of *dksA* from the P1 to additional promoters (P2 and P3). Regulation of DksA by transcription factors provides additional layers of transcriptional control. Fis, IHF (integration host factor), and Lrp (leucine responsive regulator protein) were found to negatively regulate P1 transcription,
whereas CRP-cAMP (cyclic-AMP receptor protein) stimulates P1 and CpxR (envelope stress response regulator) stimulates the P3 transcription. Therefore, it appears that the transcription of \textit{dksA} is responsive to changes in the growth and environmental conditions.

DksA expression is also controlled at the level of translation. Work in our lab identified at least 2 distinct RNA structures in the 5’-untranslated region (UTR) that function to control translation of DksA \textit{in vivo} (Trufanoff, Ou, and Osuna, Unpublished results). Thus it appears that the expression of DksA is greatly restrained at the level of translation and can potentially serve as an opportunity to modulate its intracellular levels.

\textbf{Regulation of DksA via post-translational mechanism of control}

While it is clear that DksA is subject to multiple mechanisms of transcriptional and translational control, little is known about the existence of mechanisms by which DksA may also be subject to post-translational control. DksA has been identified as a potential substrate of the protease ClpXP, containing a recognition motif in its N-terminal region (Flynn et al. 2003). This suggests that some form of post-translational regulation of DksA may be at work.

Research has shown that DksA has a relatively long half-life with reported values of \(~11-44\) min (Chandrangsu, Lemke, and Gourse 2011; Flynn et al. 2003), making it a good potential target for post-translational control. Functional regulation of DksA by the alarmone ppGpp can be considered to be a mechanism of post-translational control. DksA often acts in conjunction with and can be allosterically potentiated by ppGpp, especially during the stringent response to inhibit transcription of rRNA and ribosomal proteins (Paul et al. 2004; Paul, Berkmen, and Gourse 2005; Lemke et al. 2011) or \textit{fis} inhibition (Mallik et al. 2006). DksA has also been shown to both bind RNAP (Molodtsov et al. 2018) and regulate numerous genes independently of
ppGpp (Magnusson et al. 2007; Åberg et al. 2009). Therefore, whereas changes in cellular ppGpp levels during a response to stress can work to intensify the effects of DksA at a number of promoters, it is likely that other mechanisms may exist to control the function of DksA apart from ppGpp.

**Post-translational regulation of protein function in prokaryotes**

Prokaryotes are capable of regulating the activity or function of a protein post-translationally via a variety of mechanisms. However, this thesis will focus mainly on phosphorylation as a reversible chemical modification affecting DksA; the initial phase of this work involved an exploratory phase in which other potential mechanisms of translational control of DksA were considered, for which some preliminary observations were made.

Cleavage of a protein via proteolysis is the most common form of post-translational modification (Cain, Solis, and Cordwell 2014) and can result in an active form of the protein such as in the case of certain cell surface proteins, which rely on having their N-terminal signal peptide removed for successful secretion or translocation (Navarre and Schneewind 1999). Proteolysis can also deactivate and degrade proteins as a way to regulate function, such as in the example of the RecA/LexA proteins during the activation of the bacterial SOS response (Little 1991). Accumulation of single-stranded DNA resulting from DNA damage or mutation in bacteria promotes binding and filamentation of RecA protein. Activated RecA in turn stimulates LexA’s autocatalytic cleavage of the LexA repressor, thus relieving the repression of SOS genes.

Changes in a protein’s multimeric state is another level at which function can be regulated. While it is well established that DksA binds RNAP as a monomer in order to regulate transcription (Perederina et al. 2004; Paul et al. 2004; Ross et al. 2016; Molodtsov et al. 2018), it
is common for other transcription factors and DNA binding proteins (e.g., CRP, Fis, IHF, LacR, AraC) to require the formation of dimeric or tetrameric states in order to successfully function (Browning and Busby 2016).

Sequestration of active protein into an inactive bound state is another example of post-translational control via protein binding. A well-known example of this mode of post-translational control is in bacterial toxin-antitoxin systems such as MazEF. Also known as a suicide module, mazF encodes for a stable toxin, the presence of which leads to cell death, while the mazE encodes an anti-toxin protein, which can bind MazF, effectively neutralizing its ability to function (Engelberg-Kulka 2005). Another common example of this mode of regulation can be seen with the RNA binding proteins such as CsrA or RsmA. They can function to regulate genes post-transcriptionally by binding RNA and effecting stability or limiting access to a ribosome. While not an example of sequestration by a protein, it has been shown that the CsrB family of sRNAs can function as CsrA antagonists by providing a pool of multiple CsrA binding sites (Babitzke and Romeo 2007).

Chemical modifications represent a broad and important group of highly specific and often reversible post-translational modifications, which often modify protein function (Cain, Solis, and Cordwell 2014). Some examples of common chemical modifications in bacteria include acetylation, methylation, carboxylation, glycosylation, lipidation, nitrosylation, and phosphorylation.

Protein phosphorylation is the process of chemically modifying a protein by covalently adding a phosphate group, often by a kinase. Multiple amino acids can be targeted for modification, and addition of a phosphate is often reversible either via chemical lability or via a phosphatase enzyme, allowing for a greater degree of control over the functional changes caused
by phosphorylation. The attachment of a phosphate group on a targeted amino acid can regulate the function of a protein by altering the local surface charge, and often changes tertiary protein conformation (Mijakovic, Grangeasse, and Turgay 2016). Phosphorylation can be detected using a variety of techniques including $^{32}$P labeling of cell culture lysates separated by gel electrophoresis, Western blot detection with phospho amino acid-specific antibodies, with phosphoprotein-specific fluorescent gel stains (Pro-Q® Diamond), or with high throughput methods such as mass spectrometry. In prokaryotes Ser, Thr, and Tyr residues can have phosphates attached via the relatively stable O-phosphate bonds. However, the less stable (acid labile) N-phosphate amino acids His, Asp, Arg, Lys, and Cys are also possible, and are thought to be more common than what has been reported in the literature. Histidine and aspartic acid phosphorylations have been demonstrated to play an important role in two-component signaling. However, due to the ease with which these phosphates cleave from their amino acid in low pH conditions, phosphoamide and acyl phosphate bond forming residues have been difficult to characterize, particularly using mass spectrometry methods (Cain, Solis, and Cordwell 2014).

**Thesis outline**

In this work, I investigated the hypothesis that post-translational events modify the structure and function of DksA in *E. coli*. In chapter 2, I demonstrate that DksA is post-translationally modified by phosphorylation. We provide evidence that DksA is likely phosphorylated in multiple regions and narrow down specific amino acids that are affected by phosphorylation. Additionally, we demonstrate that loss of phosphorylation in the N-terminal region is correlated with a loss in the ability to stimulate transcription of universal stress protein A gene (*uspA*), a known target of DksA stimulation. Furthermore, a phosphomimetic mutant is capable of
restoring function. In chapter 3, we leverage the large library of DksA mutants I have generated to probe for additional functional effects of DksA. The ability of DksA to stimulate transcription of uspA is more sensitive to substitution mutations than repression of fisp transcription by DksA. Utilizing a recently published ternary crystal structure of DksA bound to RNAP along with ppGpp, we evaluate the functional effects of mutating specific amino acids in their physiological context. Our results demonstrate that ppGpp binding to site 2 is necessary for stimulation at uspA, supporting an emerging model of DksA’s function in the stimulation of transcription (Gourse et al. 2018). In addition we identify a number of additional residues that affect the ability of DksA to stimulate uspA, but not to repress fisp transcription. This suggests that distinct roles of DksA are required for stimulation and repression of transcription. Chapter 4 provides a summary and discussion of the conclusions found in this work, as well as ideas that may influence the future directions of the research initiated in this work.
Figures and Legends

Figure 1.1. Crystal Structure of DksA. DksA depicted in a ribbon model in blue (left) and a space-filling model (PDB: 1TJL; Perederina et al. 2004).
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Artsimovitch, Irina, Vsevolod Patlan, Shun-Ichi Sekine, Marina N. Vassylyeva, Takeshi Hosaka, and Kozo Ochi. 2004. “Structural Basis for Transcription Regulation by Alarmone PpGpp Inhibition of Transcription (Negative Control) of Components of Translation Machinery (e.g., rRNA, tRNA, etc.) and by Enhanced Transcription (Positive Control) Of.” *Cell* 117 (3): 299–310.


2. Phosphorylation of DksA in vivo and its effect on transcription in *E. coli*

This work was conducted in collaboration with Dustin E. Trufanoff†, Robert Osuna†, Ke Xia§, and Wilfredo Colón§.

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Abstract

DksA is a global transcriptional regulator in *E. coli* that affects transcription of hundreds of genes and, along with the small signal molecule ppGpp, plays an important role in the bacterial response to stress and starvation. While much of the previous work on DksA has focused on understanding the mechanisms of action and the numerous gene targets for transcriptional regulation, little is known about the mechanisms by which DksA expression and function may be regulated at the transcriptional and post-transcriptional levels. In this work, we provide evidence that DksA is phosphorylated in vivo, and that an N-terminal phosphorylation, likely at amino acid K26, is required for the efficient stimulation of transcription from the *uspA* promoter. DksA expressed in cells growing in low-phosphate medium in the presence of $^{32}$P is effectively labeled. Moreover, DksA readily stains with the phosphoprotein-specific gel stain (Pro-Q® Diamond), binds to a phosphoenrichment chromatography column, and results from mass spectrometry indicate that DksA may carry as many as 3 phosphates. Chemical cleavage of $^{32}$P-labeled DksA showed that both the amino- and carboxy-terminal halves of DksA are phosphorylated. The phosphorylation is acid-labile, suggesting that phosphoamide or acyl phosphate bonds are made
with positively or negatively charged residues, respectively. Amino acid substitution mutations were generated and examined for their $^{32}$P-radiolabeling in vivo, which led to an identification of several residues that affected phosphorylation. Deletion of the first 31 N-terminal amino acids resulted in a loss of phosphorylation similar to that by a Y23A K26A Y31A triple substitution located in the globular region of DksA. These mutations resulted in a decrease in the ability to stimulate transcription. Additional loss of phosphorylation was observed in the multiple substitution mutant K101A T102A K104A K105A, which defined a second phosphorylated region of DksA. However, loss of phosphorylation at this site had no observable effects on transcription. These results implicate a role for phosphorylation in the globular region of DksA as part of a mechanism to enhance its transcription regulation function in vivo.
Introduction

DksA was initially identified in *E. coli* as a multicopy suppressor of a temperature-sensitive growth and filamentation phenotype associated with a *dnaK* deletion mutation (Kang and Craig 1990). This protein is present in numerous bacterial species, acts globally as an important transcription regulator, and is required for survival under starvation (stringent response) and for the expression of virulence genes in pathogenic bacterial strains (Paul et al. 2004; Magnusson et al. 2007; Perron, Comte, and Van Delden 2005; Perederina et al. 2004; Nakanishi et al. 2006). In addition, it helps to alleviate conflicts between the DNA replication and transcription machinery, particularly upon nutritional stress (Tehranchi et al. 2010; Trautinger et al. 2005).

DksA is a 151 amino acid protein with a molecular weight of about 17.5 kDa, consisting of a coiled coil (CC) alpha helical region and a somewhat globular region, resembling in structure the transcription elongation factors GreA and GreB (Perederina et al. 2004). Indeed, like GreA and GreB, DksA binds RNA polymerase (RNAP) by inserting its CC via the secondary channel of the holoenzyme and positioning key acidic residues at the CC tip near the active site (Perederina et al. 2004; Paul et al. 2004; Molodtsov et al. 2018; Lennon et al. 2012; Lee et al. 2013). DksA binding to RNA polymerase can result in transcription repression or activation, depending on the kinetic properties of the promoter (Paul, Berkmen, and Gourse 2005).

During the stringent response, ppGpp and DksA act synergistically in vivo and in vitro to bind RNAP in order to suppress transcription of a number of genes, including those for rRNA, ribosomal proteins, and *fis*, while simultaneously up-regulating genes that are critical for survival under stress, such as *rpoS*, *uspA*, and various amino acid biosynthetic genes (Paul et al. 2004; Paul, Berkmen, and Gourse 2005; Mallik et al. 2006; Lemke et al. 2011; Magnusson et al. 2007). However, in the absence of ppGpp, DksA is able to bind RNAP (Molodtsov et al. 2018; Lennon
et al. 2009) and independently regulate the expression of numerous genes in vivo and in vitro (Åberg et al. 2009; Magnusson et al. 2007; Paul, Berkmen, and Gourse 2005). Therefore, whereas changes in cellular ppGpp levels during a response to stress can accentuate the effects of DksA at a number of promoters, it is likely that other mechanisms exist to control the function of DksA apart from ppGpp.

Western blot comparisons of *E. coli* DksA levels in exponential vs. stationary growth phase, normalized by total protein concentration in the lysates, showed little variation, suggesting that cellular DksA levels are kept more or less constant during growth (Paul et al. 2004). However, because total protein per cell changes substantially during the growth of a culture (Ali Azam et al. 1999), such normalization procedures may have inadvertently mitigated changes in cellular DksA levels during the different growth phases. When normalized per OD$_{600}$ units of cells, western blots showed that DksA levels increased over 8-fold during exponential growth (Woods and Osuna unpublished). A similar growth phase-dependent regulation pattern of DksA was observed in *Pseudomonas aeruginosa* (Perron, Comte, and Van Delden 2005). This suggests that DksA expression must be subject to positive and negative mechanisms of control to alter the intracellular concentration during various phases of growth.

Transcriptional control of DksA is achieved via the interaction of at least 3 temporally controlled promoters and several global transcriptional regulators. The primary promoter of *dksA* (P1) was shown to be subject to negative autoregulation and growth phase-dependent regulation (Chandrangsu, Lemke, and Gourse 2011). Two additional promoters were identified (P2, P3), which are primarily active during stationary phase and are dependent on the sigma factor RpoS (Woods and Osuna unpublished). Multiple weak promoters were also identified, which could also contribute to expression of DksA from a multicopy plasmid (Chandrangsu et al. 2012).
DksA, RpoS, and ppGpp play roles in switching transcription from the stronger P1 promoter to the weaker P2, P3 promoters as cells transition to stationary phase (Woods and Osuna unpublished). Whereas Fis, IHF, and Lrp were found to negatively regulate P1 transcription, CRP-cAMP (cyclic-AMP receptor protein) stimulates P1, and CpxR (envelope stress response regulator) stimulates P3. Therefore, DksA is subject to substantial transcriptional control. It is also subject to translational control, a process involving at least 2 distinct RNA structures in the 5’-untranslated region (UTR) (Trufanoff, D. E., Ou, R. W., and Osuna, R., Unpublished results). CsrA (Carbon storage regulator A) binds to the dksA 5’-UTR to exert a positive effect on translation (Edwards et al. 2011; Trufanoff, D. E., Ou, R. W., and Osuna, R., Unpublished results).

We wondered if DksA is also subject to post-translational modifications, which might act to restrain or intensify DksA function. Chemical modifications represent a broad and important group of highly specific and often reversible post-translational modifications, which can affect protein function or stability in vivo (Cain, Solis, and Cordwell 2014). More specifically, protein phosphorylation in prokaryotes is often associated with signal transduction via two-component systems and can be used to induce reversible functional changes in a series of targeted proteins as a method of regulation (Cain, Solis, and Cordwell 2014). In this work, we report our discovery that DksA is modified by phosphorylation in vivo. The phosphorylation is highly unstable under acidic conditions, indicating that residues other than serine, threonine, and tyrosine are the targets of phosphorylation. Using a combination of chemical cleavage and mutation analysis, we identify 2 phosphorylated regions in DksA, 1 of which intensifies the role of DksA as a transcriptional regulator in vivo.
Results

DksA is phosphorylated in vivo.

Our first and most convincing approach in discovering that DksA is phosphorylated in vivo was to conduct $^{32}$P labeling of growing cell cultures. *E. coli* strain RO1757 was grown in a low-phosphate medium until mid-exponential growth phase, at which point N-terminally-tagged His6-DksA was overexpressed from an IPTG-inducible plasmid (pRO552) in the presence of IPTG and $^{32}$P orthophosphoric acid. The His6-DksA in the cell lysate was purified using Ni-NTA affinity chromatography, separated by SDS-PAGE and visualized by autoradiography (Figure 2.1A). The result indicates that the His6-DksA effectively incorporates $^{32}$P in vivo.

Removal of His6-tag does not result in loss of phosphorylation.

Because the amino acid histidine can serve as a potential target of phosphorylation in prokaryotes (Cain, Solis, and Cordwell 2014), and because it is the major component of the engineered His6-tag, the included factor Xa cleavage site was utilized to remove the His6-tag and the resulting DksA was analyzed by SDS-PAGE followed by autoradiography (Figure 2.1A). Results indicate that DksA remains phosphorylated following treatment with factor Xa for 2 hours, which is sufficient to cleave the overwhelming majority of His6-tagged proteins into the “WT” DksA (Figure 2.1B).

DksA is phosphorylated during growth in LB medium.

Radiolabeling of DksA by $^{32}$P requires growth and labeling in a defined low-phosphate medium. In order to determine whether the observed phosphorylation was specific to the low-phosphate stress conditions, we turned to an independent method for detecting protein phosphorylation that could be used with cells grown in any medium, such as LB, in which $^{32}$P labeling does not work due to the presence of higher competitive phosphate concentrations in the
medium. We also wished to examine whether the N-terminal His6-tag is required for the DksA phosphorylation event to occur in vivo. Turning to an alternate method of phosphoprotein detection, we utilized a commercially available phosphoprotein stain called Pro-Q® Diamond (Thermo Fisher Scientific), which specifically stains phosphorylated proteins (Figure 2.2A). E. coli (RO1630) transformed with a non-His6-tagged DksA-expressing plasmid (pRO553) were grown until early-exponential phase, OD$_{600}$ 0.2-0.3, and DksA was induced for 1 hour by addition of IPTG. Cell lysates of IPTG-induced and -uninduced cultures were separated by SDS-PAGE and stained by Pro-Q® Diamond (Figure 2.2B). Results clearly show IPTG-induced DksA expression stains well (and above the levels of many other proteins in the cell extract) with the Pro-Q® Diamond gel stain. Therefore, DksA phosphorylation is not restricted to the low-phosphate growth medium used in the $^{32}$P-radiolabeling, nor does it require the N-terminal His6-tag (which also does not interfere with phosphorylation).

**Phosphorylated DksA can be enriched relative to total DksA.**

The detection of phosphorylation within a pool of protein can be complicated by a number of factors, including a low percent of phosphorylated protein due to the action of phosphatases that may remove the phosphates in vivo, or an inherent instability of certain phosphate bonds (Table 2.3) (Duclos, Marcandier, and Cozzone 1991; Martensen 1984). It is often possible to enrich for a greater proportion of phosphoprotein by utilizing a commercially available phosphoprotein enrichment kit (Pierce). Purified non-radiolabeled DksA (with its His6-tag removed) was passed over this column, rinsed with buffer to remove unbound protein, and then eluted as recommended by the supplier. Equal quantities DksA from preparations before and after passage through the phosphoenrichment chromatography column were separated on SDS-PAGE and stained with Pro-Q® Diamond gel stain. Afterwards, the same gel was stained with
the general protein fluorescent stain SYPRO® Ruby (Figure 2.2C). The results showed that purified His6-DksA was enriched for a higher concentration of phospho-DksA through this treatment.

Phosphor-enriched and non-enriched preparations of DksA were subjected to mass spectrometry analysis of the full-length protein (Figure 2.3). The results revealed the presence of a species that is 258 daltons heavier than a peak corresponding to unmodified DksA. The mass difference corresponds to that of 3 phosphates (H₃PO₄, 2x HPO₃). This heavier peak is more prominent in the enriched DksA sample, compared to the non-enriched sample. Therefore, these results support the idea that DksA is phosphorylated and may contain as many as 3 phosphates.

**Mass spectrometry analysis was unable to determine the location of DksA phosphorylation.**

In order to determine the location of phosphorylation, we initially turned to mass spectrometry of purified and phosphor-enriched His6-DksA. However, this technique is limited to the identification of acid stable phosphomonoesters phosphoserine, phosphothreonine, or phosphotyrosine, none of which were identified. Phosphorylations on a number of alternative amino acids result in acid labile phosphoamide or mixed anhydride bonds, which would escape detection by this method. Thus, we turned to the use of genetic and biochemical approaches to identify the location of the phosphorylated site(s).

**Two ³²P-labeled bands of unknown composition co-purify with radiolabeled His6-DksA.**

When ³²P-labeled His6-DksA is purified with a Ni-NTA immobilized metal affinity chromatography (IMAC), we consistently observe 3 radiolabeled bands that co-purify with His6-DksA (Figure 2.4). The Enzymatic cleavage by Proteinase K results in the complete loss of DksA but does not affect the other 3 bands. The largest molecular weight band was digested
completely following incubation with DNase I, while the low-molecular weight bands resisted treatment with DNase I, RNase A, or Proteinase K entirely.

**Chemical cleavage of DksA indicates multiple phosphorylations on DksA.**

In an attempt to eliminate any large, unlabeled sections of DksA from the need for further analysis, chemical cleavage was performed on radiolabeled, purified DksA and the resulting peptides analyzed by SDS-PAGE and autoradiography. $^{32}$P-labeled His6-DksA was digested via incubation with 3-Bromo-3-methyl-2-(2-nitrophenylthio)-3H-indole (BNPS-Skatole), a chemical cleavage agent that selectively cleaves proteins at tryptophan residues, of which DksA only has 1 (W47). Cleavage with BNPS-Skatole resulted in 2 $^{32}$P-labeled polypeptides roughly 5.3 and 11.7 kDa in size (Figure 2.5). These labeled peptide fragments indicate that 2 independent phosphorylation events are likely occurring both upstream and downstream of W47. Acetone precipitation is a necessary step in processing BNPS-Skatole cleaved DksA for gel electrophoresis. It was consistently observed that purified, radiolabeled protein resulted in the loss of the lowest molecular weight contaminant band following precipitation with Acetone (Figure 2.5).

Additional cleavage analysis of radiolabeled DksA peptides was performed using Cyanogen Bromide (CNBr), which cleaves proteins selectively at methionine residues. The resulting digested DksA peptides were separated by SDS-PAGE and results were analyzed by comparison of western blots using anti-DksA antibodies with autoradiographs of the gel. Results showed a labeled DksA peptide of roughly 14 kDa, consistent with a partial cleavage product resulting from a single cleavage event at M32. Acetone precipitation of the CNBr cleaved protein resulted in the loss of the lower molecular weight contaminant band (Figure 2.6).
**Phosphorylation of DksA is labile in low pH.**

The stability of a phosphate bound to an amino acid varies by side chain with certain phospho bonds showing lability in acidic or alkaline conditions (Table 2.3) (Duclos, Marcandier, and Cozzone 1991; Martensen 1984). It has been reported that phosphorylations on side chains that form phosphoamide or mixed anhydride bonds occur in prokaryotes; however, they often prove to be difficult to characterize due to their propensity to cleave in low pH conditions, such as those often used in mass spectrometry, and are therefore likely under-reported (Cieśla, Fraczyk, and Rode 2011). In order to characterize DksA’s phosphorylation stability in low pH, purified $^{32}$P-labeled His6-DksA was treated with water or 5% trifluoroacetic acid as indicated (Figure 2.7). Samples on the left were treated overnight while samples on the right were treated for 2 hours. Phosphorylation of DksA was determined by SDS-PAGE followed by autoradiography and western blot with anti-DksA antibodies. Results show a large loss in $^{32}$P activity following treatment with acid while corresponding protein levels remained unchanged (Figure 2.7), indicating phosphorylation of DksA is acid labile and likely occurring on an amino acid residue forming a phosphoamide (Arg, Lys, His) or acyl phosphate (Asp, Glu) bond (Table 2.3).

**Mutational analysis of DksA.**

While chemical cleavage of $^{32}$P-labeled DksA provided evidence for multiple cleavage events, it was unable to define a more specific region of amino acids targeted for phosphorylation. In order to narrow down the location of phosphorylation, we generated a large library of DksA mutants, systematically targeting most of the phosphorylatable amino acids (Table 2.2). DksA mutants were expressed in BL21 *E. coli* (RO1569) and tested for their ability to incorporate $^{32}$P in vivo. $^{32}$P-labeled protein was analyzed by SDS-PAGE followed by exposure
for autoradiography and, afterwards, the same gel was subjected to western blot analysis using anti-DksA antibody. Relative phosphorylation efficiencies were examined by determining the $^{32}$P signal-to-protein ratios. Results indicate that the majority of the tested DksA mutant proteins retained (in some cases improved) the ability to incorporate $^{32}$P in vivo (Figure 2.8).

A few of the tested DksA mutants were, however, reduced in their ability to be phosphorylated. Removal of the N-terminal 31 amino acids resulted in a reduction in phosphorylation by 55%. A triple substitution in this same 31 amino acid N-terminal region (Y23A K26A Y31A) similarly resulted in a reduction in $^{32}$P labeling by 56% (Figure 2.9).

The partial reduction in phosphorylation associated with disruption of a single phosphorylation site as observed in the Δ1-31 DksA or the Y23A K26A Y31A mutants is consistent with the chemical cleavage results, which point to at least 2 separate phosphorylated regions in DksA. To facilitate the identification of another phosphorylated site, additional DksA mutants were engineered on the Δ1-31 truncated form of the protein in order to identify phosphorylation sites outside of this N-terminal region. A similar screening of these additional mutants identified a multiple substitution mutant of DksA (Δ1-31 K101A T102A K104A K105A) that was completely deficient in its ability to incorporate $^{32}$P in vivo (Figure 2.9B).

**Engineered His6-tag does not interfere with the function of DksA.**

In order to facilitate a number of our experiments including probing for multimers in vitro, as well as identification of phosphorylated residues, we utilized an engineered His6-tag version of DksA. While the His6-tag is relatively small, and previous work has shown addition of a histidine tag does not interfere with DksA function or binding (Paul et al. 2004; Lennon et al. 2012), we wanted to ensure that its presence in our own construct did not interfere with the normal ability of DksA to function as a transcription regulator. β-galactosidase assays using
transcriptional reporters created by fusing a promoter region for a known target of repression by DksA (fisp), with lacZ as a reporter gene, were used to assay for loss of DksA function. β-galactosidase results indicate His6-DksA was capable of repressing transcription of fisp fully relative to WT DksA during both exponential and stationary growth phase (Figure 2.10), demonstrating no interference by the additional His6-tag.

**Calibrating plasmid-expressed DksA to levels comparable to those from chromosomally expressed DksA.**

Because some of the more subtle changes in function may be masked by overexpression of DksA, we sought to determine levels of IPTG induction that were physiologically comparable to WT, chromosomally expressed DksA concentrations. The multi-copy vector pKK223-3 drives expression of an inserted gene via the IPTG-inducible Ptac promoter. In the absence of IPTG, LacI represses transcription at this promoter; however, the promoter is strong, and leaky expression is known to occur. Chromosomally expressed concentrations of DksA were carefully measured by sampling 1 OD$_{600}$ units worth of WT MG1655 E. coli (RO 1244) cells at hour intervals and analyzed by SDS-PAGE. Known quantities of purified His6-DksA were added at a later point during electrophoresis in order to aid in the quantification of resulting western blots using anti-DksA antibodies (Figure 2.11A). DksA expression varied over time roughly 9-fold, from around 65-598ng, peaking at around 2 hours of growth.

Plasmid pKK223-3-driven DksA expression levels were also measured over a number of concentrations of IPTG. E. coli MG1655, ΔdksA transformed with a pRO552 expressing His6-DkA (RO1629) were grown until OD$_{600}$ 0.2-0.3, at which point a number of sub-cultures were induced with increasing concentrations of IPTG (Figure 2.11B). Results indicate uninduced “leaky” expression of His6-DksA resulted in 313ng of DksA, well within chromosomally expressed levels.
Phosphorylation of DksA affects its ability to function in transcriptional regulation.

DksA functions to regulate transcription of a number of genes by simultaneously up-regulating specific genes, notably those required for amino acid biosynthesis and sigma S production, while also repressing a subset of those required for growth and reproduction (Åberg et al. 2009; Magnusson et al. 2007; Paul, Berkmen, and Gourse 2005; Paul et al. 2004). Utilizing representative promoters targeted by DksA for both repression (fisp) and activation (uspA), fused to the reporter gene β-galactosidase, we assayed mutants previously identified as deficient for phosphorylation, for their ability to function.

Because the DksA mutants Δ1-31, Y23 K26 Y31, and Δ1-31 K101 T102 K104 K105 demonstrated a reduction in their ability to be phosphorylated (Figure 2.9), we sought to determine if there was a corresponding change in function for those mutants. Deletion of the first 31 N-terminal amino acids (Δ1-31) resulted in a decrease of function for both repression of fisp as well as stimulation of uspA transcription (Figure 2.12). The triple substitution mutant Y23A K26A Y31A resulted in similar loss of phosphorylation as the Δ1-31 DksA (~55%), and, likewise, appeared similarly functionally affected for regulation of both fisp and uspA. A multiple substitution mutant covering T9A S10A S11A S13A Y23A Y31A and T131A did not result in any functional changes. As K26A was the only amino acid mutated in the triple mutant Y23A K26A Y31A that was not mutated in the multiple substitution mutant T9A S10A S11A Y23A Y31A T131A, and because the potential phosphorylation of a lysine is supported by the observed acid lability of the phosphate bond, we engineered a pair of single substitution mutants; K26A as well as a K26E phosphomimetic mutant (Klose, Weiss, and Kustu 1993) and assayed them for repression and stimulation function. Unexpectedly, the K26A substitution did not impact the ability of DksA to repress fisp; however, it did result in a similarly strong loss in the
ability to stimulate \textit{usp}Ap. The phosphomimetic K26E substitution, with its negatively charged glutamic acid, is sufficient to rescue DksA function in the stimulation of \textit{usp}Ap (Figure 2.12).
Discussion

Phosphorylation is widely employed by eukaryotes and prokaryotes alike as a responsive, reversible method of post-translational regulation. This work provides evidence that DksA is phosphorylated in vivo and that a loss in ability to phosphorylate results in a functional impairment of DksA’s ability to stimulate uspA. By growing BL21 E. coli (RO1757) in low phosphate medium while adding $^{32}$P-radiolabeled phosphate upon induction of His6-DksA protein, we were able to demonstrate that it becomes reproducibly radiolabeled, indicating it is the target of phosphorylation in vivo (Figure 2.1A). Because the amino acid histidine is a known target for phosphorylation in bacteria (most notable in the case of two-component systems) we confirmed that DksA retains the phosphorylation following removal of the His6-tag via factor Xa cleavage (Figure 2.1B). Additionally, Pro-Q® Diamond fluorescent stain, a gel stain specific for phosphorylated proteins, was used to confirm WT DksA phosphorylation under rich medium growth conditions (Figure 2.2B). Phosphorylated DksA can be concentrated relative to non-phosphorylated DksA by utilizing phosphoprotein enrichment IMAC columns (Life Technologies). Analysis of DksA by Pro-Q® Diamond and SYPRO® Ruby confirmed enrichment (Figure 2.2C); mass spectrometry analysis on a protein sample enriched for phosphorylated DksA supported the presence of a species 258 daltons or roughly the mass of 3 phosphates heavier ($\text{H}_3\text{PO}_4$, 2x HPO$_3$).

Attempts to quantify the phosphorylated proportion of DksA have been complicated for a number of reasons. Partial phosphorylation of a protein pool is common, as kinase and phosphatase enzymes work together to control when and how a protein becomes phosphorylated and dephosphorylated. This partial phosphorylation of a protein pool is consistent with our ability to enrich DksA for phosphorylated proteins. While mass spectrometry results were
consistent with an increase in the larger mass peak, a peak of unmodified mass was still detected, indicating unphosphorylated DksA remains. Further complicating analysis and purification, a number of phospho bonds formed by specific residues are known to be labile under acidic or alkaline conditions (Table 2.3).

In order to determine functional effects of phosphorylation, we sought to identify which residues of DksA were necessary for phosphorylation and whether phosphorylation altered DksA’s ability to regulate transcription repression (fis\(p\)) or stimulation (usp\(4p\)). Characterizing the phosphorylated residues on DksA was complicated by the fact that phosphorylation at a number of residues results in the formation of phospho bonds that are unstable in low or high pH (Table 2.3). The inability to identify the location of phosphorylation by mass spectrometry and the demonstrated phosphate lability in low pH (Figure 2.7) indicate phosphorylation is not occurring on a Ser, Tyr, Thr, or Cys residue, which would result in the formation of the more stable phosphoester/phosphothioester bonds. Initial attempts to locate phosphorylation by eliminating large sections of DksA proved unsuccessful. Purified, radiolabeled DksA cleaved with BNPS-Skatole resulted in 2 peptides (an N-terminal fragment of roughly 5.3kDa as well as the C-terminal peptide of roughly 11.7kDA), both of which were \(^{32}\)P-labeled indicating multiple, independent phosphorylation events on DksA. Deletion of the first 31 amino acids (\(\Delta 1\)-31 DksA) resulted only in a 55% loss of phosphorylation, consistent with multiple phosphorylation sites. The triple substitution Y23A K26A Y31A resulted in a similar loss of phosphorylation as the \(\Delta 1\)-31 deletion mutant, identifying amino acids necessary for phosphorylation in the N-terminal region (Figure 2.9). This observed reduction of phosphorylation in the Y23 K26 A Y31A DksA corresponds to a similar reduction in function in both the repression of fis\(p\) as well as the stimulation of usp\(4p\) as assayed by \(\beta\)-galactosidase
activity. The N-terminal multiple substitution T9A S10A S11A S13A Y23A Y31A T131A, covering all possible phosphoester bond forming residues, did not result in a loss of function, indicating Y23A and Y31A are not responsible for the observed functional changes, suggesting K26A plays a key role in the observed functional loss (Figure 2.12). While K26A DksA did not lose its ability to function in the repression of fisp, it was functionally impaired in the ability to stimulate uspA. Furthermore, the phosphomimetic K26E mutant was fully functional for stimulation of uspA, supporting the role of this phosphorylation in the functional regulation of DksA.

Surprisingly, the reduction of phosphorylation observed in the ∆1-31 K101A T102A K104A K105A mutant did not result in any observable change in function relative to the ∆1-31 DksA control (Figure 2.12), for both fisp repression and uspA stimulation. Is it possible that phosphorylation in this more C-terminal region has an effect on regulation of transcription for promoters other than fisp and uspA, or even hereto unexplored functional roles?
Materials and methods

Chemicals and enzymes

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich or Fischer Scientific. Bacterial growth media were from Difco. All enzymes were purchased from New England Biolabs or Bio-Rad Laboratories and all oligonucleotides used for PCR and DNA sequencing were ordered from Integrated DNA Technologies. DksA antibodies were generated by ABCAM. Radioactive $^{32}$P orthophosphate ($\geq$9000 Ci/mMole) was from PerkinElmer.

Bacterial strains and plasmids

Table 2.1 lists the main bacterial strains used in this work. RO1393 was constructed by infecting RO1250 with a previously made lambda strain carrying a lacZ fusion to the fisP region from -373 to +83 relative to the start of transcription (Ball et al. 1992). RO1550 was made by transforming RO1393 with plasmids pMS421 and pKK223-3 and selecting for spectinomycin (75 $\mu$g/ml) and ampicillin (100 $\mu$g/ml) resistance. RO1554 was made by transforming RO1393 with pMS421 and selecting for resistance to 75 $\mu$g/ml spectinomycin. The dksA::tet knockout was moved by P1 transduction from RO120 into BL21 (DE3) and selected for growth in LB agar containing 15 $\mu$g/ml tetracycline to create RO1569. RO1630 is RO1393 transformed with pMS421 and pRO553; RO1757 is RO1569 transformed with pMS421 and pRO552 and selecting for resistance to 75 $\mu$g/ml spectinomycin and 100 $\mu$g/ml ampicillin.

Plasmid pRO552 was created by first amplifying the dksA gene by the polymerase chain reaction (PCR) using E. coli VH1000 chromosomal DNA as template, a downstream oligonucleotide that anneals to a region downstream of the rho-independent terminator sequence, and an upstream oligonucleotide that anneals to the beginning of the dksA gene and creates an EcoRI restriction site, a methionine start codon followed by 6 histidine codons, and sequence
encoding for a factor Xa protein recognition sequence, immediately preceding the AUG start codon for DksA. The amplified DNA product was cleaved with EcoRI, ligated into the EcoRI-Smal sites of pKK223-3, transformed into RO1554, and selected for resistance to 75 µg/ml spectinomycin and 100 µg/ml ampicillin. Plasmid pRO553 was made similarly to pRO552, except that the upstream oligonucleotide used in the PCR amplification step only carried an EcoRI recognition sequence to allow amplification of the wild-type DksA (lacking a His6 tag).

**Generation of mutant DksA library**

Targeted amino acid substitutions were accomplished via a two-step megaprimer PCR method (Barik and Galinski 1991). In the first PCR step, designed to incorporate the substitution, an oligonucleotide annealing to a nucleotide region downstream of the dksA gene was used together with an upstream-annealing oligonucleotide containing the targeted nucleotide substitutions such that 1 or more of the wild-type codons would be replaced with a high-usage codon specifying for alanine, glutamine, glutamate, asparagine, or serine, as indicated in Table 2.2. The resulting PCR product was then used as a “megaprimer” in a second PCR step together with an oligonucleotide annealing to the beginning of the dksA gene (GG GAA TTC ATG TCT CAT CAT CAT CAT CAT AGC GGC ATC GAA GGC CGC ATG CAA GAA GGG CAA AAC CGT AAA ACA) that creates an EcoRI recognition sequence, a new AUG start codon, 6 histidine codons, and codons that produce the factor Xa cleavage site just prior to the beginning of the dksA gene, such that treatment of the resulting His6-DksA protein with factor Xa would precisely remove the N-terminal His6 tag leaving an intact WT DksA protein. The resulting Pcr product from the second PCR DNA was digested with EcoRI, cloned into the EcoRI-Smal sites of pKK223-3, transformed into strain RO1569 for protein expression and purification. Plasmids were subjected to the di-deoxy nucleotide DNA sequencing method to
verify the mutations using a DNA sequencing kit (US Biochemicals) and appropriate oligonucleotide primers. Plasmids expressing WT or mutant DksA were transformed into strain RO1550 (fisp::lacZ) or strain RO1828 (uspA::lacZ) to examine the effect of the mutations on the ability to repress or stimulate transcription, respectively. The resulting plasmids and strains are listed in Table 2.2.

**Preparation of His6-tagged DksA**

Saturated cultures of *E. coli* strain RO1757 were diluted 2000-fold in a liter of Luria-Bertani (LB) medium containing 75 µg/ml spectinomycin and 100 µg/ml ampicillin and grown at 37°C in an air shaker to an early exponential growth phase (OD$_{600}$ 0.2-0.3). The cell were then induced with 0.2 mM IPTG for an additional 2 hours at 37°C with shaking. Cells were harvested by centrifugation at 6000 RPM (Sorvall GSA6 rotor) for 15 minutes and lysed in 50 ml of lysis buffer (10 mM Tris-base, pH 7.5, 80 mM NaCl, 0.1 mM phenylmethane sulfonyl fluoride [PMSF]) via sonication (Sonic & Materials Inc., ultrasonic processor VC-50) by five, 10-second pulses at 50 watts power output and 60% amplitude, while on ice. The insoluble fraction was removed by centrifugation at 10,000 rpm (Sorvall SS34 fixed angle rotor) for 15 minutes at 4°C. The resulting lysate was then passed over a 1 ml bed of Ni-NTA (Bio-Rad, Qiagen) equilibrated with 5 mM imidazole in MCAC (20 mM Tris-Cl pH 7.9, 0.5 M NaCl, 10% glycerol, 1 mM PMSF) at a rate of about 40 ml/hour. The Ni-NTA column was washed with 5 bed volumes of MCAC containing 20 mM imidazole. The His6-tagged DksA was subsequently eluted with MCAC containing 500 mM imidazole. Eluted fractions, concentration analyzed by Bradford reagent, were combined and dialyzed in dialysis buffer (20 mM Tris-base pH 7.5, 50 mM NaCl) at 4°C. For long term-storage at -20°C, proteins were dialyzed in dialysis buffer containing 50% glycerol.
\[ ^{32}\text{P} \text{ labeling in vivo} \]

*E. coli* strain RO1757 was grown in 6 ml of low phosphate media (1.3 mM sodium citrate, 0.8 mM MgSO\(_4\), 2.5 \(\mu\)M Fe\(_3\), 7.5 mM (NH\(_4\))\(_2\)SO\(_4\), 0.2 mM KH\(_2\)PO\(_4\), 100 mM Tris-base, pH 7.4, 0.4% D-Glucose, 15 \(\mu\)M thiamine, 1 mM arginine, 0.2% casamino acids, 98 \(\mu\)M tryptophan) containing 100 \(\mu\)g/ml ampicillin and 75 \(\mu\)g/ml spectinomycin at 37\(^\circ\)C in a shaking water bath until early exponential growth phase (OD\(_{600}\) 0.2-0.3). DksA expression was then induced for 2 hours at 37\(^\circ\)C with 0.2 mM IPTG along with 50 \(\mu\)l of 5 mCi \(^{32}\text{P}\)-labeled orthophosphoric acid. Cells were then harvested by centrifugation and lysed by vortex in 300 \(\mu\)l bacterial protein extraction reagent (B-PER; Thermo Scientific) in the presence of 10 \(\mu\)l protease inhibitor cocktail (Sigma-Aldrich), 30 \(\mu\)l phosphatase inhibitor cocktail (Sigma-Aldrich), 10 \(\mu\)l of DNase I (1000 units/ml) (Sigma-Aldrich), and 2.5 \(\mu\)l RNase A (10,000 units per ml) (Affymetrix). The resulting lysate was clarified by centrifugation and the soluble fraction mixed with 2.7 ml of MCAC containing 5 mM imidazole. The sample was then passed through a disposable gravity-flow chromatography column pre-packed with 200 \(\mu\)l of Ni-NTA bead slurry (Qiagen) that had been equilibrated in MCAC made with 5 mM imidazole. The bound protein was washed with MCAC containing 20 mM imidazole, and eluted with MCAC made with 500 mM imidazole.

**Factor Xa cleavage**

The N-terminal His6-tag was removed from \(^{32}\text{P}\)-labeled His6-DksA by incubating with factor Xa (Novagen) for 1 to 4 hours under the conditions outlined by the manufacturer. Samples were removed every hour, mixed with Xarrest Agarose (Novagen) and centrifuged to remove factor Xa. Removal of the His6-tag was verified by 15% SDS-PAGE, in which the protein bands were visualized by coomassie blue staining or autoradiography.
**Pro-Q® Diamond phosphoprotein staining**

The commercially available Pro-Q® Diamond protein stain (Invitrogen) is a fluorescent gel stain that is selective for phosphorylated proteins. Protein samples separated by 15% SDS-PAGE were stained according to the manufacturer’s recommendations and imaged on a Typhoon 9400 using a 532 nm laser and 560 nm long pass filter. SYPRO® Ruby total protein fluorescent gel stain was subsequently used on the same gels following the manufacturer’s instructions and imaged on a Typhoon 9400 using a 457 nm laser and 610 nm band pass filter.

**Phosphor-enriching phosphorylated protein**

The Pierce Phosphoprotein Enrichment kit from Thermo™ was used as directed by the manufacturer to enrich the phosphorylated pool of purified His6-DksA. When using this procedure to prepare proteins for mass spectrometry analysis, we successfully used an in-house buffer (100 mM Sodium Phosphate, pH 7.5, 50 mM NaCl) that lacks the detergent CHAPS, which interferes with mass spectrometry.

**Chemical cleavage of 32P-labeled DksA**

For BNPS-Skatole cleavage, 100 µl of purified 32P-labeled DksA (~27 µg/100 µl) was precipitated with 4 volumes of ice-cold acetone at -20°C for 15-20 minutes, centrifuged at 12,000 rpm for 10 minutes, and the resulting protein pellet resuspended in 99 µl glacial acetic acid, with 1 mM PMSF. One µl of 3-Bromo-3-methyl-2-(2-nitrophenyl)sulfanylindole (BNPS-Skatole; 50 µg/µl) solution was added and allowed to react for 1 hour at 42°C in the dark. The resulting cleaved DksA was precipitated once more in acetone at -20°C for 15–20 minutes before being resuspended in gel loading buffer (50 mM Tris pH 6.8, 2% w/v SDS, 200 mM β-mercaptoethanol, 10% v/v glycerol, 0.2% w/v bromophenol blue) and separated by SDS-PAGE on a 15% polyacrylamide gel and visualized by autoradiography.
For CNBr Cleavage, 100 µl of purified DksA was acetone precipitated in 4 volumes of ice-cold acetone at -20°C for 15–20 minutes, centrifuged, and the resulting protein pellet resuspended in 30 µl CNBr buffer (0.1 M Tris base, pH 8.0, 3% w/v SDS, 1 µM dithiothreitol, 20 mM 2-hydroxyethyl disulfide) and heated to 100°C for 2 minutes. We then added 70 µl of 70 µg/µl CNBr made in formic acid and allowed to cleave for 4 hours at room temperature in the dark. The reaction was stopped by heating to 50°C and adding of 200 µl N-ethylmorpholine. Cleaved DksA was precipitated once more in 4 volumes of acetone at -20°C for 15–20 minutes, centrifuged, and the pellet resuspended in gel loading buffer, separated by SDS-PAGE on a 15% polyacrylamide gel and visualized by autoradiography.

Western blot

Proteins separated by SDS polyacrylamide gel electrophoresis were electro-blotted onto 0.2 µm nitrocellulose filter paper in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% v/v methanol, pH 8.3) for 16 hours at 15 volts. The nitrocellulose blot was washed for 5 minutes in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4, 0.1% v/v TWEEN 20) twice before blocking for 1 hour in a solution of 3% w/v freeze-dried milk in PBST. Following an additional 2 rinses in PBST, the blots were incubated for 1.5 hours in a primary antibody solution (PBST with 1% bovine serum albumin, and 1:1000 dilution of rabbit, anti-DksA antibody). The blots were washed twice again in PBST and incubated in a secondary antibody solution (PBST with 1% bovine serum albumin, and 1:3000 dilution goat anti-rabbit horse radish peroxidase-conjugated antibody [Bio-Rad]) for 1 hour. Colorimetric development was performed using the Bio-Rad Opti-4CN™ development kit, as recommended by the manufacturer.
Phosphorylation analysis by autoradiography

All DksA mutants (Table 2.2) were $^{32}$P-labeled in vivo, purified as described above, and separated by 15% SDS-PAGE. The gel was exposed to a phosphor-screen and the $^{32}$P-signal quantified by phosphorimaging. The same gel was then submitted to western blot analysis using anti-DksA antibody. Relative band intensities were measured using ImageQuant software and the amount of mutant protein present in each lane was determined by comparison with the bands intensities of the WT DksA control. To assess the relative level of protein phosphorylation in vivo, the $^{32}$P signal-to-protein ratio is averaged among 3 or 4 samples and compared to that of the WT protein.

β-galactosidase assays

β-galactosidase assays were performed as previously described (Miller 1992). Saturated cell cultures were diluted 100-fold in LB medium containing 75 µg/ml spectinomycin and 100 µg/ml ampicillin and grown at 37°C with shaking for 4 hours. The results are expressed as an average from 4 independently-grown cultures ± standard deviations. For the transcription repression assay, the percent transcription repression attained by each of the mutant DksA proteins tested is obtained using the equation:

$$% \text{ transcription repression} = 100 \times (\beta\text{-galactosidase activity in absence of DksA} - \beta\text{-galactosidase activity in presence of mutant DksA})/(\beta\text{-galactosidase activity in absence of DksA} - \beta\text{-galactosidase activity in the presence of WT DksA}).$$

For the transcription stimulation assay, the percent transcription stimulation attained by each of the mutant DksA proteins tested is obtained using the equation:
**Figures and Legends**

**Figure 2.1. DksA is Phosphorylated In Vivo.** *E. coli* BL21 transformed with pRO552 (RO1757) was grown in low-phosphate growth medium at 37°C. His6-DksA expression was induced by treatment with 0.2 mM IPTG in the presence of $^{32}$P orthophosphoric acid. Purified radiolabeled His6-DksA was incubated with factor Xa for up to 2 hours at room temperature and electrophoretically separated in 15% SDS polyacrylamide gel. (A) Autoradiography of in vivo-labeled $^{32}$P-DksA. Lane 1: purified $^{32}$P-labeled His6-DksA; Lane 2: $^{32}$P-labeled DksA after 2 hours of factor Xa treatment. (B) Coomassie blue staining of purified His6-DksA before factor Xa treatment (Lane 1) and after 1 hour (Lane 2) and 2 hours (Lane 3) of factor Xa treatment.
Figure 2.2. In vivo DksA phosphorylation occurs in rich medium with or without the His6-tag. (A) Specificity of Pro-Q® Diamond staining. PeppermintStick™ molecular weight standards containing 2 phosphorylated proteins (45 kDa ovalbumin and 23.6 kDa β-casein) and 4 non-phosphorylated proteins were electrophoretically separated in a 15% SDS polyacrylamide gel and stained with Pro-Q® Diamond (phosphoprotein gel stain) and subsequently with SYPRO® Ruby (for general protein fluorescent staining), as indicated beneath the lanes. (B) Detection of in vivo-phosphorylated non His-tagged DksA. E. coli strain RO1630 was grown in LB medium to an OD600 of 0.2 and treated with 0.2 mM IPTG for 1 hour to induce expression of wild-type DksA (without a His6-tag). Cell lysates prepared before (-) and after (+) IPTG treatment were separated in a 15% SDS polyacrylamide gel and stained with the Pro-Q® Diamond phosphoprotein gel stain. A band corresponding to DksA is indicated with an arrow. (C) Phosphorylated His6-DksA is enriched by metal ion chromatography. Purified, His6-DksA was enriched for phosphorylated protein using a commercially available phosphoenrichment chromatography column (Pierce). Samples taken prior to and following phosphoenrichment were separated by SDS-PAGE and stained with Pro-Q® Diamond and subsequently with SYPRO® Ruby as recommended by the manufacturer. Fluorescently stained proteins were visualized using a Typhoon 9400 laser scanner.
A

Phosphorylated Proteins

SYPRO Ruby
Pro-Q Diamond

B

IPTG-Induction
-
+

Pro-Q Diamond

C

Phospho-enrichment of His6-DksA
-
+

Pro-Q Diamond
SYPRO Ruby
Figure 2.3. Multiple DksA Phosphorylations Detected by Mass Spectrometry. Comparison of purified, His6-DksA prior to (un-enriched) and after enrichment with a commercially available phosphoenrichment chromatography column (contributed by Ke Xia, Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY)
Figure 2.4. Two $^{32}$P-Labeled Bands of Unknown Content Consistently Co-Purify with His6-DksA. *E. coli* BL21 transformed with pRO552 (RO1757) was grown in low-phosphate growth medium at 37°C. His6-DksA expression was induced by treatment with 0.2 mM IPTG in the presence of $^{32}$P orthophosphoric acid. Chromatographically purified, $^{32}$P-labeled His6-DksA was visualized by SDS polyacrylamide gel followed by autoradiography, routinely revealing the co-purification of 3 additional unknown radiolabeled bands. Enzymatic digestion was utilized to determine the macromolecular content of these bands with factor Xa along with either RNase, DNase or Proteinase K added as indicated. The radiolabeled contaminant bands that co-purify with DksA are indicated by *. 
Figure 2.5. Cleavage of DksA by BNPS-Skatole Indicates Two Separately $^{32}$P-labeled Regions of DksA. *E. coli* BL21 transformed with pRO552 (RO1757) was grown in low phosphate growth medium at 37°C. His6-DksA expression was induced by treatment with 0.2 mM IPTG in the presence of $^{32}$P orthophosphoric acid and the resulting protein purified by Ni$^{2+}$-IMAC. Purified His6-DksA was treated by factor Xa, acetone or BNPS-Skatole as indicated. Following treatment, samples were analyzed by 15% SDS-PAGE and visualized by autoradiograph.
Figure 2.6. Cleavage of $^{32}$P-labeled His6-DksA by CNBr Results in a Phosphorylated Peptide Roughly 14 kDA in Size. *E. coli* BL21 transformed with pRO552 (RO1757) was grown in low phosphate growth medium at 37°C. His6-DksA expression was induced by treatment with 0.2 mM IPTG in the presence of $^{32}$P orthophosphoric acid and the resulting protein purified by Ni$^{2+}$-IMAC. Purified, radiolabeled His6-DksA was cleaved by CNBr as indicated, and the resulting DksA peptides separated by SDS-PAGE and exposed to autoradiography.
Figure 2.7. Phosphorylation of DksA is labile in low pH. *E. coli* BL21 transformed with pRO552 (RO1757) was grown in low-phosphate growth medium at 37°C. His6-DksA expression was induced by treatment with 0.2 mM IPTG in the presence of $^{32}$P orthophosphoric acid and the resulting protein purified by Ni$^{2+}$-IMAC. Purified, radiolabeled His6-DksA was incubated in H$_2$O or 5% TFA as indicated for 2 hours (right set) or overnight (left set). Samples were precipitated with acetone as indicated, and subsequently analyzed by SDS-PAGE followed by autoradiography and western blot using anti-DksA antibodies.
Figure 2.8. Most Mutations Did Not Result in a Reduction in $^{32}$P Labeling. Over 38 different mutants (covering 101 amino acids) of DksA were analyzed for their ability to $^{32}$P label in vivo. (A) A number of *E. coli* BL21 strains expressing a library of mutant DksA were grown in low-phosphate growth medium at 37°C. His6-DksA expression was induced by treatment with 0.2 mM IPTG in the presence of $^{32}$P orthophosphoric acid and the resulting protein purified by Ni$^{2+}$-IMAC. Purified, radiolabeled DksA was analyzed by SDS-PAGE and the resulting gel visualized by autoradiograph and western blot with anti-DksA antibodies. (B) Quantification of the relative $^{32}$P signal per protein. WT and mutant DksA $^{32}$P and western blot signals were quantified from experiments shown in panel A and supplemental Figure 2.S.1 that were done in quadruplicate sets and shown as a ratio of $^{32}$P/protein signal normalized to the WT. The WT ratio was arbitrarily assigned a value of 100 and all other ratios are shown relative to this one. The results are given as an average of 4 ratios and the error bars represent standard deviation. * denotes p-values<0.05; ** denotes p-values<0.01; *** denotes p-values<0.001.
**Figure 2.9. Some Mutations Result in the Loss of Phosphorylation.** (A & B) *E. coli* BL21 strains expressing mutant DksA as indicated were grown in low-phosphate growth medium at 37°C. His6-DksA expression was induced by treatment with 0.2 mM IPTG in the presence of $^{32}$P orthophosphoric acid and the resulting protein purified by Ni$^{2+}$-IMAC. Purified, radiolabeled DksA was analyzed by SDS-PAGE and the resulting gel visualized by autoradiograph and western blot with anti-DksA antibodies. (C) Quantification of the relative $^{32}$P signal per protein. The signals in panel A and B were quantified as described in Figure 2.8B.
A

B

C

WT DksA

Δ1-31 DksA

Δ1-31

Y23A

K26A

Y31A

Δ1-31

K101A

T102A

Y31A

Δ1-31

K104A

K105A

32P/Protein
Figure 2.10. Engineered His6-tag does not interfere with the function of DksA. Saturated cultures of RO1550 E. coli strain carrying a fisp::lacZ fusion and plasmid-expressed WT (pRO553), His6-DksA (pRO552), or vector only (pKK223-3) were diluted 100-fold in LB medium containing 100 µg/ml ampicillin and 75 µg/ml spectinomycin, and assayed for β-galactosidase activity during both exponential and stationary growth phases. (A) Schematic of the DNA reporter construct expressing β-galactosidase, consisting of the fis promoter region (fisp) fused to lacZ. The arrow shows the direction of transcription. (B) β-galactosidase activities during both stationary (green, left) and mid exponential (gray, right) growth phases were averaged from 4 independent trials and plotted as indicated.
Figure 2.11. “Leaky” Expression from the pKK223-3 High Expression Vector is within the Physiological Range of Chromosomally Expressed DksA. (A) Saturated cultures of E. coli MG1655 were diluted 100-fold in LB medium and grown at 37°C. At the intervals of time indicated beneath the gel, equivalent cell quantities (based on OD_{600} units) were harvested, lysed, and separated by SDS-PAGE along with the indicated quantities (ng) of purified His6-DksA (shown at the top of the gel), which were loaded 2 hours after electrophoresis was initiated. The resulting gel was subjected to a western blot using anti-DksA antibody. (B) Saturated cultures of E. coli MG1655 ΔdksA transformed with pRO552 (His6-DksA) were diluted 100-fold in LB medium containing 100µg/l ampicillin and grown at 37°C until early exponential growth phase. Cells were then treated with the IPTG concentrations indicated below the gel for 1 hour. Samples were separated by SDS-PAGE along with the indicated quantities at the top of the gel (ng) of purified His6-DksA, which were loaded 2 hours after electrophoresis was initiated. The resulting gel was subjected to western blotting using anti-DksA antibody.
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### Diagrams

**A**
- **His6-DksA**
- **DksA**

**B**
- **His6-DksA**
- **His6-DksA**
- **His6-DksA**
Figure 2.12. The N-terminal Phosphorylation Reduces the Ability of DksA to Function as a Transcriptional Stimulator of *uspA*. Saturated cultures of RO1550 *E. coli* strain carrying a *fisp::lacZ* fusion (A) or RO1828 *E. coli* strain carrying an *uspA::lacZ* fusion (B) and plasmid-expressed WT or mutant DksA previously shown to be deficient in their ability to $^{32}$P label, were diluted 100-fold in LB medium containing 100 µg/ml ampicillin and 75 µg/ml spectinomycin, grown for 4 hours at 37°C, and assayed for β-galactosidase activity. β-galactosidase activities were averaged from 4 independently grown cultures and used to determine the percent of WT DksA repression activity using the following equation. Error bars represent standard deviation. * denotes p-values<0.05; ** denotes p-values<0.01; *** denotes p-values<0.001:

\[
\% \text{ transcription repression} = 100 \frac{(\beta\text{-galactosidase activity in absence of DksA} - \beta\text{-galactosidase activity in presence of mutant DksA})}{(\beta\text{-galactosidase activity in absence of DksA} - \beta\text{-galactosidase activity in the presence of WT DksA})}
\]
A

Relative expression under stimulation and repression conditions for different strains.

B

Relative expression under stimulation and repression conditions for different strains.
Figure 2.13. Structural Relationship of DksA Residues Likely Targeted for Phosphorylation. DksA (light brown) and ppGpp (pink) bound to RNAP (gray). PDB: 5VSW; (Molodtsov et al. 2018)
Supplemental Figure

Figure 2.S.1. Most mutations did not result in a reduction in $^{32}$P-labeling. Complete set of DksA mutants assayed for loss of phosphorylation by $^{32}$P autoradiography and western blot. All mutants analyzed are grouped by gel, and loss of phosphorylation is measured relative to the “WT” (full length or Δ1-31 DksA as indicated). Analyses of figures not included in Figure 2.8 are included here.
Table 2.1. Bacterial Strains and Plasmids Used in this Work

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<td><em>E. coli</em> K12, F- λ- lacI lacZ</td>
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† Unless otherwise indicated, listed strains and plasmids were generated as part of this work.
### Table 2.2. List of Plasmids and Strains Carrying Wild Type and Mutant DksA

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† This information described in Duclos, Marcandier, & Cozzone 1991; Martensen 1984.
References


3. **Distinct roles of DksA are required for stimulation and repression of transcription in vivo**

This work was conducted in collaboration with Sui Tung Cheung and Robert Osuna.

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**Abstract**

DksA is a bacterial gene regulator that functions synergistically with the stress alarmone ppGpp to mediate the stringent response. It also functions independently of ppGpp to regulate transcription of a number of genes. Its function is dependent on its binding affinity to RNA polymerase and requires specific interactions between RNAP and catalytic amino acids located on the coiled coil tip, D74 and A76. In this work, we examined the effects of a number of DksA substitution mutants and 2 deletion mutants, including some affecting key residues known to be involved in ppGpp binding, on the ability to repress transcription of the fis promoter (fisp) or to stimulate transcription of uspA promoter (uspAp) in vivo. Our results show that DksA tolerates a large number of mutations with minimal effects on its ability to repress transcription in vivo, suggesting that the integrity and stability of the DksA structure may involve extensive intramolecular interactions not easily disturbed by point mutations. However, we also found several DksA mutations (e.g., E21Q E25Q, K26A, R91A, K94A K97A K98A, C114A C117A, S116A, D137N, K139A, E143Q, R145A, E146Q) that strongly affect the ability to stimulate transcription but have little or no effect on its ability to repress transcription, demonstrating that certain functions of DksA are unique to its transcription stimulatory role. Some of these residues (R91, K94, K98, K139) are seen to be involved in ppGpp binding at the ppGpp site 2 of RNA
polymerase, according to recent crystallographic evidence. Other residues not interacting with ppGpp are also required for transcription stimulation. Some interact with RNA polymerase in a different conformational state and others form 2 clusters in a solvent-exposed surface of DksA in the ternary complex, suggesting that interactions with other factors besides ppGpp are required for efficient transcription stimulation.
Introduction

Bacteria respond to changing environmental conditions by rapidly altering transcription of hundreds of genes, utilizing a network of transcription factors (Browning and Busby 2016). Unlike most classic transcription factors, which function as DNA-binding proteins, DksA regulates transcription by binding RNA polymerase (RNAP) via the secondary channel (Paul et al. 2004; Perederina et al. 2004). DksA contains 151 residues that fold into a structure similar to that of other RNAP secondary channel-binding proteins, such as GreA and GreB, which form a globular head region and a coiled-coil (CC) region with an acidic tip. Transcriptional regulation by DksA is required for cellular responses to stress or amino acid starvation, in the prevention of transcriptional conflicts with DNA replication machinery, and for virulence of various pathogenic bacteria (Lee et al. 2012; Nakanishi et al. 2006; Paul et al. 2004; Perederina et al. 2004; Tehranchi et al. 2010).

DksA function can be potentiated by the small molecule alarmone guanosine pentaphosphate and guanosine tetraphosphate (collectively referred to here as ppGpp) (Paul et al. 2004; Paul, Berkmen, and Gourse 2005; Perederina et al. 2004; Molodtsov et al. 2018). During the stringent response, ppGpp and DksA act synergistically in vivo and in vitro to bind RNAP and repress transcription of a number of genes, including those for ribosomal RNA, ribosomal proteins, and the nucleoid-associated protein Fis, while simultaneously up-regulating genes that are critical for survival under stress, such as those for *iraP* and *drsA* (whose gene products promote RpoS expression), sigma 24, *uspA*, and various amino acid biosynthetic genes (Girard et al. 2017; Lemke et al. 2011; Magnusson et al. 2007; Mallik et al. 2006; Paul, Berkmen, and Gourse 2005; Paul et al. 2004). In the absence of ppGpp, DksA is able to bind RNAP, albeit with relatively lower affinity (Lennon et al. 2009; Molodtsov et al. 2018), and independently regulate
the expression of numerous genes in vivo and in vitro (Åberg et al. 2009; Magnusson et al. 2007; Paul et al. 2005). Moreover, the gene regulatory functions of DksA may differ in the presence or absence of ppGpp.

*E. coli* RNAP contains 2 binding sites for ppGpp. Site 1, located at the junction of the β’ and ω subunits, is DksA-independent. Site 2 is roughly 60 Å away from site 1 near the secondary channel of RNAP in close proximity to DksA and is only formed when DksA is bound to the RNAP (Molodtsov et al. 2018; Ross et al. 2016; Ross et al. 2013). The ternary crystal structure of RNAP, DksA, and ppGpp bound to site 2 reveals several DksA residues that are in contact with the phosphate groups of ppGpp, suggesting that these interactions may be required for the synergistic effects between ppGpp and DksA. Indeed, several of the residues involved (L95, K98, R129, and K139) were shown to be essential for ppGpp binding at site 2. However, they were not required for transcription repression of *rrnBP1* by DksA in vitro. DksA R91 is also involved in ppGpp binding to site 2. However, a mutation at this residue affected the ability of DksA to repress transcription independently of the presence of ppGpp (Ross et al. 2016). Hence, the functional significance of the interactions between DksA and ppGpp remain elusive.

In this work, we examine the effects of a number of DksA mutations, including some targeting key residues thought to be involved in ppGpp binding, on the ability to regulate transcription repression of the *fis* promoter (*fisp*) or stimulation of the *uspA* promoter (*uspAp*). We find that DksA tolerates a large number of mutations with minimal effects on its ability to repress transcription, suggesting that this protein is structurally and functionally robust. Some mutations (e.g., Δ1-31, D71A D74A, R125A, K139A K147A) affect the ability of DksA to both repress and activate transcription, indicating common roles of DksA in these regulatory functions. However, we also identify numerous DksA mutations (e.g., E21Q E25Q, K26A, E29Q
E30Q E34Q, R91A, R93A, K94A, K98A, S116A, T131A, D137N, K139A, T140A, E143Q, R145A, E146Q) that specifically affect its ability to stimulate transcription but not to repress transcription, demonstrating that certain functions of DksA are unique to its transcription stimulatory role. Some of these residues (R91, K94, K98, K139) are involved in ppGpp binding at the ppGpp site 2. In addition, several residues located near the N-terminal region of DksA are required for transcription stimulation even though they are not seen to be in contact with RNAP or ppGpp in the crystal structure. This suggests that additional molecular interactions with DksA residues or conformational changes not depicted in current structural models are required for transcription stimulation of uspA in vivo.
Results

**Mutations affecting over 60 percent of DksA were generated for functional analysis.**

Single or multiple mutations and 2 amino-terminal deletions were generated that collectively affect 92 of the 151 amino acids within DksA. Most of the targeted amino acids are solvent-exposed and well distributed throughout the entire protein (Figure 3.1). Two amino-terminal deletions remove the DksA residues 1 through 13 (∆1-13) or residues 1-31 (∆1-31) and are stably expressed in vivo as N-terminal His6-tagged proteins. Given the extensive alpha helical structure of DksA, most of the replacements were made to alanine so as to minimize effects on the local structure (Spek et al. 1999). Glutamic acid residues were conservatively replaced with glutamine. All DksA varieties were expressed from the plasmid pKK223-3 under control of the Ptac promoter. We have found by western blot analysis that DksA expression from this plasmid under non-induced conditions results in cellular DksA levels comparable to those generated from the chromosomal DksA (chapter 2).

**Mutational effects on fisp repression.**

The *fis* gene, encoding the nucleoid-associated protein Fis (factor for inversion stimulation) is transcribed from a single promoter (*fisp*) as an operon together with the upstream gene *dusB* (also known as *yhdG*), encoding tRNA dihydrouridine synthetase B (Ball et al. 1992; Mallik et al. 2004). Previous work demonstrated that DksA negatively regulates *fis* transcription in vivo and in vitro (Mallik et al. 2006). That work showed that the *fisp* repression effect by DksA in vivo was greatest after about 4 hours of outgrowth from saturated cultures in LB medium, when cells reached early stationary phase. Thus, in order to examine the effects of our entire set of DksA mutations on the ability to repress *fisp* transcription in vivo, saturated cultures of RO1550
(fisp:\lacZ) cells expressing wild-type or mutant DksA were diluted 100-fold in fresh LB growth medium, grown for 4 hours at 37°C, and examined for β-galactosidase activity (Figure 3.2). Remarkably, the majority of the examined mutations did not result in an appreciable loss in ability to repress fisp transcription (> 80% repression function). This could suggest that there are just a few critical residues that are specifically required for RNAP binding and transcription repression in vivo, or that multiple DksA residues play redundant roles in interacting with RNA polymerase to bring about binding and repression. Previous work has implicated a conserved D74 residue on the CC tip that comes near the active site on RNAP and is necessary for transcription repression (Lee et al. 2013). Our results are consistent with this finding, as DksA with dual substitutions at D71A and D74A resulted in a 60% loss of fisp repression compared to the WT DksA (Figure 3.2B). A deletion of the first 13 residues resulted in a modest (-12%) decrease in repression function in vivo, while deletion of the first 31 residues resulted in a greater (-33%) reduction in fisp repression function. Western blot analysis indicated that these deleted proteins were expressed at levels comparable to the WT DksA in vivo, suggesting that deletion of the first 31 residues does not appreciably affect the stability of the protein. These results suggest that 1 or more residues among the first 31 residues of DksA could be involved in fisp repression. Alternatively, the Δ1-31 deletion may cause a conformational change that affects the interactions between the remaining DksA and RNA polymerase.

The single mutation R125A strongly reduced the repression activity in vivo, resulting in a 68% loss of fisp transcription repression function and the single mutation E143Q resulted in a moderate reduction (-24%) in repression function (Figure 3.2B and D). However, most of the single point mutations and several of the double and triple mutations showed no appreciable loss in fisp repression function in vivo (Figure 3.2B). Even a DksA mutant containing 7 alanine...
substitutions (T9A S10A S11A S13A Y23A Y31A T131A) showed no loss in fisp repression function.

Given the high tolerance of DksA transcription repression function to a number of single, double, and triple mutations, we opted to test the effects of various other multiple DksA mutations engineered on a truncated Δ1-31 form of the protein, which was moderately deficient in repression function. It was hoped that this approach could help uncover DksA regions involved in transcription repression that might not be otherwise uncovered through the use of single mutations. To determine if the additional mutations affected the transcription repression function beyond what is observed with Δ1-31 DksA, we compared their effects on transcription repression to that of Δ1-31 DksA (Figure 3.2C). Moderate reductions in transcription repression function were observed for Δ1-31 R48A R52A E54Q R57A (32% reduction), Δ1-31 K94A K97A K98A (29% reduction), Δ1-31 E115Q E120Q (24% reduction), and Δ1-31 C114A C117A (23% reduction). The cysteines C114 and C117 are seen in the crystal structure to participate in the coordination of zinc together with C135 and C138 (Molodtsov et al. 2018; Perederina et al. 2004). The observation that their replacements with alanine caused modest reductions in transcription repression suggests that the zinc coordination function of these cysteines is not an absolute requirement for RNAP binding and repression in vivo. The multiple mutation Δ1-31 R87A R91A E92A R93A resulted in a stronger reduction (63%) in fisp repression function. However, the single mutations R91A and R93A present on the full-length DksA protein caused minimal effects on transcription repression (Figure 3.2B). This suggests that R87 and/or E92 play a role in repression. Alternatively, whereas individual mutations might make relatively small contributions to the repression function, collectively they result in a substantial loss of function. The mutation Δ1-31 K139A, K147A resulted in a severe loss (80%) of repression function.
function compared to Δ1-31 DksA (Figure 3.2C and D). However, the single mutation K139A showed no reduction in transcription repression function (Figure 3.2B), suggesting that the severe loss of function detected by Δ1-31 K139A K147A is predominantly caused by the K147A mutation (Figure 3.2D).

**Mutational effects on usp4p stimulation.**

In addition to its ability to repress transcription at a subset of genes, DksA also functions to stimulate transcription of an alternative set of genes including the universal stress protein A gene, *uspA* (Åberg et al. 2009). Transcription from the *uspA* promoter (*usp4p*) increases during growth inhibition caused by various conditions of stress including entry into the stationary phase (Farewell et al. 1996; Nyström and Neidhardt 1994). Its transcription is stimulated by DksA and ppGpp (Gummesson, Lovmar, and Nyström 2013). In order to determine the effects of our set of DksA mutations on the ability to stimulate *usp4p* transcription in vivo, saturated cultures of RO1828 (*usp4p::lacZ*) cells expressing wild type or mutant DksA were diluted 100-fold in fresh LB growth medium, grown for 4 hours at 37°C, and examined for β-galactosidase activity (Figure 3.3). Unlike the ability of DksA to repress *fisp*, the ability to stimulate transcription of *usp4p* was reduced to varying extents by as many as 27 different DksA mutants examined. Mutations of the acidic residues at the tip of the CC region of DksA (D71 and D74) were shown to severely reduce the ability of DksA to function in vivo (Lee et al. 2013; Perederina et al. 2004). This finding is validated in our *usp4p* stimulation assay because the D71A D74A mutant DksA was reduced by 72% in its ability to stimulate transcription in vivo (Figure 3.3B). Consequently, any DksA mutation in our set causing greater than a 70% reduction in transcription stimulation was considered to be severely affected in this function.
A deletion of the initial 13 N-terminal amino acids (Δ1-13) showed no loss of transcription stimulation function, while deletion of the initial 31 residues (Δ1-31) resulted in considerable reduction (55%) in the ability to stimulate uspA (Figure 3.3A). Confirmation for this effect came from examining single and multiple substitutions of residues within this region (E21Q E25Q, K26A, E29Q E30Q E34Q), which resulted in about a 30 to 45% reduction in the ability to stimulate transcription. The nearby mutation H39A similarly resulted in a modest reduction in transcription stimulation function. These results indicate that the amino-terminal region of DksA from residues 21-39 contains several residues that contribute to the full transcription stimulation function in vivo.

The mutation S116A exhibited a wide range of effects, depending on the context of other mutations present. For instance, whereas S116A showed about a 42% loss in function and S83A showed no appreciable loss in transcription stimulation function, the S83A S116A double mutation resulted in a complete loss of transcription stimulation function (Figure 3.3B). The latter effect cannot be attributed to a loss in protein stability in vivo both because western blot analysis indicate that these mutant proteins are expressed to levels similar to that of WT DksA (not shown) and because they are all able to bind RNAP to repress transcription at near-wild type levels in vivo (Figure 3.2B). Thus, while an effect of S83A mutation by itself is not detected in our in vivo transcription stimulation assay, it exacerbates the effect of S116A. On the other hand, the mutation Y113A, which by itself shows no appreciable loss in transcription stimulation function, is able to avert the effect of S116A (Figure 3.3B). Several positively charged residues in the DksA region from residues 91 to 98 were found to be required for the transcription stimulation function in vivo. Alanine replacements of R91 and K94 severely affected the ability to stimulate transcription (Figure 3.3B & D). The mutations R93A and K94A were also deficient
in the transcription stimulation function. Three of these residues (R91, K94, and K98) face the ppGpp bound at site 2 in the ternary complex (Figure 3.3D).

Residues closer to the C-terminal region of DksA also affected the ability to stimulate transcription to various degrees. The single alanine substitutions T137A and T140A each showed a moderate decrease in stimulation function (Figure 3.3B). Both are located in the C-terminal helix and their side chains extend outward into the solvent. The single mutations D137A, R145A, and E146Q each suffered a relatively strong reduction in the ability to stimulate transcription. These residues are also located in the C-terminal helix. The side chains of D137 and R145 extend outward toward the solvent. The side chain of E146 extends toward the pocket harboring the DksA-bound ppGpp but is too far (6.2 Å) from ppGpp in the crystal structure in direct contact with it. Three other single mutations—R125A, K139A, and E143Q—suffered severe reductions in the transcription stimulatory role of DksA. The side chain of K139 is closely associated with the DksA-bound ppGpp in the crystal structure (Figure 3.3D). The side chains of R125 and E143 extend into the DksA pocket harboring ppGpp but separated by 9.9 Å and 6 Å, respectively, β from ppGpp.

Several mutations affecting residues in the CC region of DksA (E65Q, R48A R52A E54Q R57A) caused moderate reductions in transcription stimulation (Figure 3.3C). The double mutation E115Q E120Q moderately reduced transcription stimulation. These residues are located in the globular region in a loop guarding the coordinated zinc. The double mutation C114A C117A, which showed only a moderate reduction in transcription repression (Figure 3.2C), was severely defective in the transcription stimulation function (Figure 3.3C). These residues are implicated in the coordination of zinc in the DksA structure. However, C135 and C138 also participate in the coordination of zinc but, surprisingly, the double mutation C135A C138A
showed no appreciable effect on the transcription stimulation function (Figure 3.3C). This raises a question concerning the importance of the zinc coordination in the transcription regulation function and suggests that C114 and/or C117 play a role in the transcription regulation function apart from their role in coordinating zinc.
Discussion

In order to successfully regulate transcription in *E. coli*, DksA must bind the secondary channel of RNAP as a monomer, making specific contacts between DksA and RNAP, which bring the catalytic residues at the CC tip close to the bridge helix at the active site (Lee et al. 2013; Lennon et al. 2012; Molodtsov et al. 2018). Although DksA is capable of acting without ppGpp to regulate the transcription of a large number of genes, the presence of ppGpp can potentiate the role of DksA, have no measurable effect, or have an opposite effect to that of DksA in vivo (Åberg et al. 2009; Magnusson et al. 2007).

Two distinct ppGpp binding sites have been identified in RNAP. Site 1 is bound by ppGpp in a DksA independent manner, while binding to site 2 requires DksA (Vrentas et al. 2005). The ppGpp bound to RNAP at site 2 is positioned in close proximity to DksA in the ternary structure formed with RNA polymerase, DksA and ppGpp, and the DksA residues R91, K94, K98, and K139 are seen to be well-positioned to form electrostatic interactions with the phosphate groups of ppGpp (Molodtsov et al. 2018; Ross et al. 2016; PDB: 5VSW). Moreover, mutations at these residues reduced the ppGpp binding affinity at site 2. However, such mutations were still well capable of repressing transcription of *rrnB* P1 in vitro (Ross et al. 2016), thus suggesting that these interactions between DksA and RNAP are not essential for repression.

Transcription repression at *rrnB* P1 in vitro can occur with ppGpp alone through its binding to site 1 on RNAP (Gourse et al. 2018; Ross et al. 2013). Transcription of *rrnB* P1 can also be repressed by DksA alone in vitro. In addition, ppGpp bound at site 2 in conjunction with DksA potentiates the transcription repression of *rrnB* P1 (Ross et al. 2016). In contrast, for transcription stimulation, the *iraP* (encoding 1 of several anti-adaptor proteins involved in the stabilization of the stress sigma factor RpoS) requires DksA and ppGpp bound at site 2 and is
unaffected by ppGpp binding to site 1 (Gourse et al. 2018; Ross et al. 2016). These observations have led to an emerging hypothesis that DksA may play different roles in the transcription stimulation and repression processes.

**The functional requirements for DksA are different for transcription stimulation and repression.**

The results presented in our work, using in vivo transcription repression and stimulation assays, provide persuasive support for the notion that DksA plays different roles in the transcription stimulation and repression processes. We found that alanine substitutions of DksA residues R91, K94, K98, and K139, which are required for ppGpp binding at site 2 and are shown in the crystal structure to be interacting with ppGpp (Molodtsov et al. 2018; Ross et al. 2016), are uniquely required for transcription stimulation of *uspA* but not for transcription repression of *fis* in vivo (Figure 3.4). Both of these in vivo transcription assays were conducted during the early stationary phase, when cellular ppGpp levels are known to increase (Buckstein, He, and Rubin 2008; Murray, Schneider, and Gourse 2003; Varik et al. 2017), yet DksA interactions with ppGpp were only critical for *uspA* stimulation (Figure 3.4). Thus, DksA appears to play a distinct role in positioning and stabilizing ppGpp at site 2 of RNAP during transcription stimulation in vivo.

Crystallographic studies show that there are conformational changes that occur in the promoter free DksA-RNAP-ppGpp ternary structure compared to the promoter free DksA-RNAP binary structure (Molodtsov et al. 2018). This suggests that different conformational states of a RNAP-DksA complex are required for the transcription stimulation compared to repression. This would not be surprising, given the different molecular events that must occur during these 2 processes. Promoters that are sensitive to DksA repression are characterized by their inability to form stable open transcription initiation complexes and by their requirement for high
concentrations of the transcription initiation NTP to increase the stability of open complexes (Mallik et al. 2006; Paul et al. 2005; Paul et al. 2004). During transcription repression at these promoters, DksA binds the secondary channel of RNAP to bring the CC tip near the active site of the holoenzyme and interacts with residues in the trigger loop of RNAP (Lennon et al. 2012; Perederina et al. 2004). Allosteric changes in RNAP induced by DksA binding (even in the absence of ppGpp) are detected on the basis of DksA-dependent changes in the RNAP-promoter footprinting patterns. Such allosteric changes may interfere with the formation of stable promoter complexes (Rutherford et al. 2009). It is also feasible that the positioning of the CC tip in this complex sterically hinders the association of the initiating NTP with the template DNA at the active site to prevent it from stabilizing the open complex. Hence, in this view, a principal function of DksA in repressing transcription is to properly bind the secondary channel of RNAP to present its CC tip within the active site and trigger an allosteric change in RNAP.

Promoters that are stimulated by DksA tend to be characterized by relatively weak RNAP-promoter binding to form closed complexes. It is proposed that, in order to stimulate transcription at these promoters, DksA binding to RNAP acts to lower the energy barrier required to transition from a closed complex to a relatively stable intermediate RNAP-promoter complex (Paul et al. 2005). Once formed, these stable intermediate complexes can then proceed to initiate transcription faster than they can dissociate, hence resulting in higher transcription activity.

Why is ppGpp binding at site 2 required for transcription stimulation? One possibility is that the transcription stimulation process demands a more stable association between DksA and RNAP that could endure the transitions through 1 or more intermediate complexes on the path to the productive initiation of transcription. The interactions between ppGpp bound at RNAP site 2
and DksA can provide this needed stability. It has been shown that ppGpp increases the binding affinity of DksA for RNAP (Molodtsov et al. 2018). Over-expression of DksA in vivo can partially stimulate transcription of various promoters in the absence of ppGpp (Magnusson et al. 2007), suggesting that an equilibrium shift toward the bound state of DksA can favor the transcription stimulation process. In addition, a DksA mutation (N88I) that increases its binding affinity to RNAP was shown to stimulate transcription of the *livJ* promoter in the absence of ppGpp (Blankschien et al. 2009).

It is also possible that the change in conformational state observed in the presence of ppGpp bound at site 2 (Molodtsov et al. 2018) additionally facilitates the transition toward productive initiation complexes. This may be aided by new interactions formed between DksA and RNAP in the presence of ppGpp. For instance, ppGpp binding at site 2 allows for the formation of a contact between DksA Gln63 with Arg1242 of the β' subunit of RNAP, DksA Asn68 with Lys598 in the β' subunit, DksA Asp71 at the CC tip with Arg1106 in the β subunit, and DksA R93 with the backbone carbonyl group of I937 in the β' subunit. We found that DksA R93A was considerably reduced (~46%) in its ability to stimulate transcription of *uspA*P, but retained its full ability to repress *fisp* transcription (Figure 3.2 and Figure 3.3), demonstrating that this interaction plays a unique role in transcription stimulation. In contrast, DksA R125 is able to make contact with D674 in the β' subunit both in the presence and absence of ppGpp. Previous work (Parshin et al. 2015), utilizing R125A mutants, found them to be reduced for both RNAP binding as well as for *rrnBP1* repression. When combined with the N88I “super DksA” mutation, which improves binding to RNAP (R125A/N88I), they were able to rescue the deficiency of R125A. In our assays, DksA R125A was defective for both repression of *fisp* and stimulation of *uspA*P transcription (Figure 3.2 and Figure 3.3), which is consistent with a loss of a DksA-RNAP
interaction that is common to both processes. It is not clear why the effect of R125A is more severe for the stimulation than the repression function in our assays. One possibility is that transcription stimulation by DksA demands a higher binding affinity and is therefore more sensitive to the DksA-RNAP binding affinity than the repression function. Another example is seen with the DksA E143 residue, which binds the backbone amino group of Gly676 in the β' subunit of RNAP both in the presence and absence of DksA. The DksA E143Q mutant is again defective for both the repression and stimulation function, albeit more severely in the latter (Figure 3.2 and Figure 3.3).

Two other DksA mutations, T131A and E146Q, are defective for transcription stimulation but not repression (Figure 3.2 and Figure 3.3). Neither of these residues is seen to be interacting with other components of the ternary structure (Figure 3.4B). However, their side chains are within 6 and 6.2 Å from 1 of the 5’ phosphates of ppGpp. Thus, it is conceivable that 1 or both of these residues are required to interact with ppGpp once the ternary complex binds the promoter and transitions through various intermediate states toward the open complex to initiate transcription. Allosteric changes in the ternary complex during this process may bring into play these 2 residues.

**Two solvent-exposed regions are involved in transcription stimulation.**

A remarkable observation arising from our studies is that a number of residues clustered in 2 regions at or near the solvent-exposed globular region of DksA were found to be required for efficient transcription stimulation but not for transcription repression (Figure 3.5). Region 1 includes C114, S116, C117, D137, T140, and R145, near the position of the coordinated zinc. Region 2 includes E21, E25, K26, E29, E30, E34, and H39. Mutations in these residues were only affected, or were much more severely affected, in their loss of transcription stimulation
compared to repression. We suggest that both of these regions may serve as contact surfaces for intermolecular interactions not revealed in the current crystal structure, which are required for transcription stimulation in vivo. Region 1 is ~19 Å away from residues 267-272 in the β subunit of RNA polymerase. Hence, these regions are unable to interact in the current structure, unless a sizeable conformational change occurring during transcription stimulation brings these regions together. Region 2 is fully solvent-exposed at the globular extremity of the DksA structure. Central to this region is K26, which we have shown is involved in phosphorylation of DksA in vivo (chapter 2). Several observations pointed to K26 as a likely target of phosphorylation: (1) the mutation Y23 K26 Y31 resulted in a decrease in phosphorylation and transcription stimulation but Y23 and Y31, in the context of several other mutations, did not affect phosphorylation or transcription stimulation; (2) DksA phosphorylation is acid-labile, as may be expected for a phosphorylated lysine; and (3) a decrease in transcription stimulation observed for the K26A mutation was fully restored with the phosphomimetic mutation K26E. A phosphorylated lysine and 5 glutamic acid residues in this region comprise a fully exposed negatively charged surface that could serve as a binding target of a positively charged soluble factor. This hypothetical factor could well be another protein that binds to region 2 in vivo and perhaps also to other regions of RNA polymerase to further stabilize the association of DksA with RNAP. The crystal structure of DksA is that of a decamer, suggesting that DksA may form a quaternary structure (Perederina et al. 2004). Indeed, we have observed that DksA is able to multimerize into various quaternary states in vitro and in vivo (Appendix). One of the intermolecular interactions involves the globular domain harboring region 2. Thus, it is not unreasonable to envision a second DksA protein interacting with region 2 and other parts of the
RNAP surface in this region to further stabilize the complex and efficiently stimulate transcription.

In summary, we have identified a number of DksA residues that are uniquely or predominantly required for transcription stimulation compared to repression, strongly suggesting DksA uses different mechanisms to achieve these 2 functions in vivo. A subset of these residues is involved in binding the ppGpp at site 2. Another subset is involved in contacting RNAP in a different conformation. A third subset of residues forms 2 solvent-exposed regions that may invite additional intermolecular interactions with additional factors to stimulate transcription in vivo.
Materials and methods

Chemicals and enzymes

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Bacterial growth media were from Difco. All enzymes were purchased from New England Biolabs or Bio-Rad Laboratories and all oligonucleotides used for PCR and DNA sequencing were ordered from Integrated DNA Technologies. DksA antibodies were generated by ABCAM.

Bacterial strains and plasmids

The main bacterial strains used in this work are listed in Table 3.1. RO1393 was constructed by infecting RO1250 with a previously made lambda strain carrying a lacZ fusion to the fisP region from -373 to +83 relative to the start of transcription (Ball et al. 1992). RO1550 was made by transforming RO1393 with plasmids pMS421 and pKK223-3 and selecting for spectinomycin (75 µg/ml) and ampicillin (100 µg/ml) resistance. RO1554 was made by transforming RO1393 with pMS421 and selecting for resistance to 75 µg/ml spectinomycin. RO1569 was created by first amplifying the dksA::tet knockout by P1 transduction from RO120 into BL21 (DE3) and selecting for growth in LB agar containing 15 µg/ml tetracycline to create RO1569. RO1630 is RO1393 transformed with pMS421 and pRO553; RO1757 is RO1569 transformed with pMS421 and pRO552 and selecting for resistance to 75 µg/ml spectinomycin and 100 µg/ml ampicillin.

Plasmid pRO552 was created by first amplifying the dksA gene by the polymerase chain reaction (PCR) using E. coli VH1000 chromosomal DNA as template, a downstream oligonucleotide that anneals to a region downstream of the rho-independent terminator sequence, and an upstream oligonucleotide that anneals to the beginning of the dksA gene and creates an EcoRI restriction site, a methionine start codon followed by 6 histidine codons, and sequence
encoding for an factor Xa protein recognition sequence, immediately preceding the AUG start codon for DksA. The amplified DNA product was cleaved with EcoRI, ligated into the EcoRI-Smal sites of pKK223-3, transformed into RO1554, and selected for resistance to 75 µg/ml spectinomycin and 100 µg/ml ampicillin. Plasmid pRO553 was made similarly to pRO552, except that the upstream oligonucleotide used in the PCR amplification step only carried an EcoRI recognition sequence to allow amplification of the wild-type DksA (lacking a His6 tag).

**Generation of mutant DksA library**

Targeted amino acid substitutions were accomplished via a two-step megaprimer PCR method (Barik and Galinski 1991). In the first PCR step, designed to incorporate the substitution, an oligonucleotide annealing to a nucleotide region downstream of the dksA gene was used together with an upstream-annealing oligonucleotide containing the targeted nucleotide substitutions such that 1 or more of the wild-type codons would be replaced with a high-usage codon specifying for alanine, glutamine, glutamate, asparagine, or serine, as indicated in Table 3.2. The resulting PCR product is then used as a “megaprimer” in a second PCR step together with an oligonucleotide annealing to the beginning of the dksA gene (GG GAA TTC ATG TCT CAT CAT CAT CAT CAT CAT AGC GGC ATC GAA GGC CGC ATG CAA GAA GGG CAA AAC CGT AAA ACA) that creates an EcoRI recognition sequence, a new AUG start codon, 6 histidine codons, and codons that produce the factor Xa cleavage site just prior to the beginning of the dksA gene, such that treatment of the resulting His6-DksA protein with factor Xa would precisely remove the N-terminal His6 tag leaving an intact WT DksA protein. The resulting PCR products from the second PCR DNA were digested with EcoRI, cloned into the EcoRI-Smal sites of pKK223-3, and transformed into strain RO1569 for protein expression and purification. Plasmids were subjected to the di-deoxy nucleotide DNA sequencing method to
verify the mutations using a DNA sequencing kit (US Biochemicals) and appropriate oligonucleotide primers. Plasmids expressing WT or mutant DksA were transformed into strain RO1550 (fisP::lacZ) or strain RO1828 (uspA::lacZ) to examine the effect of the mutations on the ability to repress or stimulate transcription, respectively. The resulting plasmids and strains are listed in Table 3.2.

**β-galactosidase assays**

β-galactosidase assays were performed as previously described (Miller 1992). Saturated cell cultures were diluted 100-fold in LB medium containing 75 µg/ml spectinomycin and 100 µg/ml ampicillin and grown at 37°C with shaking for 4 hours. The results are expressed as an average from 4 independently-grown cultures ± standard deviations. For the transcription repression assay, the percent transcription repression attained by each of the mutant DksA proteins tested is obtained using the equation:

\[
\text{% transcription repression} = 100\times(\beta\text{-galactosidase activity in absence of DksA} - \beta\text{-galactosidase activity in presence of mutant DksA})/\beta\text{-galactosidase activity in absence of DksA} - \beta\text{-galactosidase activity in the presence of WT DksA}).
\]

For the transcription stimulation assay, the percent transcription stimulation attained by each of the mutant DksA proteins tested is obtained using the equation:

\[
\text{% transcription stimulation} = 100\times(\beta\text{-galactosidase activity in presence of mutant DksA} - \beta\text{-galactosidase activity in absence DksA})/\beta\text{-galactosidase activity in presence of wild type DksA} - \beta\text{-galactosidase activity in the absence of WT DksA}).
\]
Figures and Legends

Figure 3.1. Mutated residues in DksA mutant library. *E. coli* DksA crystal structure (PDB: 5VSW; Molodtsov et al. 2018) highlighting all mutated residues represented in our DksA mutant library. Targeted amino acids are colored by type as indicated and the coordinated zinc ion is represented in magenta. The N-terminal region extending from residues 1-31 is shown in lavender.
Figure 3.2. Effect of DksA mutations on fisp transcription repression in vivo. Saturated cultures of RO1550 E. coli strain carrying a fisp::lacZ fusion and plasmid-expressed WT or mutant DksA were diluted 100-fold in LB medium containing 100 µg/ml ampicillin and 75 µg/ml spectinomycin, grown for 4 hours at 37°C, and assayed for β-galactosidase activity. (A) Schematic of the DNA reporter construct expressing β-galactosidase, consisting of the fis promoter region (fisp) fused to lacZ. The arrow shows the direction of transcription. (B) The effect of DksA mutations on the ability to repress fisp transcription relative to the WT DksA. For each type of DksA protein examined, β-galactosidase activities were averaged from 4 independently grown cultures and used to determine the percent of WT DksA repression activity using the following equation:

\[
\% \text{ transcription repression} = 100 \left( \frac{\beta\text{-galactosidase activity in absence of DksA} - \beta\text{-galactosidase activity in presence of mutant DksA}}{\beta\text{-galactosidase activity in absence of DksA} - \beta\text{-galactosidase activity in the presence of WT DksA}} \right)
\]

Green, yellow, orange, and red bar colors represent 4 ranges of fisp repression activity, as indicated. Error bars represent standard deviations. * denotes p-values<0.05; ** denotes p-values<0.01; *** denotes p-values<0.001. (C) The effect of mutations in Δ1-31 DksA on the ability to repress fisp relative to Δ1-31 DksA. Percent of Δ1-31 DksA repression was determined and represented as in panel B but, in this case, 100% repression was assigned to the effect produced by Δ1-31 DksA. (D) DksA structure (PDB: 5VSW; Molodtsov et al. 2018) showing the positions of mutated residues and their effects on fisp repression. Mutations resulting in nearly normal repression function (>80% activity) are shown in green; moderate effects (60-80%) are shown in yellow; strong effects (30-60%) are shown in orange; severe effects (0-30%) are shown in red. A space-filling model of ppGpp is colored lavender.
A fis repress by DksA

B

C

D

E143
K147
R125

180°

ppGpp

ppGpp

D74
D71

R63
R81
R87
R92
R91

100% - 80%
80% - 60%
60% - 30%
30% - 0%
Figure 3.3. Effect of DksA mutations on *uspA* transcription stimulation in vivo. Saturated cultures of RO1828 *E. coli* strain carrying a *uspA*p::lacZ fusion and plasmid-expressed WT or mutant DksA were diluted 100-fold in LB medium containing 100 μg/ml ampicillin and 75 μg/ml spectinomycin, grown for 4 hours at 37°C, and assayed for β-galactosidase activity. (A) Schematic of the DNA reporter construct expressing β-galactosidase, consisting of the *uspA* promoter region (*uspA*p) fused to *lacZ*. The arrow shows the direction of transcription. (B) The effect of DksA mutations on the ability to stimulate *uspA*p transcription relative to the WT DksA. β-galactosidase activities were averaged from 4 independently grown cultures and used to determine the percent of WT DksA transcription stimulation activity using the following equation:

\[
\text{\% transcription stimulation} = 100 \times \frac{\text{β-galactosidase activity in presence of mutant DksA}}{\text{β-galactosidase activity in absence of DksA}} \times \frac{\text{β-galactosidase activity in presence of WT DksA}}{\text{β-galactosidase activity in the absence of DksA}}.
\]

Green, yellow, orange, and red bar colors represent 4 ranges of *uspA*p transcription stimulation activity, as indicated. Error bars represent standard deviations. * denotes p-values<0.05; ** denotes p-values<0.01; *** denotes p-values<0.001. (C) The effect of mutations in Δ1-31 DksA on the ability to stimulate *uspA*p transcription relative to Δ1-31 DksA. Percent of Δ1-31 DksA transcription stimulation activity was determined and represented as in panel B but, in this case, 100% transcription stimulation was assigned to the effect produced by Δ1-31 DksA. (D) DksA structure (PDB: 5VSW; Molodtsov et al. 2018) showing the positions of mutated residues and their effects on *uspA*p transcription stimulation. Mutations resulting in near WT levels of transcription stimulation (>80% activity) are shown in green; those resulting in moderate loss of stimulation (60-80%) are shown in yellow; those resulting in strong reductions in transcription stimulation (30-60%) are shown in orange; those resulting in severe reductions (0-30%) are shown in red. A space-filling model of ppGpp is colored lavender.
AuspA stimulation by DksA

Relative uspA stimulation
- 0%
- 30%
- 60%
- 80%
- 100%

B

C

D

180°
Figure 3.4. DksA residues involved in ppGpp binding are uniquely required for transcription stimulation. Magnified view of DksA amino acid interactions with the phosphate groups of ppGpp (PDB: 5VSW; Molodtsov et al. 2018). The ppGpp phosphate groups are shaded in red. (A) DksA structure showing the position of mutated residues and their effect on transcription repression. Two images are shown rotated 180° relative to each other. Polar contacts are represented with dotted lines. Only DksA side chains for the residues targeted for mutation are shown using the same coloring scheme as in Figure 3.2. (B) DksA structure showing the position of mutated residues and their effect on transcription stimulation. DksA side chains for the residues targeted for mutation are shown using the same coloring scheme as in Figure 3.3. All other representations are as in panel A.
Figure 3.5. Position of DksA residues affecting transcription repression and stimulation in the ternary crystal structure. The residue-coloring scheme is as in Figure 3.2 and Figure 3.3. (A) Crystal structure of DksA, ppGpp, and RNA polymerase showing the effect of mutated residues on transcription repression of fisp. (B) Crystal structure of DksA, ppGpp, and RNA polymerase showing the effect of mutated residues on transcription stimulation of uspA. The view is the same as in panel A. (C) A rotated view of the structure in panel A. (D) A rotated view of the structure in panel B. This view is the same as in panel C.
**Figure 3.6. Residues at or near the globular region of DksA are required for transcription stimulation.** DksA bound to the secondary channel of RNA polymerase together with ppGpp. A patch of residues forming region 1 (blue) and region 2 (red) are indicated. K26 is colored in magenta.
# Tables

## Table 3.1. Bacterial Strains and Plasmids Used in this Work

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<th>Strain</th>
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<td>VH1000, <em>dksA::tet</em></td>
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</tr>
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<tr>
<td>RO1828</td>
<td>RO1250, <em>λuspAp::lacZ, pMS421, pKK223-3</em></td>
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<tr>
<td>VH1000</td>
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<td>Pharmacia</td>
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<td><em>pSC101 (lacI StrR SpcR)</em></td>
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Table 3.2. List of All Mutants and Strains Generated for this Work

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References


DksA Prevents Conflicts between DNA Replication and Transcription Machinery.” *Cell* 141 (4): 595–605.


4. **Discussion and Future Work**

The role of DksA as a stress response global gene regulator has become well characterized over the past decade. While it is clear that the function of DksA can be modulated to a large degree by the small alarmone ppGpp (Paul et al. 2004; Paul, Berkmen, and Gourse 2005; Perederina et al. 2004), it is also clear that DksA acts independently of ppGpp and sometimes in opposition to it (Magnusson et al. 2007; Åberg et al. 2009). This led to the supposition that other strategies, aside from the potentiation effects offered by ppGpp, may exist to modulate the function of DksA *in vivo*. It has been reported that DksA levels are constant throughout growth (Paul et al. 2004). However, work in our lab has shown that cellular DksA levels vary over 8-fold during different growth phases. Thus it is possible that, by modulating the intracellular levels of DksA, the ability of DksA to regulate transcription may be fine-tuned to meet the needs of the cell at any given time of growth. Our lab has also obtained evidence that the *dksA* mRNA 5′-untranslated region is able to fold into structures that hinder the translation of DksA, indicating that the expression of DksA is also controlled at the translational level (Trufnoff, D. and Osuna, R., unpublished results). However, at the time that this work was initiated, nothing was known about post-translational events that might affect the function of DksA. Thus, the original goal of my graduate research was to investigate and characterize potential post-translational events affecting DksA structure and their roles in regulating the DksA function in *E. coli*.

Bacteria are known to utilize a variety of reversible and non-reversible post-translational control mechanisms (Cain, Solis, and Cordwell 2014). Non-reversible processes include
proteolysis, deamidation, and elimination. Reversible processes include a large number of events such as protein multimerization, phosphorylation, methylation, acetylation, glycosylation, isoprenylation, AMPylation, and AMP ribosylation. The reversible nature of these processes makes them suitable strategies for functional control. In this work, we investigated the possibilities that protein cleavage, multimerization, or phosphorylation events may exist that could potentially affect the function of DksA.

We found that WT DksA and a number of substitution mutants of DksA exhibit distinct, reproducible proteolytic cleavage patterns that are affected by the growth media and the genetic background of the *E. coli* strain. However, most of the observed DksA cleavage patterns were circumvented by the addition of a protease inhibitor cocktail during processing of the cell lysate (Appendix, Figure A.4). Thus, whereas different proteases are potentially capable of cleaving DksA under different growth conditions, much of the proteolytic activity that we detected was restrained in the growing cultures of *E. coli*. It is possible that they occur in vivo at levels low enough to elude our detection in the presence of protease inhibitor cocktail but sufficiently high enough to affect the half-life of DksA. However, we concluded that DksA proteolysis is unlikely to be a salient strategy to regulate DksA function.

DksA is currently modeled to function as a monomer by inserting its CC region into the secondary channel of RNAP (Perederina et al. 2004; Paul et al. 2004). DksA was first crystallized as a multimer consisting of 10 monomers (Perederina et al. 2004). The monomers of DksA appeared to be arranged in a few repeating dimeric orientations (Appendix, Figure A.1). This suggested the possibility that DksA adopts a quaternary state in solution. If so, this could provide a mechanism for controlling the availability or stability of active monomeric DksA.
This work showed that purified His6-DksA separated by PAGE under non-denaturing conditions readily forms bands of increasingly higher molecular weight, indicating the presence of various multimeric states in vitro. Numerous multimeric interactions can also be observed by glutaraldehyde crosslinking (Appendix, Figure A.2). The higher molecular weight bands can be disrupted with increasing urea concentrations, indicating that the stability of DksA multimers relies on secondary and tertiary structural properties of DksA. While fewer discrete multimeric states of DksA are detected in vivo, at least 2 prominent states of DksA structure are observed. Interestingly, the ratio between these 2 states of DksA changes, depending on the growth conditions (Appendix, Figure A.3). When cells were grown more slowly under limited carbon source conditions, the higher DksA molecular weight complex becomes more prominent compared to cells rapidly growing in rich medium. Because DksA is currently modeled to bind RNAP as a monomer in order to function (Molodtsoy et al. 2018), multimeric DksA could represent a mechanism for maintaining an inactive “pool” of DksA in vivo that could potentially affect its ability to function.

A novel discovery of this work was the finding that DksA is phosphorylated in vivo. This was demonstrated by the covalent incorporation of radioactive $^{32}$P onto DksA in vivo and by the specific staining of DksA with the phosphoprotein stain Pro-Q® Diamond. The potential impact of this finding resulted in it becoming the focus of my graduate research (chapter 2). Protein phosphorylation represents a major class of chemical modification in which a phosphate group is attached at specific amino acids in order to alter the function of the target protein. The addition of a negatively charged phosphate group can trigger an allosteric effect on DksA that could potentially promote or interfere with intermolecular interactions (Mijakovic, Grangeasse, and Turgay 2016). Phosphorylation is often associated with the amino acids serine, threonine, or
tyrosine, which result in the formation of a relatively stable phosphoester bond. However, in bacteria, phosphorylation also occurs on an additional subset of amino acids that includes arginine, lysine, and histidine (resulting in formation of a phosphoamide bond) or aspartic acid and glutamic acid (resulting in formation of a mixed anhydride bond) (Table 2.3). Phosphorylations that produce phosphoamide and mixed anhydride bonds are unstable at low pH, complicating a number of downstream biochemical analyses (Cieślą, Fraczyk, and Rode 2011).

Repeated attempts to locate the site(s) of DksA phosphorylation by mass spectrometry of peptide digests were unsuccessful. This was most likely due to the use of fluoroacetic acid during workup, which we showed removes the $^{32}$P-labeled phosphates from DksA (Figure 2.7). Hence, we turned to a mutational approach to identify the DksA residues involved in phosphorylation. To this end, we generated a series of targeted amino acid substitutions covering 68 of 151 amino acids as well as a pair of deletions targeting the first 13 or first 31 N-terminal residues (Table 2.2). While the majority of the generated DksA mutations did not result in an appreciable loss of DksA phosphorylation (Figure 2.8), several of the mutations resulted in a phosphorylation deficiency. These pointed to 2 phosphorylation regions in DksA, 1 in the region from residue 23 to 31 and the other in the region from residue 101 to 105. This observation was consistent with mass spectrometry analysis of full-length DksA, which indicated that more than 1 phosphate (and as many as 3 phosphates) may be associated with DksA. It is also consistent with chemical cleavage analysis of DksA, which showed that phosphorylation occurred in both the amino-terminal 47 residues and in the carboxy-terminal 104 residues.

The DksA deletion mutant Δ1-31, but not Δ1-13 DksA, resulted in over a 50% loss of phosphorylation, suggesting that the region from residues 14-31 served as a target for
phosphorylation. The triple substitution Y23A K26A Y31A resulted in a similar loss of phosphorylation, further narrowing the region of potentially targeted residues. Because only a partial loss of phosphorylation was associated with this N-terminal region, we had expected that mutations affecting another phosphorylated site would also result in a partial loss of phosphorylation. Thus, to facilitate the identification of another phosphorylated site, an additional set of substitution mutants was engineered onto the Δ1-31 deletion mutant, which had removed 1 of the phosphorylated sites. We found that the multiple substitution mutant Δ1-31 K101A T102A K104A K105A resulted in the complete loss of phosphorylation, identifying the region from residues 101-105 as a second target of DksA phosphorylation (Figure 2.9).

We wanted to know if phosphorylation affects the ability of DksA to function in its roles as a transcription stimulator or as a transcription repressor. Phosphorylation-deficient DksA mutants were assayed for their ability to function in the transcription repression of the fis promoter (fisp) or stimulation of the uspA promoter (uspAp) in vivo. The Δ1-31 DksA deletion mutant resulted in a moderate loss of repression and a strong loss in ability to stimulate. The multiple substitution T9A S10A S11A Y23A Y31A T131A, which mutates all potential phosphoester-forming residues (Thr, Ser, Tyr) in the 1-31 region, had no appreciable effect on either repression or stimulation of transcription. The triple mutant Y23A K26A Y31A, which was roughly as deficient in its ability to phosphorylate as Δ1-31 DksA, was also similarly affected in its ability to function in the repression and stimulation of transcription. This suggested that the loss of phosphorylation in the amino-terminal region of DksA can affect its ability to regulate transcription.

As K26A was the only amino acid mutated in the triple mutant Y23A K26A Y31A that was not mutated in the multiple substitution mutant Δ1-31 T9A S10A S11A Y23A Y31A T131A,
and because the potential phosphorylation of a lysine is supported by the observed acid lability of the phosphate bond, we engineered a pair of single substitution mutants, K26A as well as K26E, with a glutamate residue in place of lysine designed as a phosphomimetic substitution (Klose, Weiss, and Kustu 1993), and assayed them for function in transcriptional repression and stimulation. The K26A DksA mutant showed a strong decrease in its ability to stimulate transcription. However, the phosphomimetic K26E mutant restored the uspA stimulation function (Figure 2.12). Taken together, these results led us to strongly suspect a role of K26 in phosphorylation in this N-terminal region, and that this phosphorylation is important to the ability of DksA to stimulate transcription.

Curiously, the K26A substitution did not impair the ability of DksA to repress transcription. The K26E substitution also resulted in WT DksA levels of transcription repression. While the interpretation of these results is not clear, the results point to a discrepancy in the requirements of DksA in the processes of transcription repression and transcription stimulation. It is conceivable that, in addition to disrupting phosphorylation, the triple mutation Y23A K26A Y31A results in a local conformational change that may reduce the ability to repress transcription and that the phosphorylation of K26 is only required for transcription stimulation. Another interesting possibility is that the triple mutation Y23A K26A Y31A may actually disrupt 2 phosphorylated targets, 1 at K26 and another at an unidentified nearby residue, whereas the K26A mutation only prevents phosphorylation at residue 26. In this scenario, it would be possible that the ability to repress transcription is only affected by the loss of 2 phosphorylation sites in this region, whereas loss of phosphorylation at K26 is sufficient to affect the ability to stimulate transcription. The notion of 2 phosphorylated sites in the N-terminal is not far-fetched. Mass spectrometry analysis indicated the existence of 3 phosphorylated sites on DksA, but deletion of the region from 1-31
in combination with the multiple mutations K101A T102A K104A K105A completely abolishes DksA phosphorylation. Furthermore, cleavage of phosphorylated DksA with BNPS-Skatole resulted in an N-terminal 5 kDa and a C-terminal 12 kDa fragment, both of which were phosphorylated (Figure 2.5). However, the 5 kDa fragment (representing the DksA region from residues 1-47) contained about twice the $^{32}$P signal intensity as the 12 kDa fragment (representing the region from residues 48-151), suggesting that the N-terminal region contained twice the phosphates as the C-terminal region.

We were only able to attribute a functional role to the N-terminal region phosphorylation. The multiple substitution mutant K101A T102A K104A K105A is functional for both fis repression and uspA stimulation (Figure 2.12). It is possible that the loss of phosphorylation associated with this mutant plays a role in some other function of DksA. It would be interesting to test the effect of these mutations on the role of DksA on pausing transcription elongation, as part of mechanisms that make use of tagging proteins for degradation (like Lon protease), or some other aspect of the life of DksA. Additionally, a possible role—not tested in our assay—that this phosphorylation may play within DksA-RNAP-ppGpp complexes cannot be ruled out. Given the proximity of this region to bound ppGpp at site 2 (Figure 2.13), it is conceivable that the $\gamma$ phosphate of a pppGpp bound to this site could be cleaved by DksA and this is a phosphate residual from such an event. In other words, this region of DksA might be phosphorylated in response to pppGpp binding during the process of transcription stimulation. Such a hypothetical function would not only enable DksA to access the intracellular pool of pppGpp and process it for proper function in transcription activation, but would also suggest a role for DksA in contributing to the intracellular pool of ppGpp.
DksA functions to both stimulate and repress a large set of genes, and this function can be potentiated, unaffected, or even reduced by ppGpp (Åberg et al. 2009; Magnusson et al. 2007; Paul, Berkmen, and Gourse 2005). PpGpp is capable of binding RNAP at 2 distinct binding sites located roughly 60Å apart. Site 1, formed by the β' and ω subunits of RNAP (Ross et al. 2013) is DksA-independent (Vrentas et al. 2005), while Site 2 requires specific contacts with DksA to facilitate ppGpp binding (Ross et al. 2016; Molodtsov et al. 2018). While DksA mutations at L95, K98, R129, and K139 disrupt ppGpp binding at site 2, this disruption did not impair the ability of DksA to repress transcription of rrnB P1 in vitro (Ross et al. 2016), suggesting that the binding of ppGpp at site 2 does not affect the repression function of DksA. Conversely, transcriptional stimulation of iraP by DksA has been shown to require ppGpp binding at site 2, leading to an emerging model that there may be distinct functional requirements for stimulation or repression by DksA. Our results support and expand on this model by demonstrating that the disruption of DksA residues participating in the binding of ppGpp at site 2 severely affects the ability of DksA to stimulate uspAp while largely unaffected repression of fisP in vivo (Figure 3.4). Additionally, a recently published pair of ternary and binary crystal structures of DksA bound to RNAP with and without ppGpp, respectively (Molodtsov et al. 2018), allowed us an opportunity to evaluate the functional effects of our DksA mutant library in the context of these structures. We identify 2 regions of DksA that differentially affect uspAp stimulation but not fisP repression, independently of ppGpp interactions.

The binding of ppGpp at site 2 induces conformational changes that occur in the DksA-RNAP-ppGpp ternary structure compared to the DksA-RNAP binary structure (Molodtsov et al. 2018). Is it possible that the functions of transcriptional repression and stimulation by DksA require different conformational states, or residue interactions of an RNAP-DksA complex? It
was observed that the conformational changes induced by ppGpp bound at site 2 allow for novel contacts at a number of amino acid positions. For example, the substitution of R93A, one of such residues contacting RNAP only when ppGpp is bound, strongly decreased the ability to stimulate uspAp transcription while not affecting the ability to repress fisp.

Two distinct, solvent-exposed regions of DksA were found to be sensitive to amino acid substitutions for the stimulation of uspAp but were not affected in the repression of fisp. Region 1, consisting of residues C114, S116, C117, D137, T140, and R145, is located near the coordinated zinc atom. Region 2 is positioned on the globular domain, roughly centered on residue K26, and includes residues E21, E25, K26, E29, E30, E34, and H39 (Figure 3.5). Given the likelihood that K26 is phosphorylated, region 2 could represent a negatively charged surface, positioned in a solvent-exposed manner, that could act to bind an as of yet unidentified molecule. One of the previously observed dimeric interfaces formed by crystallized DksA molecules involves this solvent-exposed globular region of DksA (Appendix, Figure A.1B). Is it possible that a second DksA associates with the RNAP-bound DksA to help stabilize the DksA-RNAP-ppGpp complex in vivo? Or perhaps a different protein altogether joins this complex in vivo through its association with the globular region of DksA. The observation that μM concentrations of DksA are typically required to observe transcription regulation in vitro may suggest that one or more additional factors normally associated with the DksA-RNAP complex in vivo may be missing in the in vitro transcription assays.

Our results provide novel evidence that DksA is phosphorylated in vivo, and that this phosphorylation potentiates the transcription stimulatory function of DksA and not repression (Figure 4.1). Because the phosphorylation event is reversible, there is an opportunity for regulation of the protein function. The combined effects of kinases and phosphatases establish a
balance between the phosphorylated and unphosphorylated states, which may, in turn, be affected by some extracellular or intracellular signal to modulate the activity of DksA as necessary. A systematic mutational analysis allowed us to identify DksA residues important for binding ppGpp at site 2, or for binding RNAP that only affects the stimulation function and not the repression ability of DksA. Finally, two regions of clustered residues were identified on the globular head of DksA that are necessary for the stimulation function but not repression of transcription.

Beyond the initial binding step of RNAP with the promoter to form a closed complex, several intermediate complexes are formed on the path towards open promoter complex formation (Record et al. 1996). The formation of at least one of the intermediate complexes involves a relatively slow step. Upon formation of an open complex, RNAP engages in successive cycles of abortive initiation before it finally clears the promoter as a transcription elongation complex. Hence, the process whereby DksA stimulates the formation of productive transcription initiation complexes may demand a more stable interaction between DksA and RNAP compared to the process whereby DksA facilitates the kinetically fast dissociation of RNAP from promoter complexes that are already unstable. Moreover, the transition toward open complex formation requires allosteric changes in RNAP that are distinct from those involved in the dissociation pathway. The additional interactions required between DksA and ppGpp, DksA and RNAP, and possibly between DksA and as of yet unidentified factors in vivo, might all work together to meet the more stringent requirements of DksA during transcription stimulation compared to repression.

It is possible that the loss of stimulatory function observed when disrupting the N-terminal phosphorylation at K26, ppGpp binding at site 2, or specific contacts formed by DksA-RNAP,
results from a decrease in the stability of DksA bound to RNAP, which may suffice in its role as a repressor but not in its role in stimulating transcription. In a similar way, the two regions observed in the globular head of DksA could be participating in some kind of binding event with a yet unknown cellular factor (there is evidence DksA binds DNA non-selectively [Perron, Comte, and Van Delden 2005; Figure 2.4], and that DksA multimerizes), providing the required increase in stability to promote the stimulatory function of DksA (Figure 4.1).

As a conserved global transcription regulator, DksA is necessary for a number of important bacterial functions, including the initiation of pathogenesis (Nakanishi et al. 2006), biofilm formation (Sherlock et al. 2004), or the survival of a number of conditions of stress (Paul et al. 2004, Paul, Berkmen, and Gourse 2005). Given its part in such a diverse, and often critical set of roles, it would be hypothesized that DksA expression and function would be fine-tuned and controlled at a number of regulatory levels. Indeed, our lab has found DksA is controlled at the level of transcription, involving 3 promoters that are temporarily expressed as well as a number of cellular factors that regulate its transcription (Woods, Ou, Trufanoff, Stark, and Osuna, Unpublished results). It is also regulated at the level of translation, involving 2 alternate structures in the 5’ UTR region of the mRNA (Trufanoff, Ou, and Osuna, Unpublished results). My work adds another layer of fine-tuned control at the level of protein phosphorylation, which offers a strategy for a very rapid control of DksA function, particularly for transcription stimulation. This strategy for regulating DksA function most likely improves the bacteria’s timely response under conditions of stress to support its survival and fitness.
Future Work

Identifying the DksA residues that are targeted for phosphorylation proved to be more challenging than originally anticipated. While phosphoester and phosphothioester bonds formed by the phosphorylation of Thr, Ser, Tyr, or Cys are stable under conditions of low pH, phosphorylation at other residues such as Arg, Lys, His, Asp, or Glu forms bonds that are unstable in acid. The phosphorylation of DksA is acid labile, complicating attempts at traditional mass spectrometry and biochemical techniques. It would be worthwhile to follow up on attempts at mass spectrometry by employing a modified, acid-free methodology. In addition, functional analysis of K26A as well as the phosphomimetic K26E, implicates K26 as a likely target for phosphorylation. Radiolabeling experiments as described in chapter 2, with the newly generated K26A mutant, would directly address whether K26 is a target of phosphorylation. Radiolabeling of single substitution DksA mutants K101A, T102A, K104A, and K105A would also provide more insight into the nature of the phosphorylation in that area of DksA.

The reduction of phosphorylation associated with the N-terminal Y23A, K26A, Y31A triple mutant affected transcription stimulation efficiency by 50% and repression by 64%. Is it possible that this reduction observed for both functions was due to a loss in DksA binding affinity for RNAP? Because the binding of RNAP by DksA is expected to be a function of the intracellular concentration of available DksA, we can vary the in vivo expression levels by IPTG induction to determine if the transcription regulation function of the phosphorylation-deficient DksA is more sensitive to changes in intracellular concentrations compared to the WT protein. Previous work has shown the DksA N88I mutation increases the binding affinity to RNAP (Blankschien et al. 2009). Thus, we could engineer the triple mutation Y23A K26A Y31A together with N88I to ensure stronger RNAP binding, and determine if that can rescue the
transcription regulation function of DksA in vivo. As follow-up work, in vitro DksA-RNAP binding assays have been developed (Blankschien 2009) that can be used to compare binding affinities of phosphor-enriched DksA with a phosphorylation-deficient mutant.

The region consisting of K101-K105 is of special interest because of our inability to identify any functional changes caused by the loss of phosphorylation observed with our Δ1-31 K101A T102A K104A K105A mutant. Given the proximity of this location to ppGpp bound at site 2, we wonder if a pppGpp bound to this site could be cleaved by DksA, resulting in a phosphate bound to this site. While highly speculative, this idea would be easy to test experimentally by assaying the phosphorylation of Δ1-31 DksA in vivo in an E. coli relA, spoT background and comparing with WT DksA as a control. If a pppGpp bound at site 2 is the source of phosphorylation for the K101-K105 region, we would expect to see a partially phosphorylated WT DksA and a loss of phosphorylation by Δ1-31 in a relA spoT strain.

It is known that ΔDksA strains are unable to survive a number of stress conditions (e.g. temperature, pH, amino acid starvation). Is the ability to phosphorylate DksA necessary for successful function in surviving these conditions? For example, the survival of amino acid limitation requires functional DksA for the stimulation of certain amino acid biosynthesis genes. Using the phage lambda-derived Red recombination system, we can replace the chromosomally expressed WT DksA with a phosphorylation-deficient mutant and test the resulting strains’ ability to survive conditions of amino acid limitation.

It would also be informative to determine if phosphorylation provides a fitness advantage. This can be done by growing together a mixture of E. coli strains expressing the WT DksA or a phosphodeficient DksA mutant under different conditions of stress. If each strain
carries a gene that confers a specific antibiotic resistance (for example, $kan^R$ vs. $spec^R$), at various points during their growth, they can be plated on agar LB medium containing their respective antibiotics and the colony-forming units would inform us of their relative fitness under the various growth conditions. As controls, strains expressing WT DksA and resistance to either kanamycin or specinomycin are grown together under the same experimental conditions and plated on selective agar media. If phosphorylation of DksA does indeed confer a survival benefit under these conditions, WT $E. coli$ would be expected to outcompete the mutant over time.
Figure 4.1. Model of DksA stimulation vs. repression functions.

Signal?

Phosphorylation

Dephosphorylation

ppGpp contacts
Additional RNAP contacts
Role for the globular head

Transcription Repression

Transcription Stimulation

Other Factors:
proteins?
DNA?
References


DksA multimerizes in vitro.

Purified DksA crystallizes as a complex of 10 subunits exhibiting 3 distinct forms of intermolecular interactions (Figure A.1) (Perederina et al. 2004). While multimerization may have been an artifact of the crystallization process, it could not be ruled out that multimerization occurs in solution as well. Since DksA functions by binding RNA polymerase as a monomer (Molodtsov et al. 2018), multimeric DksA could represent an inactivate state. To determine if DksA multimers are formed in solution, we conducted non-denaturing polyacrylamide gel electrophoresis of purified His6-tagged DksA. The purified His6-DksA gave a single band when denatured and separated by SDS PAGE (Figure A.2A). However native gel electrophoresis of DksA from the same protein preparation revealed over 8 bands representing DksA protein complexes of varying molecular weights. When this purified DksA preparation was treated with 6 M urea and separated on 6 M urea gels, most of the high molecular weight complexes were dissociated into several of the lower molecular weight complexes. These observations indicate that DksA forms numerous multimeric states in solution, most of which rely on non-covalent chemical interactions that can be weakened by the presence of urea.

To use a different approach, a preparation of His6-DksA was treated with various concentrations of glutaraldehyde. Because of its short spacer length of about 5 Å, glutaraldehyde covalently crosslinks amino groups of proteins that are within close proximity, such as those located within dimeric interfaces. Intermolecular crosslinking of DksA could be readily detected using SDS PAGE (Figure A.2B). In the absence of glutaraldehyde, monomeric His6-DksA and a
45 kDa protein contaminant are seen in this preparation. Upon addition of 0.1 % and 0.5% glutaraldehyde, the monomeric DksA band decreases in intensity while 4 higher molecular weight crosslinked products of approximately 38, 58, 84, and 140 kDa can be observed. These sizes roughly correspond in size to multimers of the 19 kDa His6-DksA. The band corresponding to the 45 kDa protein does not decrease in intensity with the glutaraldehyde treatment, indicating that it is not crosslinked into higher molecular weight product, as may be expected for a protein that does not dimerize. These results suggest that DksA forms various multimeric states in solution and is consistent with the results of the native gel electrophoresis.

**DksA likely multimerizes in vivo.**

While purified DksA readily forms potential multimers in vitro, it is unknown whether DksA is also capable of forming multimers in vivo. Western blot analysis of cell lysates separated by native PAGE were used to assay DksA multimerization over a different phases of growth and in limited or rich growth media. MG1655 *E. coli* were grown in LB and M9 + glycerol with equal OD$_{600}$ cell units taken at various growth phases. Samples were separated by native PAGE and western blotted with anti-DksA antibody (Figure A.3). Two distinct bands were detected, possibly corresponding to monomeric and dimeric DksA in the separated cellular extracts, while the purified His6-DksA formed additional multimeric bands. Interestingly, the ratio of the 2 DksA states was shown to vary with the growth phase, showing an eventual loss of 1 of the 2 bands in late stationary phase. Growth media also appeared to affect the ratio of these 2 bands, with *E. coli* grown in LB appearing to favor the formation of the lower band, while cells grown in M9 + glycerol heavily favored formation of the upper band.
**Proteolytic cleavage of DksA is relieved by addition of protease inhibitor cocktail during lysis.**

DksA has been previously demonstrated as a target for post-translational processing by the protease ClpXP (Flynn et al. 2003); however, it was noted that it was likely the target of additional proteases. Observations of western blots of purified protein or cell lysates in our lab periodically show a DksA peptide fragment estimated around 11 kDa in size in certain strain backgrounds (MG1655; RO1838); interestingly, cleavage is often pronounced when DksA is purified from cells grown in low phosphate media (Figure A.4A). Expression of DksA from the same plasmid (pRO552) in a BL21 strain, commonly used for protein expression (RO1757), was sufficient to relieve this 11 kDa proteolysis of His6-DksA, however, a new cleavage product of ~18 kDa appears instead. Additionally, in the course of generating, and characterizing a large number of mutant DksA varieties, I noticed an increase in cleavage during processing such as lysis for western blots or purification regardless of using the BL21 background for expression. Inclusion of a protease inhibitor cocktail (Sigma Aldrich) during lysis is sufficient to prevent these observed cleavages, indicating they may not be occurring in vivo.
Figures and Legends

Figure A.1. DksA Crystallizes as a Decamer. (A) Crystal of 10 *E. coli* DksA molecules (B) Three repeating potential dimeric interfaces identified from the *E. coli* DksA crystal structure. (PDB: 1TJL; Perederina et al. 2004).
Figure A.2. Purified His6-DksA Forms Multiple Discrete Bands When Separated by Non-Denaturing PAGE. (A) Purified DksA separated by SDS-PAGE as well as non-denaturing PAGE stained by coomassie brilliant blue. Purified His6-DksA runs as a discrete band on a 15% SDS-polyacrylamide gel (2). Increasing concentrations (6, 8, 10 µg) of native His6-DksA (3-5), and the effect of 6M urea (6-8). (B) Glutaraldehyde crosslinking of purified His6-DksA confirms the formation of multimeric states in vitro. Purified His6-DksA was incubated with increasing amounts of glutaraldehyde and separated by SDS-PAGE followed by western blot with anti-DksA antibody. Various multimeric states are indicated based on molecular weight. An unknown protein contaminant was co-purified with His6-DksA and is indicated by *. The glutaraldehyde crosslinking was conducted by Dustin E. Trufanoff, Department of Biology University at Albany, SUNY.

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Figure A.3. The Effect of Growth Phase and Media Conditions on the Formation of Potential DksA Dimers in vivo. Samples of WT (RO1244) and ΔDksA (RO1250) *E. coli* were taken at increasing cell densities/different growth phases in both LB and M9 + glycerol media as indicated. Lysates were separated by non-denaturing PAGE followed by western blot with anti-DksA antibody. These western blots were conducted by Dustin E. Trufanoff, Department of Biology, University at Albany, SUNY.
Figure A.4. Effects of strain and growth media on DksA cleavage are prevented by protease inhibitor cocktail. (A) Effect of strain and growth media on DksA cleavage. His6-DksA purified from 2 different strains (RO1838 and RO1757) that were grown to mid exponential growth phase in LB or low phosphate medium was separated by SDS PAGE and visualized by western blot. The strains and growth medium are indicated at the top. His6-DksA and an 11 kDa cleavage product are indicated with arrows. (B) Protease inhibitor cocktail prevents proteolysis associated with cell lysis. His6-DksA grown in MG1655 (RO1838) as well as BL21 (RO1757) *E. coli* strains, lysed with and without protease inhibitor cocktail (Sigma Aldrich). Western blot analysis was performed with anti-DksA antibodies.