Genetic networks controlled by the bacterial replication initiator and transcription factor DnaA in *Bacillus subtilis*

By

Tracy Washington

B.S., Biochemistry
University of Oklahoma, 2007

Submitted to the Computational and Systems Biology Graduate Program in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the Massachusetts Institute of Technology
September 2013

Signature of Author: _____

Tracy Washington
Computational and Systems Biology Graduate Program
August 13, 2013

Certified by: _____

Alan D. Grossman
Professor of Biology
Thesis Supervisor

Accepted by: _____

Christopher Burge
Professor of Biology
Director, Computational and Systems Biology Graduate Program

Genetic networks controlled by the bacterial replication initiator and transcription factor DnaA in *Bacillus subtilis*

By

Tracy Washington

Submitted to the Computational and Systems Biology Graduate Program on August 13, 2013 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

ABSTRACT

DnaA is the bacterial replication initiator, which also functions as a transcription factor to regulate gene expression. In *B. subtilis*, DnaA has previously been shown to repress its own transcription and has also been implicated in directing part of the transcriptional response to replication stress. Because *dnaA* is essential, most of DnaA's potential effects on gene expression have been determined through indirect methods, which have implemented perturbations in replication and sequence analyses to predict direct effects of DnaA transcriptional regulation.

Below, I take a more direct approach to assay DnaA's effect on gene expression and specific transcriptional regulatory networks by deleting *dnaA* in an *oriN*$^+$ *ΔoriC* strain background, which renders *dnaA* non-essential. Isogenic *dnaA*$^+$ cells were constructed similarly and have *dnaA* constitutively expressed from an ectopic locus. In this background, DNA replication no longer depends on *dnaA* and is initiated instead by a plasmid replicon, *oriN*. The native origin of replication, *oriC*, is also deleted to eliminate differences in replication between *ΔdnaA* and *dnaA*$^+$ cells. Consequently, I can directly compare differences in gene expression due to the presence versus absence of *dnaA*.

Deletion of *dnaA* results in approximately 463 significant differences in gene expression, most of which I show are due to DnaA direct activation of the gene *sda*. Many of these genes lie downstream of Sda activity and comprise several regulons, such as the Spo0A, AbrB, and SinR regulons. These regulons are known to become active during the transition from exponential growth to stationary phase.

In addition to the many effects on gene expression, I show that deletion of *dnaA* results in lowered competence development. I also revisit the transcriptional response to replication stress and show that some of the previously predicted targets of DnaA respond to replication stress in a DnaA-dependent manner.

Lastly, in collaboration with others, I have studied the relationship between a DnaA regulator, YabA and a nucleoid binding protein Rok. YabA and Rok associate at some of the same chromosomal regions, and at these regions YabA absolutely depends on Rok for its association. We are currently trying to understand the functional relationship between YabA, Rok, and DnaA.

Thesis Supervisor: Alan D. Grossman
Title: Professor of Biology

Acknowledgments

I would like to thank my adviser Alan for his unwavering support and encouragement during my time spent in his lab. He has taught me a lot about how to do science and the importance of keeping things "simple" whenever possible. I've always appreciated his feedback regarding my project, but also his emphasis on developing the skills necessary for not only a successful career in research, but also successful career in general.

I would also like to thank my committee members, past and present, Michael Laub, Steve Bell, Ernest Fraenkel, and Ann Hochschild for their helpful advice, thoughts, and suggestions.

I would also like to acknowledge fellow members of the Grossman lab, both past and present, who have showed me the ropes and taught me the ins and outs of working with *Bacillus*. I would like to thank C. Lee in particular for helping me with my rotation project and for being always ready and willing to help out with just about anything. I'd also like to acknowledge Houra Merrikh and Carla Bonilla, former postdocs in the lab who also worked on various aspects of DnaA and who were very supportive in teaching me new lab techniques, offering advice, and being enthusiastic collaborators. I'd also like to thank Kayla Menard for being a very wonderful and supportive labmate and for the many fun conversations we shared about our respective projects.

Lastly, I would like to thank all of my friends and family for their love and encouragement throughout the years and for helping make my time at MIT an enjoyable one.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Introduction

The initiation of replication and transcription are both highly regulated processes in most organisms. In bacteria, the widely conserved replication initiation protein DnaA also functions as a transcription factor. DnaA is a AAA+ ATPase present in virtually all bacteria and homologous to several subunits of ORC, the origin recognition complex in eukaryotes. DnaA binds and hydrolyzes ATP, and also binds ADP with similar affinity. DnaA-ATP is needed for replication initiation and appears to be more active in binding DNA (Sekimizu et al., 1987; Speck et al., 1999). DnaA contains four domains: 1) an N-terminal domain that promotes interactions with other DnaA molecules and the replicative helicase (at least in *E. coli*), 2) a flexible linker domain, 3) a AAA+ ATPase domain, which binds and hydrolyzes ATP to modulate DnaA oligomerization and DNA binding, and 4) a C-terminal helix-turn-helix DNA binding domain, which in *Escherichia coli* recognizes and binds to the consensus sequence 5'-TTATNCACA-3' (DnaA box). (Messer et al., 1999; Schaper and Messer, 1995; Seitz et al., 2000; Sutton and Kaguni, 1997)

DnaA as the replication initiator

To initiate replication, DnaA binds to a specific chromosomal region known as the origin of replication (*oriC*). Origins of replication are stretches of DNA typically ranging from ~200 to ~1000 bp in bacteria. DnaA binds to several sites in *oriC* and causes local unwinding of an AT-rich region, the DNA unwinding element or DUE (Fig. 1) (Mott and Berger, 2007). The DUE is the site of replisome assembly and was originally defined by origin sensitivity to digestion by a single-strand specific nuclease (Bramhill and Kornberg, 1988).

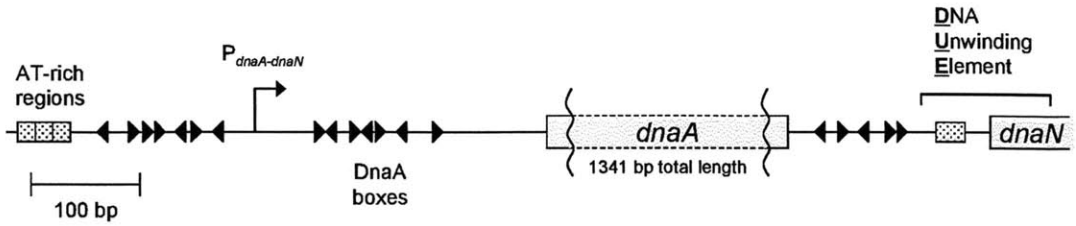

Figure 1. The *B. subtilis* origin of replication *oriC*.

Pictured are components of the *B. subtilis* origin of replication. The origin is comprised of three clusters of DnaA boxes, denoted by black triangles, the *dnaA-dnaN* promoter region and upstream portion of the *dnaA-dnaN* operon, and local AT-rich regions, one of which comprises the DUE, located between *dnaA* and *dnaN*. Figure adapted from Krause et al.

Replication origins are normally indispensable for autonomous chromosomal replication. In *E. coli*, much is known about the individual steps leading up to origin melting and subsequent replisome assembly. First, DnaA recognizes and binds to DnaA boxes at the origin. This binding process occurs in an ordered, sequential manner, and is dependent on DnaA binding of ATP. DnaA boxes at the origin vary with respect to their relative affinities for DnaA, and it is those with stronger affinity that are first occupied during initiation. Stronger affinity DnaA boxes do not appear to have a preference for either DnaA-ATP or DnaA-ADP. Weaker affinity sites, however, preferentially bind DnaA-ATP and lie interspersed between stronger affinity sites. Once DnaA-ATP levels become sufficient for initiation, DnaA binds to the weaker affinity DnaA boxes, and the DnaA-origin complex achieves a final state in which all DnaA boxes are occupied. Given the close spacing of DnaA boxes and interactions between individual DnaA protomers, the DnaA-origin complex adopts a helical, filamentous structure in which the DnaA oligomer wraps around the duplex DNA (Fig 2) (Mott and Berger, 2007).

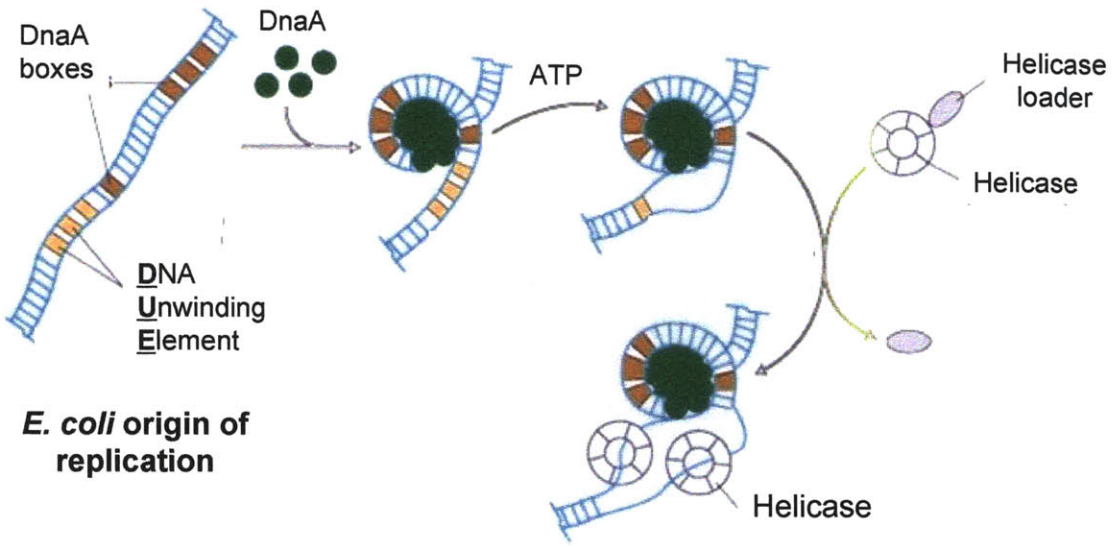

Figure 2. Steps of replication initiation at *oriC* in *E. coli*.
Upon initiation, DnaA binds to the DnaA box clusters, represented as brown boxes at the origin of replication. Oligomerization of DnaA occurs in an ATP-dependent manner and promotes local unwinding of the DUE and subsequent recruitment of helicase and its associated helicase loader. Figure adapted from Bramhill and Kornberg.

This final structure then gives rise to an active origin by exerting sufficient strain on the DNA to induce local melting of an AT-rich region called the DNA unwinding element (DUE). Newly created ssDNA at the DUE is stabilized by DnaA-ATP, Ssb (single strand binding protein), and additional proteins (Krause and Messer, 1999). The helicase loader protein DnaC then assembles helicase onto the Ssb-coated ssDNA. Following helicase loading is that of the beta-processivity factor (DnaN) and finally DNA polymerase (PolC). After complete assembly of the replisome, bidirectional replication can proceed.

In *B. subtilis* and likely many other Gram-positive bacteria, helicase loading at *oriC* differs from that in *E. coli* (Bruand et al., 2005). Instead of one helicase loading protein, DnaA is required to recruit three to the origin: DnaD, DnaB, and DnaI. DnaI exists in a complex with the replicative helicase (Smits et al., 2010). Note that the nomenclature of replisomal proteins is not

conserved between *E. coli* and *B. subtilis*. Also, in *B. subtilis* DnaA interacts directly with DnaD, but is not known to interact with DnaC.

Regulation of DNA replication initiation

Most known mechanisms regarding regulation of replication converge on DnaA and *oriC* function during initiation. Several key factors and proteins involved in regulating initiation have been identified in *E. coli*; however, most other organisms lack these proteins and likely employ other methods of replication control. Below, I describe known mechanisms of replication control in *E. coli* and contrast them with those known in *B. subtilis*.

Regulation of replication initiation in *E. coli*

In *E. coli*, three main mechanisms controlling replication initiation are known to exist. One, a process called RIDA, which stands for regulatory inactivation of DnaA, ultimately results in DnaA ATP hydrolysis (Camara et al., 2005; Riber et al., 2006; Su'etsugu et al., 2004). It involves two proteins Hda, a homolog of DnaA, and the beta-processivity factor DnaN. Hda localizes to emerging replication forks via its interaction with DnaN and promotes DnaA ATP hydrolysis at the origin. ATP hydrolysis depletes the necessary DnaA-ATP pools required for initiation and consequently inhibits re-initiation of replication.

A second mechanism of replication control involves Dam methylation and origin sequestration by the protein SeqA. In *E. coli*, DNA exists in a chemically modified form, in which the adenine at many palindromic GATC sequences is methylated by the enzyme Dam methyltransferase (Lobner-Olesen et al., 2005). Newly synthesized DNA strands containing GATC sequences exist temporarily unmodified before Dam methylation occurs, and because of the semi-conservative nature of replication, emerging duplex DNA at replication forks exist in a

hemi-methylated state in which the old template strand is methylated and the newly synthesized strand is unmethylated. SeqA recognizes and binds to hemi-methylated GATC DNA and shields it from immediate recognition by other proteins such as DnaA. Since GATC sites are in close proximity to DnaA boxes and enriched at *oriC*, origin sequestration by SeqA results in prevention of over-initiation (Kaguni, 2006; Nievera et al., 2006; Waldminghaus and Skarstad, 2009).

A third mechanism of replication control involves titration of DnaA by the locus *datA*. The *datA* region contains a cluster of DnaA boxes, which acts to titrate DnaA away from the origin (Morigen et al., 2003; Ogawa et al., 2002). Since *datA* is located relatively close to the origin, it is replicated soon after initiation. After its replication, *datA* exists in multiple copies and can then exert an even more pronounced effect on DnaA at the origin

Regulation of replication initiation in *B. subtilis*

Control of replication initiation in *B. subtilis* differs from that in *E. coli*. *B. subtilis* lacks Hda, Dam methylation, and SeqA. Although *B. subtilis* also lacks the *datA* locus, it contains several other sites with clustered DnaA boxes that have been shown to act similarly as *datA* (Okumura et al.). *B. subtilis* contains several other regulators that have been shown to affect binding of DnaA to *oriC* during growth and sporulation.

YabA, a negative regulator of replication, has been shown to interact with both DnaA and DnaN (Cho et al., 2008; Noirot-Gros et al., 2006). It affects DnaA activity at the origin by influencing DnaA binding. *In vitro* YabA has been shown to lower the cooperativity of DnaA binding at the origin; *in vivo*, overexpression of YabA also results in lower levels of DnaA binding at the origin (Merrikh and Grossman, 2011). Also, YabA activity is presumably regulated via its interaction with DnaN, which removes YabA from the origin as replication

forks progress along the DNA (Katayama et al., 2010). YabA has also been shown to localize to regions outside the origin, some of which are also bound by DnaA and/or the nucleoid binding protein Rok (Appendix).

DnaD, one of the helicase loading proteins, which interacts directly with DnaA, has also been shown *in vitro* to lower cooperativity of DnaA binding at the origin (Bonilla and Grossman, 2012). Like YabA, it also localizes to other regions bound by DnaA outside the origin (Smits et al., 2011).

Soj, like YabA, has been shown to interact directly with DnaA. It has been shown to inhibit DnaA oligomerization via interaction with DnaA's AAA+ ATPase domain (Scholefield et al., 2012). SirA, which is active only during sporulation, also interacts directly with DnaA and acts to inhibit its binding to the origin (Rahn-Lee et al., 2009; Rahn-Lee et al., 2011).

DnaA as a transcription factor

DnaA also functions as a transcription factor, the first indication of which was its auto-regulatory activity. DnaA autoregulation has been characterized in both *B. subtilis* and *E. coli*, and in both organisms, DnaA has been shown to bind to DnaA boxes within its promoter region to repress its own transcription (Braun et al., 1985; Ogura et al., 2001). As a result, DnaA autorepression helps maintain DnaA levels to carefully control the frequency and timing of replication initiation.

In addition to regulating its own expression, DnaA is known to regulate the expression of several other genes. One additional target of DnaA in *E. coli* is the *nrdAB* operon, which is responsible for the reduction of ribonucleotides to deoxyribonucleotides. *In vitro* DnaA binding to the *nrdAB* promoter has been shown to be distinct for the two nucleotide-bound forms of

DnaA (DnaA-ATP vs. DnaA-ADP). DnaA-ATP binds to the *nrdAB* promoter with higher affinity than DnaA-ADP and at high concentrations appears to repress *nrdAB* expression. At lower concentrations, DnaA-ATP activates *nrdAB* expression (Olliver et al.). After initiation, DnaA-ATP levels drop due to Hda activity (RIDA), and as a result, expression of *nrdAB* increases (Gon et al., 2006). Given the role of *nrdAB* in synthesizing substrates necessary for DNA replication, many have proposed that DnaA may act to synchronize deoxyribonucleotide synthesis with replication and the cell cycle, and it is likely that DnaA also acts to control *nrdAB* expression in other organisms (Gon et al., 2006; Herrick and Sclavi, 2007; van Sinderen et al., 1995).

In *B. subtilis*, DnaA is involved in the transcriptional response to replication stress and directly controls the expression of the following genes: *sda, ywlC, yydA, ywcI, vpr*, and *dnaA-dnaN*. Each of these genes contains a cluster of DnaA boxes within its respective promoter regions to which DnaA directly binds. Also, in the presence of replication stress, association of DnaA at these sites dramatically increases, and the expression of surrounding operons changes accordingly (Breier and Grossman, 2009; Goranov et al., 2005). The function of one of these genes, *sda*, is described in more detail below, and my work shows that *sda* is involved in affecting a transcriptional reguatory network controlled indirectly by DnaA. The other genes whose functions have also been determined include *ywlC* and *vpr*. The essential gene *ywlC* has recently been shown to catalyze a threonylcarbamoyl tRNA modification, which is involved in enhancing codon-anticodon pairing during translation to prevent frameshift mutations (Lauhon, 2012). The gene *vpr* encodes an extracellular serine protease, and is induced by replication stress, DNA damage, and phosphate starvation (Allenby et al., 2005; Sloma et al., 1991). Functions for *ywcI* and *yydA* have yet to be determined.

DnaA is also known to bind an additional chromosomal region that lies immediately downstream of the gene *gcp*. DnaA, however, does not appear to function as a transcription factor at this site. DnaA binding at *gcp* and the sites described above have also been implicated in replication control (Okumura et al., 2012). They may also serve as a sink for DnaA, much like *datA* in *E. coli*, to modulate its activity at *oriC* (Okumura et al.).

Regulation of the transcription factor activity of DnaA

Many of the above regulators of replication initiation in *B. subtilis* seem to employ a similar mechanism of controlling DnaA activity by inhibiting its cooperative binding and oligomerization at the origin. Some, including YabA and DnaD, have also been shown to associate with DnaA at regions outside the origin, and as a result, are thought to also modulate DnaA's transcriptional regulatory activity at these sites.

DnaA and the nucleoid associated protein Rok

Recent work showed that DnaA and YabA both localize to some regions bound by the nucleoid binding protein Rok (Appendix). Rok has been shown to preferentially bind to AT-rich regions in the genome and acts as a general repressor of gene expression (Smits and Grossman, 2010). It is known to repress expression of *comK*, the master regulator of competence development and also controls the expression of various genes involved in cell surface modifications and peptide secretion (Albano et al., 2005). Given the co-localization of DnaA, YabA, and Rok to various chromosomal regions, we sought to explore the relationship among their binding. I discuss this issue more in the Appendix section.

DnaA and sda

sda, a small open reading frame of 159 bp, encodes a cell cycle checkpoint protein, which halts entry into sporulation during perturbations in replication. *sda*, which stands for suppressor of *dnaA*, was originally discovered in a genetic screen for mutants that suppress a sporulation defect in *B. subtilis* cells carrying a *dnaA(ts)* allele (Burkholder et al., 2001). Expression of *sda* is activated by DnaA, and during normal cell growth, increases in DNA content correlate with increases in *sda* expression (Veening et al., 2009). Also, the *sda* promoter preferentially binds DnaA-ATP ($K_d \sim 0.15$ μM) versus DnaA-ADP ($K_d \sim 0.51$ μM) by approximately four-fold (communicated by Janet Smith). Coupling Sda activity to DnaA activity, i.e., delay of sporulation during DNA replication, appears to promote inheritance of a fully intact chromosome by the *B. subtilis* endospore.

Sda inhibits activation of the key stationary phase and sporulation transcription factor Spo0A. Spo0A is a response regulator that when active binds DNA to directly control the expression of several genes necessary for sporulation. Activation of Spo0A occurs by its phosphorylation via an upstream phosphorelay that is comprised of a set of histidine kinases that transfer phosphate to Spo0F (Fig 3).

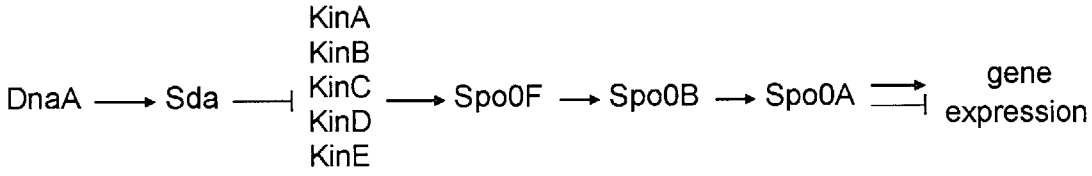

Figure 3. Diagram of phosphorelay
Spo0A is activated by a phosphorelay involving multiple histidine kinases. Under appropriate conditions, they autophosphorylate, and each can then transfer phosphate to the response regulator Spo0F. Spo0F, in turn phosphorylates Spo0B, which ultimately phosphorylates Spo0A. Once phosphorylated, Spo0A becomes active and can then regulate downstream gene expression. DnaA is connected to Spo0A activation via one of its direct targets Sda, which preferentially inhibits KinA and KinB autophosphorylation.

The phosphate from Spo0F is then transferred to Spo0B, and then from Spo0B to Spo0A (Hilbert and Piggot, 2004; Hoch, 1993). Sda directly acts on KinA, and probably KinB to prevent their autophosphorylation and hence activation of Spo0A (Cunningham and Burkholder, 2009; Veening et al., 2009; Whitten et al., 2007) (Fig 3).

Spo0A is also involved in transcriptional changes during entry into stationary phase and not just sporulation. Spo0A directly inhibits expression of a stationary phase regulator AbrB, which controls many genes related to nutrient deprivation and cell growth (Furbass et al., 1991; Strauch et al., 1990). Spo0A also affects activity of the transcription factor SinR, which is involved in controlling cell motility, chaining, and biofilm formation (Chai et al., 2010).

Transcriptional regulatory networks controlled by DnaA

To study the effects of DnaA on gene expression, methods have traditionally focused on perturbing replication to affect DnaA activity and downstream gene expression. This is because *dnaA* is normally essential, and without a functional copy of *dnaA*, cells are unable to replicate their genomes and hence unable to grow and divide. Moreover, DnaA activity is believed to be highly coupled to replication initiation, and as a result, DnaA's role as replication initiator may convolute any DnaA-dependent transcriptional effects. Consequently, isolating genes whose transcription is directly influenced by the presence and/or absence of DnaA has proven difficult.

In this work, I take a more direct approach to assay DnaA's effects on global gene expression. Building on previous studies, I compare gene expression between a non-essential *ΔdnaA* mutant and a cognate *dnaA*+ strain. Within these strains, DnaA's role in replication is completely abolished by deleting *oriC* and providing a plasmid replicon *oriN* in trans. *oriN* encodes it own replication initiator *repN*, which acts in lieu of DnaA (Moriya et al., 1997). By

separating DnaA's roles in replication initiation and transcriptional regulation, we can directly probe DnaA-dependent gene expression without the complicating effects of replication on DnaA activity and gene expression.

Thesis summary

As both replication initiator and transcription factor, DnaA is at the interface of two fundamental molecular and cellular processes: DNA replication and gene expression. Here, I discuss DnaA's role in the coordination of these two processes; however, I focus mainly on DnaA regulation of gene expression. I've found that during normal growth, loss of *dnaA* results in ~463 significant effects on gene expression and that most of these effects are indirect and occur through the DnaA direct target, Sda. Overexpression of *sda* suppresses many of the global effects on transcription of a *dnaA* deletion. I also show that DnaA has an effect on competence development. I conclude that DnaA direct control of its target gene expression results in a much broader effect on global gene expression and cell phenotypes than previously anticipated. Finally, I present some preliminary data regarding a recently discovered connection between a negative regulator of replication YabA and the nucleoid binding protein and transcription factor Rok.

Chapter 2

Genetic networks controlled by the bacterial replication initiator and transcription factor DnaA in *Bacillus subtilis*

Tracy Washington and Alan D. Grossman

This chapter is being prepared for publication.

Abstract

DnaA is a widely conserved bacterial AAA+ ATPase that functions as both replication initiator and transcription factor. Though the role of DnaA in initiation of DNA replication has been extensively characterized, its role in regulation of gene expression remains less understood. Previous work has shown DnaA to mediate part of the transcriptional response to replication stress. In addition, we show that in the absence of replication stress, DnaA controls the expression of several regulons, some including the Spo0A, AbrB, and SinR regulons, which are generally active during the transition from exponential growth to stationary phase. Expression of these genes, among others, is affected by a well-characterized DnaA-activated gene, *sda*, and overexpression of *sda* is able to overcome many effects of loss of *dnaA*. In addition, we show that a *dnaA* null mutation results in lowered competence development. Together, these effects imply that DnaA plays an important role in regulating cell physiology, especially during late exponential and early stationary phases. DnaA regulation of global gene expression during exponential growth, however, seems to occur primarily through *sda*.

Introduction

DnaA is the widely conserved bacterial replication initiation protein. It is a AAA+ ATPase and DNA binding protein (Duderstadt and Berger, 2008; Iyer et al., 2004). During replication initiation, DnaA binds to several sites (DnaA boxes) in the chromosomal origin of replication (*oriC*), causes unwinding of an AT-rich region in *oriC*, and facilitates recruitment of the replication machinery. DnaA also binds to several sites throughout the genome outside of *oriC*. DnaA functions as a transcription factor at many of these sites, activating expression of some genes and repressing expression of others (Breier and Grossman, 2009; Goranov et al., 2005).

We were interested in determining the effects of DnaA-mediated transcriptional regulation on global gene expression in *Bacillus subtilis*. Due to DnaA's dual roles in replication and transcriptional regulation, perturbations in DnaA activity would likely lead to gene expression changes resulting from DnaA control of replication. These effects would be independent of DnaA transcriptional regulatory activity. To circumvent these potential replication-dependent transcriptional changes, we created a system in which DnaA no longer acts as a replication initiator but instead functions solely as a transcription factor. As a result, we were able to determine the effects of DnaA on global gene expression in *B. subtilis*, separate from any possible effects on replication initiation. We describe this system below.

We wished to determine the effects of DnaA on global gene expression in *B. subtilis*, separate from any possible effects on replication initiation. We also sought to define which part of the transcriptional response to replication stress was dependent on *dnaA* (directly or indirectly). We used strains in which *dnaA* and *oriC* are non-essential (Hassan et al., 1997; Moriya et al., 1997). In these strains, replication initiates from a heterologous origin of replication (*oriN*) using its cognate initiator (*repN*) that are integrated near the position of *oriC*. *dnaA* can be expressed in these strains without altering replication since there is no functional *oriC*.

We found that *dnaA* was required for some of the changes in gene expression in response to replication stress. In addition, we found that DnaA affects expression of a large network of genes, and the majority of these genes do not appear to be direct targets of DnaA. Most of these indirect effects were traced back to the effects of DnaA on the checkpoint gene *sda*. *sda* was originally identified as a suppressor of the sporulation defect of a *dnaA(ts)* mutant (Burkholder et al., 2001). The *sda* gene product is a small protein that inhibits histidine protein kinases that are

required for activation of the stationary phase and sporulation transcription factor Spo0A (Cunningham and Burkholder, 2009; Rowland et al., 2004; Whitten et al., 2007). Our results indicate a large role for DnaA in transcriptional networks during growth, cellular responses to replication stress, and likely during entry into stationary phase.

Results

Approach

To determine the effects of *dnaA* on gene expression during exponential growth, we compared mRNA levels for virtually all open reading frames (ORFs) in cells with and without *dnaA*. We also determined if *dnaA* was required for changes in gene expression in response to inhibition of replication (replication stress). We used strains in which *dnaA* and *oriC* are non-essential. In these strains, the origin of replication (*oriN*) and its replication initiator (*repN*) from plasmid pLS32 are integrated into the *B. subtilis* chromosome near *oriC* (Hassan et al., 1997; Moriya et al., 1997). Normally, *dnaN*, encoding the processivity clamp, is in an operon with *dnaA* in the *oriC* region, and its transcription is repressed by DnaA (Moriya et al., 1985; Ogura et al., 2001). To remove this regulation, we expressed *dnaN* from a xylose-inducible promoter (Pxyl-*dnaN*) at an ectopic site (*amyE*) in the chromosome. *dnaA*, under control of an IPTG-inducible promoter (Pspank-*dnaA*) was inserted into the chromosome at the non-essential *lacA* (Methods). We used two strains, *oriN*+ Δ*oriC* Δ(*dnaA-dnaN*) Pxyl-*dnaN*, (strain AIG200; indicated as *oriN* Δ*dnaA*) and *oriN*+ Δ*oriC* Δ(*dnaA-dnaN*) Pxyl-*dnaN* Pspank-*dnaA* (strain TAW5; indicated as *oriN dnaA*+) (Fig 1; Table 1), to determine the effects of *dnaA* on gene expression. Where indicated, replication elongation was arrested by addition of hydroxyl-phenyl-

azo-uracil (HPUra), a compound that binds to the catalytic subunit, PolC, of *B. subtilis* DNA polymerase III and blocks replication elongation (Brown, 1970).

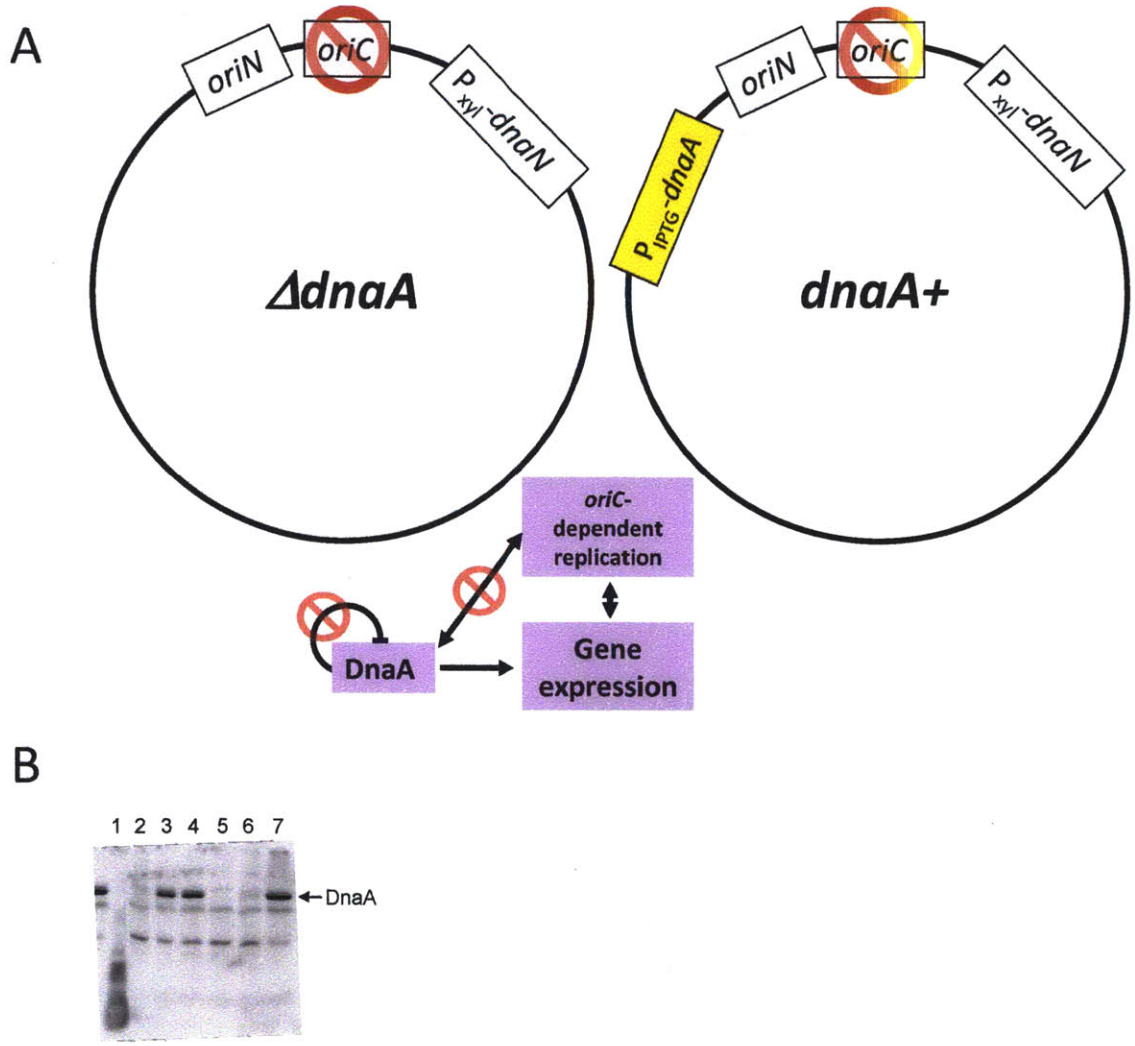

Figure 1. Diagram of non-essential *dnaA* strains and Western blot of relative DnaA levels.

(A) In these strains, the native origin of replication *oriC* is deleted and replaced with *oriN*, a plasmid replicon, which encodes its own replication initiator *repN*. In the absence of *oriC*, replication proceeds from *oriN*, independently of *dnaA*. The sliding clamp *dnaN* is expressed ectopically under control of a xylose-inducible promoter. In the *dnaA*$^{+}$ strain, *dnaA* is expressed ectopically under control of an IPTG-inducible promoter. Under these conditions, effects of DnaA on gene expression can be studied independently of its role in replication initiation. (B) Western blot of DnaA. Lanes: 1) Novex protein standards, 2) *ΔdnaA* (AIG200), 3) wildtype (AG174), 4) PcomG-*lacZ* (JMS289), 5) *ΔdnaA* PcomG-*lacZ* (TAW28), 6) Pspank-*dnaA* PcomG-*lacZ* (TAW35) -IPTG, 7) Pspank-*dnaA* PcomG-*lacZ* (TAW35) +IPTG

Effects of *dnaA* on gene expression during exponential growth

We evaluated the effects of loss of *dnaA* on gene expression during exponential growth. We compared mRNA levels for virtually all open reading frames in cells replicating from *oriN*, either in the absence or presence of *dnaA*. Relative amounts of mRNA in a *dnaA* null mutant were plotted versus the relative amounts in an isogenic *dnaA*+ strain (Fig. 2). We identified 463 genes whose expression was significantly altered in the absence of *dnaA* (q-value ≤ 0.003). Of these genes, expression of 125 decreased and 338 increased in the absence of *dnaA* (Fig. 2). These results indicate that expression of 125 genes is normally activated and that of 338 genes is normally repressed by DnaA, either directly or indirectly.

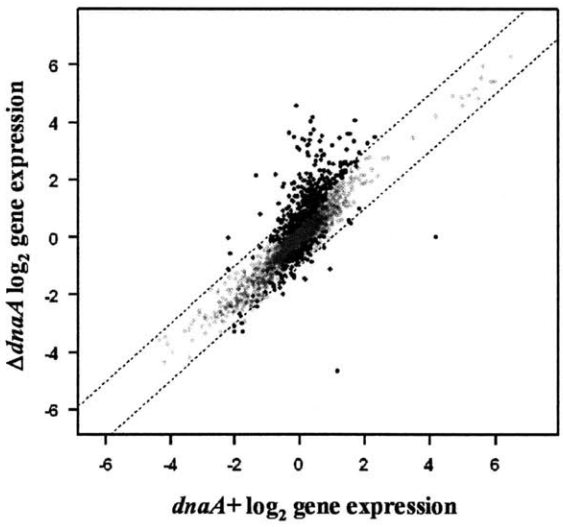

Figure 2. Differential gene expression between Δ*dnaA* and *dnaA*+ cells.

All gene expression measurements were made relative to a common reference as described in the Materials and Methods section. In black are genes (n = 463) that are considered differentially expressed (q-value ≤ 0.003). In gray are genes that do not meet the statistical criterion for differential expression. The dotted lines signify 2-fold differences in expression between Δ*dnaA* and *dnaA*+ cells; points outside the dotted lines correspond to differences greater than 2-fold, and points inside the dotted lines correspond to differences less than 2-fold.

The effects of *dnaA* on expression of most of these genes was likely indirect. Previous analyses of DnaA binding and differential gene expression in the presence versus absence of replication stress revealed approximately 55 potential direct targets of DnaA (Breier and Grossman, 2009; Goranov et al., 2005), and of the 463 genes affected by loss of *dnaA*, only 13 were previously inferred to be controlled directly by DnaA (Table 2). Most of DnaA's effects on gene expression therefore appear to be indirect.

Network analysis of genes affected by *dnaA*

To better characterize the indirect effects of DnaA on gene expression, we searched for known regulons overrepresented within the set of genes differentially expressed between *ΔdnaA* and *dnaA*$^+$ cells. Enrichment of a given regulon would imply DnaA to act, somehow, through the corresponding regulator of said regulon. We expected genes from a given regulon to be randomly distributed (hypergeometrically) between the sets of differentially and non-differentially expressed genes. If genes of a given regulon, however, were present among the set of differentially expressed genes in numbers significantly exceeding those expected by chance (q-value ≤ 0.05), then we considered the set of differentially expressed genes enriched for that given regulon. We found that genes in the Spo0A, AbrB, PhoP, SinR, Btr, and YvrH regulons were significantly affected by *dnaA* (Table 3). Intriguingly, the regulons identified are involved in controlling gene expression associated with nutrient limitation and entry into stationary phase.

Spo0A is the master regulator of stationary phase gene expression and the initiation of sporulation. Spo0A is active in the phosphorylated form (Spo0A~P) and controls expression of many genes, both directly and indirectly (Molle et al., 2003). Phosphorylation of Spo0A occurs through a phosphorelay. Several histidine protein kinases autophosphorylate and donate

phosphate to the response regulator Spo0F. Phosphate is then transferred from Spo0F to Spo0B, and finally from Spo0B to Spo0A (Fig. 3).

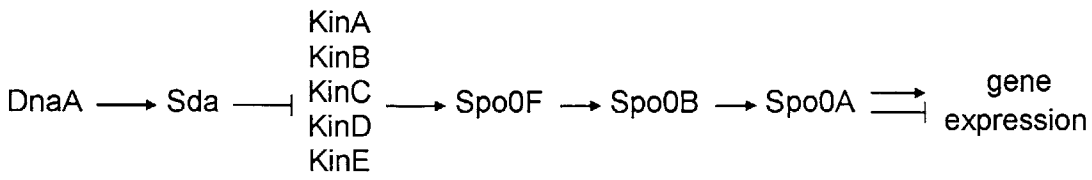

Figure 3. Diagram of phosphorelay.

Spo0A is activated by a phosphorelay involving multiple histidine kinases. Under appropriate conditions, they autophosphorylate, and each can then transfer phosphate to the response regulator Spo0F. Spo0F, in turn phosphorylates Spo0B, which ultimately phosphorylates Spo0A. Once phosphorylated, Spo0A becomes active and can then regulate downstream gene expression. DnaA is connected to Spo0A activation via one of its direct targets Sda, which preferentially inhibits KinA and KinB autophosphorylation.

The activation (phosphorylation) of Spo0A is affected by DnaA via the direct transcriptional activation of the sporulation inhibitory gene *sda* by DnaA. *sda* encodes a small checkpoint protein that inhibits phosphorylation of Spo0A and the initiation of sporulation by inhibiting the kinases that are primarily responsible for activation of Spo0A (Cunningham and Burkholder, 2009; Veening et al., 2009; Whitten et al., 2007). As a result, stationary phase gene expression and initiation of sporulation are inhibited when expression of *sda* is increased and elevated when expression of *sda* is low. Several of the regulons affected by DnaA, including AbrB, SinR, and PhoP, are regulated by Spo0A via its effect on the expression of each regulator, thereby providing a possible link to DnaA via *sda* (Fig. 4).

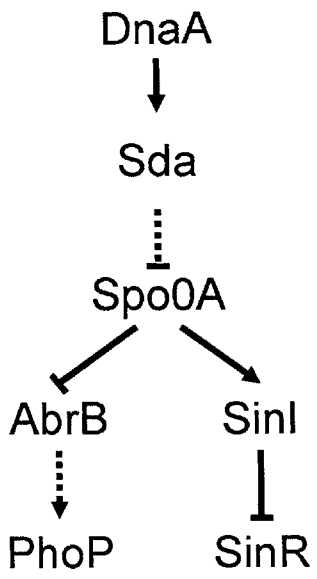

Figure 4. Regulatory connections downstream of DnaA.

DnaA activates expression of *sda*, the product of which inhibits activation (phosphorylation) of Spo0A. Spo0A~P inhibits expression of *abrB*, which when deleted lowers expression of the PhoP regulon. Spo0A~P also activates expression of *sinI*, the product of which inhibits activity of the transcription factor SinR.

AbrB functions mostly as a repressor of gene expression, and as cell growth approaches stationary phase, its levels become reduced causing de-repression of its target genes. AbrB is affected by Spo0A~P in two ways. Transcription of *abrB* is repressed by Spo0A~P (Furbass et al., 1991; Strauch et al., 1990), and Spo0A~P activates transcription of *abbA*, encoding an inhibitor of AbrB (Banse et al., 2008).

SinR is a transcription factor that controls genes involved in cell motility and biofilm formation (Chu et al., 2006; Kearns et al., 2005). SinR is inhibited by SinI (Bai et al., 1993) and SlrR (Chu et al., 2008), and transcription of these is controlled by Spo0A and AbrB (Chai et al., 2008; Chu et al., 2008).

PhoP, a transcription factor activated upon phosphate starvation, activates transcription of genes related to phosphate uptake and utilization (Hulett, 2002). Expression of *phoP* is affected

by AbrB and a transcription factor called ScoC (Hulett, 2002; Kaushal et al., 2010; Sun et al., 1996), both of which are affected by Spo0A.

Btr is a transcriptional activator involved in iron homeostasis (Gaballa and Helmann, 2007), and YvrH is a response regulator transcription factor that controls genes involved in cell wall processes (Salzberg and Helmann, 2008; Serizawa et al., 2005). There are no known connections, however, between Spo0A and either regulator.

Role of *sda* on gene networks controlled by DnaA

Based on the known connections among DnaA and transcription of *sda*, the effects of Sda on Spo0A activity, and the effects of Spo0A on other regulators, we postulated that the control of *sda* by DnaA could account for a significant portion of differential gene expression between *ΔdnaA* and *dnaA*$^+$ cells. If so, then ectopic expression of *sda*, independently of *dnaA*, should suppress the effects of loss of *dnaA*. In addition, since DnaA activates transcription of *sda*, loss of *sda* should cause many of the same or similar effects as loss of *dnaA*. We tested these predictions using a fusion of *sda* to the IPTG-inducible promoter Pspank (Pspank-*sda*) and a loss of function allele of *sda*.

We compared gene expression between *ΔdnaA* and *dnaA*$^+$ cells that have *sda* either ectopically expressed or deleted. Overexpressing or deleting *sda* within both *ΔdnaA* and *dnaA*+ cells suppresses many of the effects of a *dnaA* null mutation (Fig. 5; columns 1, 3, and 4).

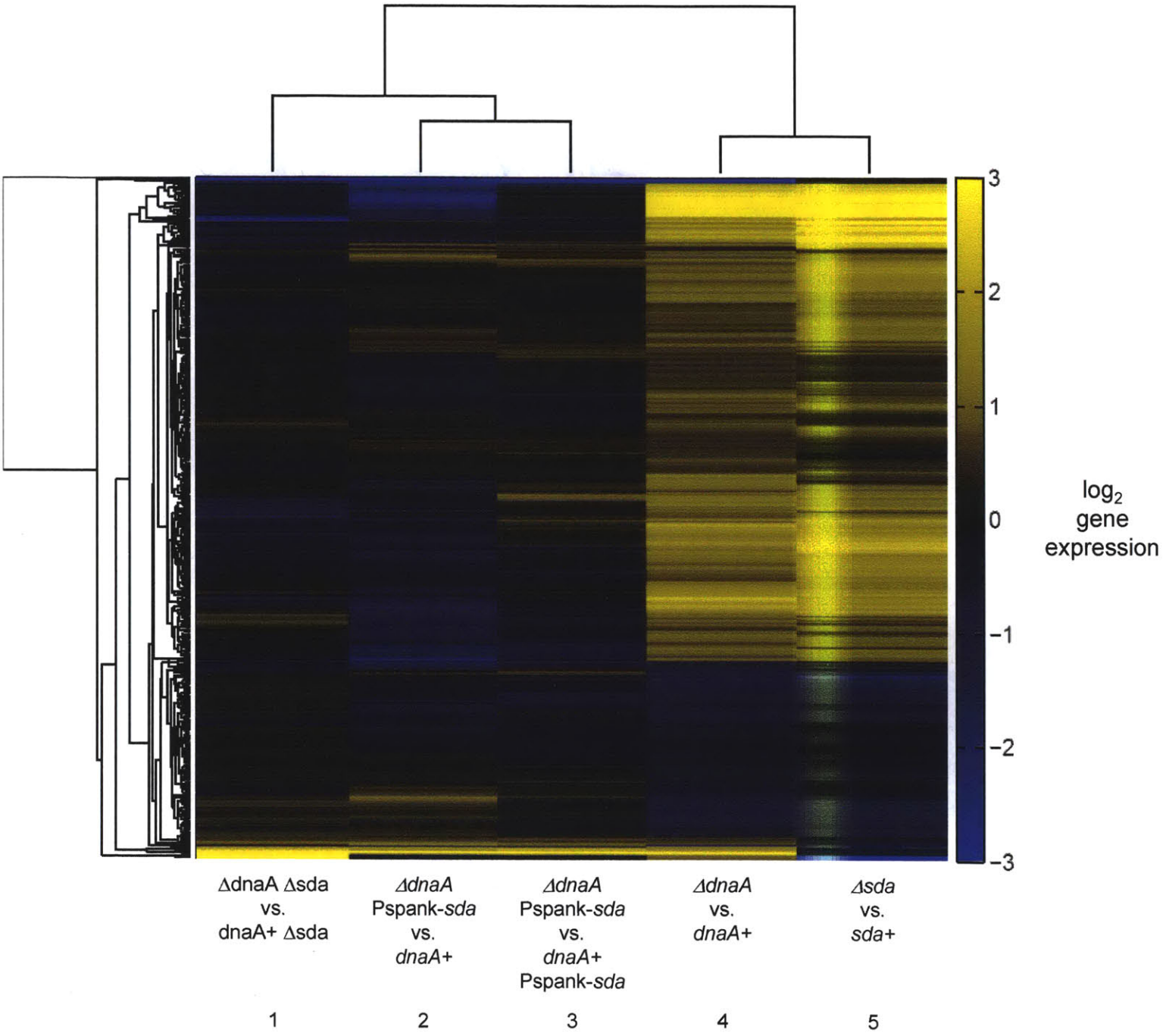

Figure 5. Gene expression profiles of 463 *ΔdnaA* vs. *dnaA*+ differentially expressed genes across various Pspank–sda and *Δsda* strain backgrounds.
Only genes differentially expressed (q-value ≤ 0.003) between *ΔdnaA* vs. *dnaA*+ cells (column 4) are shown. $\log_2$ gene expression values are depicted in shades of yellow, black, and blue. Some genes, though differentially expressed, appear as black because of a statistical significance cutoff vs. fold-change cutoff. Also, though off scale, values greater than or equal to an 8-fold change in gene expression appear as yellow or blue. Genes (rows) are hierarchically clustered by

Euclidean distance. Strain comparisons (columns) are hierarchically clustered by correlation coefficient. Differential gene expression changes markedly depending on relative levels of *sda* expression between *ΔdnaA* vs. *dnaA+* cells. Overexpression of *sda* in a *ΔdnaA* mutant reverses expression differences between *ΔdnaA* vs. *dnaA+* cells (columns 2 and 3 vs. column 4). Deletion of *sda* in both *ΔdnaA* vs. *dnaA+* cells likewise negate expression differences observed between *ΔdnaA* vs. *dnaA+* cells (column 1 vs. column 4). Profiles of differential gene expression between *Δsda* vs. *sda+* cells and *ΔdnaA* vs. *dnaA+* cells appear very similar (column 5 vs. column 4). Therefore, most expression changes observed between *ΔdnaA* vs. *dnaA+* cells are likely due to DnaA activation of *sda*.

Of the 463 differentially expressed genes that occur between *ΔdnaA* and *dnaA+* cells, we conservatively estimate at least 149 to be regulated by *sda* and at least 9 to be regulated independently of *sda*. We consider a gene to be *sda*-controlled if *sda* overexpression or deletion significantly alters the effects of a *ΔdnaA* mutation. Likewise, for expression to be considered independent of *sda*, expression changes in a *ΔdnaA* mutant must remain relatively constant despite *sda* overexpression or deletion. Among *sda*-controlled genes are those belonging to regulons described in Table 3; however, only the Spo0A, AbrB, SinR, and PhoP regulons remain significantly enriched. The Btr regulon was not represented among these genes and is unlikely controlled by Sda and its effects on known downstream regulators. DnaA-dependent expression changes that are independent of *sda* include known direct targets *ywlC, gidA,* and *yqeF* (immediately upstream of *sda*).

We also found that loss of *sda* caused many changes in gene expression that were similar to those observed in the absence of *dnaA* (Fig. 5; columns 4 and 5). Again, these results are consistent with the inference that many of the effects of loss of *dnaA* are mediated through decreased expression of *sda*.

The effects of *dnaA* on the transcriptional responses to replication stress

Several previously proposed DnaA targets were shown to change expression in response to replication stress. In addition, some of these targets, due to replication stress, also bound increased amounts of DnaA (Breier and Grossman, 2009; Goranov et al., 2005). Since we found relatively few of DnaA's putative direct targets (Breier and Grossman, 2009; Goranov et al., 2005)differentially expressed between *ΔdnaA* and *dnaA*+ cells (Table 2), we suspected the expression of some direct targets to depend also on replication stress. We decided to compare gene expression between the *ΔdnaA* and *dnaA*+ strains with and without replication stress.

Specifically, we compared the response to HPUra within the Δ*dnaA* strain to that within the *dnaA*+ strain. Considering these two responses, we grouped genes into two categories: those with an HPUra response independent of *dnaA* (457 genes) and those with an HPUra response dependent on *dnaA* (55 genes). Among the former are genes that constitute part of the well-characterized RecA/LexA-dependent DNA damage response, which are listed in Table 4. Genes among the latter are listed with expression values in Table 5 and include sporulation killing factors (*skfBCGH*), ribonucleotide reductase and associated factors (*nrdIEF-ymaB*), pyrimidine biosynthesis machinery (*pyrAAABBCDEFKP*), and components of the integrative and conjugative element *ICEBs1* (*yydGH*) and temperate phage *SPβ*. Some of these and others that also respond to HPUra in a *dnaA*-dependent manner were previously predicted to be DnaA direct targets (Breier and Grossman, 2009; Goranov et al., 2005) and include *dhbE*, *nrdIEF-ymaB*, *pyrAAABBCDEFKP*, *ywlC*, and *yyzF* (immediately upstream of *yydA*). The genes *ywlC* and *yyzF* are both well-established direct targets of DnaA (Breier and Grossman, 2009; Goranov et al., 2005). Clusters of potential DnaA binding sites exist within the promoter regions and coding sequences of both genes, and DnaA has been shown to bind to these regions *in vivo* (Breier and

Grossman, 2009; Ishikawa et al., 2007). *dhbE, nrdIEF-ymaB*, and *pyrAAABBCDEFKP*, however, contain fewer putative DnaA binding sites within their regulatory regions and have not definitively been shown to bind DnaA *in vivo*. It is likely that DnaA's effect on their transcriptional response to HPUra and that of other genes not shown to be directly regulated by DnaA is indirect and mediated by additional regulators (Table 5). These regulators likely include Sda and those downstream of Sda (e.g. Spo0A, AbrB), since association of DnaA at and transcription from the *sda* promoter markedly increase during replication stress (Burkholder et al., 2001). Genes regulated by PyrR, PurR Fur, LexA, ResD, and RapI appear also within Table 5; however, the mechanism by which DnaA may be affecting these regulators remains unknown.

Effects of *dnaA* on competence development

While working with various *oriN* strains, we noticed that they were difficult to transform using standard protocols (Spizizen, 1958) and wondered if they were defective in competence development. To assay their relative timing and levels of competence development, we introduced into these strains a fusion of *lacZ* to a competence-specific promoter (PcomG-*lacZ*). This fusion, expressed only during competence development, is a proxy for competence development (Magnuson et al., 1994). Note that we could not reliably infer effects on competence gene expression from the microarray data presented above since competence development does not coincide with the time at which samples for mRNA analyses were taken.

We found that expression of PcomG-*lacZ* was reduced in the absence of *dnaA* (Fig. 6).

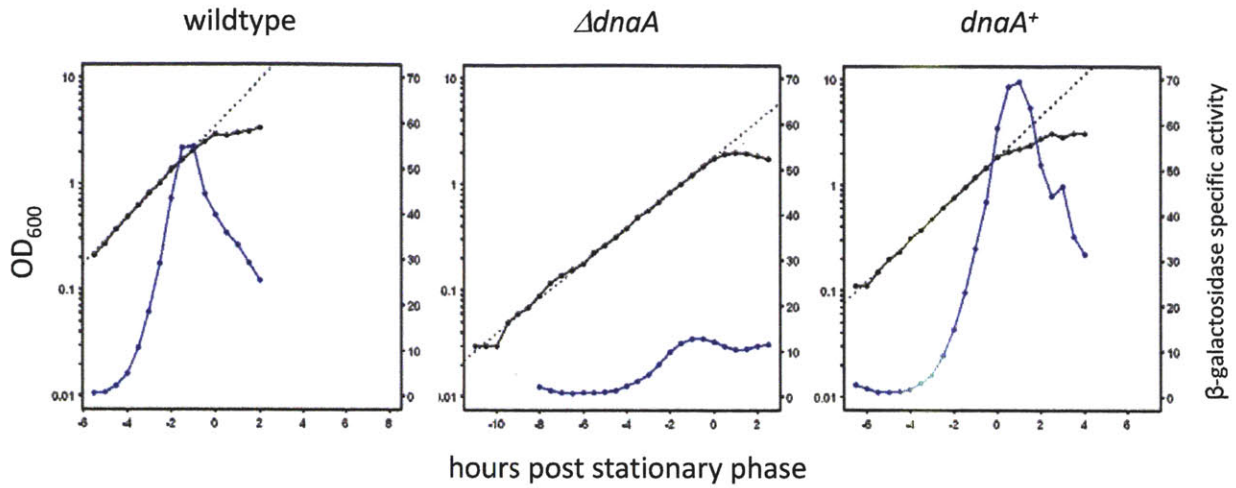

Figure 6. Competence development is reduced in a ΔdnaA mutant.

A PcomG-*lacZ* reporter was introduced to wildtype, *ΔdnaA*, and *dnaA*$^+$ strain backgrounds to assay competence development over time. *comG* is a competence gene expressed late in the competence development pathway and encodes for a component of the DNA uptake machinery. Cell density is depicted in black, while β-galactosidase specific activity is depicted in blue. In wildtype and *dnaA*$^+$ strain backgrounds, expression of *comG* increases steadily as cells grow and peaks as cells transition from exponential growth to stationary phase. In a *ΔdnaA* mutant, the same trend occurs; however, the relative activity of the *comG* promoter is greatly reduced, resulting in an approximately five-fold decrease in β-galactosidase specific activity.

In the *oriN dnaA*+ strain, PcomG-*lacZ* expression was low at low cell densities and increased during exponential growth as the cell density increased. This pattern of gene expression in defined minimal medium with arabinose and xylose was similar to that of cells growing in defined minimal medium with glucose (van Sinderen et al., 1995). In contrast, in the *oriN* Δ*dnaA* strain, expression of PcomG-*lacZ* was reduced by approximately five-fold. This reduction was consistent with the decreased transformation efficiencies observed in *oriN* Δ*dnaA* strains. Given the known roles of Spo0A and AbrB in competence development (Hahn et al., 1995), the simplest explanation of these results is that the effects of *dnaA* on the Spo0A and AbrB regulons is causing the decrease in competence gene expression and development.

Discussion

Using a *dnaA* null mutant, we analyzed the effects of DnaA on gene expression during exponential growth in *B. subtilis*. Some effects we observed are likely direct; however, most appear to be indirect and occur through activity of the direct target and cell cycle checkpoint gene *sda* and Spo0A, the master regulator of stationary phase and sporulation initiation. We also found that some of the changes in gene expression during replication stress were dependent on *dnaA*, verifying previous predictions (Goranov et al., 2005). There were also several DnaA-dependent changes in gene expression that did not appear to be through *sda* and Spo0A, indicating previously unrecognized connections between DnaA and other transcription factors. Lastly, we found that *dnaA* is needed for normal competence development and that in the absence of *dnaA*, competence levels are greatly reduced. The effect of DnaA on competence development is likely to occur via *sda* and downstream targets, some of which are well known regulators of competence development (e.g. Spo0A, AbrB). The effect of DnaA on global gene expression, spans multiple regulons and is much more widespread than previously thought. Although uncovered by assessing the effects of a *dnaA* null mutation, we suspect that this control likely serves to coordinate multiple cell processes, including competence development, with DnaA activity and hence DNA replication, as is the case for the effects of DnaA on transcription of *sda* (Veening et al., 2009).

DnaA and Sda

The effects of Sda on global gene expression are striking. Of the ~460 genes affected by loss of DnaA, >32% are also affected by loss or overexpression of *sda*. Sda is highly unstable, with a half-life of ~1 minute during exponential growth (Ruvolo et al., 2006). As a result, Sda protein

levels correlate strongly with respective transcript levels and thus DnaA activity. Transcription of *sda* varies through a cell division and replication cycle, likely through its regulation by DnaA (Veening et al., 2009). This coupling between expression of *sda* and the replication cycle likely results in cyclical activation of Spo0A and cyclical expression of many of the indirect targets of DnaA. If any of these gene products are unstable or transiently activated (like Spo0A), then the cyclical nature of their transcription is likely to result in alterations in protein levels and/or activity. Based on the genes affected by loss of *dnaA*, affected processes could include: competence development, cell division, motility, and biofilm formation.

The effects of *dnaA* on competence development likely occur through the effects of DnaA on *sda*. Preliminary results indicate that overexpression of *sda* in a *dnaA* null mutant is sufficient to restore competence gene expression to a level similar to that of an isogenic *dnaA*+ strain (unpublished results). Spo0A activity affects expression of *comK*, the master regulator of competence development and gene expression. Low levels of Spo0A~P activate *comK*, whereas high levels of Spo0A~P repress *comK* (Hahn et al., 1995). Our results indicate that Sda likely influences entry into the competence pathway in addition to entry into sporulation.

It is not yet known how many genes are affected by DnaA in other organisms. DnaA is widely conserved, and its role as a transcription factor is also conserved. However, *sda* is found only in Bacilli. There is an array of DnaA binding sites upstream of *sda* in the organisms that have it, indicating that in these bacteria, the global effects of DnaA are likely conserved. However, if DnaA is having such widespread effects on gene expression on other bacteria, then these effects are likely mediated through other target genes.

Summary

We observed a multitude of DnaA-dependent effects on gene expression, a large portion of which can be attributed to DnaA direct activation of the KinA inhibitor Sda. Expression of *sda* has previously been shown to inhibit entry into sporulation, and we infer from our observations that it also plays an important role in maintaining normal levels of competence development upon exiting exponential growth. These results imply an important cell cycle input signal for not only sporulation but also competence development, and given the regulatory networks involved in these processes, we suspect effects of Sda to extend to other stationary phase-dependent cell phenotypes.

Materials and Methods

Growth conditions

Cells were grown in batch culture at 37 °C in minimal S750 salts supplemented with 1% (w/v) arabinose, 0.5% (w/v) xylose, 0.1 mM IPTG, 40 μg/ml tryptophan, and 40 μg/ml phenylalanine. If cells were also threonine auxotrophs, then 120 μg/ml threonine was also added to the medium. If cells were to be arrested for replication elongation, HPUra was added to the medium at a final concentration of 38 μg/ml.

RNA and cDNA preparation and microarray hybridization and scanning

Cells were harvested during mid-exponential growth (OD_{600} ~ 0.4), fixed with an equivalent volume of cold methanol, and centrifuged at 4 °C. Supernatants were decanted and cell pellets stored at -80 °C until further use. For cell lysis, pellets were thawed at room temperature, re-suspended in 10 mg/ml lysozyme in TE buffer (pH 8), and incubated at 37 °C. RNA was isolated from lysates using the Qiagen RNeasy mini kit and quantified using a NanoDrop ND-1000. Both sample and reference RNA (Goranov et al., 2005; Goranov et al., 2006) were reverse transcribed, and cDNA product purified using the Qiagen MinElute PCR purification kit; washes were performed with 75% ethanol to minimize cross-reactivity during subsequent dye coupling. Cy3 and Cy5 dyes were coupled to reference and sample cDNA, respectively, and reactions were quenched with 4 M hydroxylamine. Separate reference and sample cDNA labeling reactions were pooled and cleaned up using the Qiagen MinElute PCR purification kit. Salmon testes DNA and yeast tRNA were added to each pool of cDNA; cDNA aliquots were then hybridized at 42 °C overnight to microarrays including >95% of annotated *B. subtilis* ORFs and multiple

intergenic regions (Britton et al., 2002). Subsequently, arrays were washed in SSC buffer and then scanned on an Axon Instruments GenePix 4000B scanner.

Array data analysis

Raw data was obtained from the resulting image files using the Genepix Pro software package and normalized using the R statistical software package limma (Smyth, 2005). Gene expression values were corrected for multiple hypothesis testing using the Benjamini-Hocherg correction option in limma. Genes were called differentially expressed such that within a given set of genes the expected number of FP < 1. The set of differentially expressed genes was analyzed for enriched regulons as follows. Known regulons and their associated regulators were extracted from files available in the BsubCyc database version 1.5 (Caspi et al.) and used to generate a "background distribution" of regulons within the *B. subtilis* genome. Enriched regulons were determined using Fisher's exact test with the Benjamini-Hochberg multiple hypothesis testing correction ($q \leq 0.05$).

β-galactosidase assays

Cells were grown under the conditions described above. For each sample, 1 ml of cell culture was harvested, and cells were made permeable by addition of 2 drops of toluene and vigorous mixing. Samples were stored at -20 °C until future use. Samples were thawed at 37°C and ß-galactosidase assays were done essentially as described (Jaacks et al., 1989; Miller, 1972) Specific activity is expressed as the (ΔA420 per min per ml of culture per OD600 unit) x 1000.

Tables.

Table 1. Strains Used in This Study	
Strain	Relevant genotype (comment and/or reference)
AG174	*phe trp* (Perego et al., 1988)
AIG200	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, phe trp*$^+$ (Goranov et al., 2005)
BB668	*Δsda phe trp* (Burkholder et al., 2001)
TAW5	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, lacA::Pspank-dnaA-tet, phe trp*$^+$
TAW86	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, thrC::Pspank-sda-mls, phe trp*$^+$
TAW97	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, lacA::Pspank-dnaA-tet, thrC::Pspank-sda-mls, phe trp*$^+$
TAW106	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, lacA::Pspank-dnaA-tet, spo0A::mls, phe trp*$^+$
TAW118	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, Δsda spoIVC::mls, phe trp*$^+$
TAW121	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, lacA::Pspank-dnaA-tet, Δsda spoIVC::mls, phe trp*$^+$
JMS289	*thrC::PcomG-lacZ-mls, phe trp* (Magnuson et al., 1994)
TAW28	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, thrC::PcomG-lacZ-mls, phe trp*$^+$
TAW35	*ΔdnaA-oriC-dnaN::spc, amyE::P_{xylA}-dnaN-cm, spoIIIJ::oriN-kan, lacA::Pspank-dnaA-tet, thrC::PcomG-lacZ-mls, phe trp*$^+$

Table 2. Previously Inferred Direct DnaA Targets

ID	Name	Description	$\log_2$ *ΔdnaA /dnaA*$^+$	q-value
BSU00010	dnaA	chromosomal replication initiator protein DnaA	-5.849	0
BSU05940	gcp	Metalloprotease	-0.332	0.002816
BSU41010	gidA	tRNA uridine 5-carboxymethylaminomethyl modification enzyme	0.842	0
BSU17390	nrdF	ribonucleoside-diphosphate reductase (minor subunit)	0.389	0.001209
BSU38070	sacT	transcriptional antiterminator	1.783	0
BSU25690	sda	check point factor coupling initiation of sporulation and replication initiation	-0.682	0.000177
BSU21480	sunA	sublancin 168 lantibiotic antimicrobial precursor peptide in SPBeta prophage	1.33	2.00E-06
BSU21470	sunT	Sublancin 168 lantibiotic transporter	1.035	0
BSU38090	vpr	extracellular serine protease	1.609	2.00E-06

BSU38080	ywcI	hypothetical protein	2.238	0
BSU36950	ywlC	putative ribosome maturation factor; RNA binding protein	1.386	0
BSU40230	yydA	conserved hypothetical protein	0.694	5.10E-05
BSU40220	yydB	putative phosphohydrolase	0.682	0.000224

Table 3. Top 10 Enriched Regulons

Regulator	Enriched targets	q-value
AbrB	abrB, aslA, asnH, dppA, dppB, dppC, dppD, dppE, pbpE, racX, rbsA, rbsB, rbsC, rbsD, rbsK, rbsR, rsiW, sboA, sdpA, sdpB, sdpC, sdpI, sdpR, sigH, sigW, sinI, skfA, skfB, skfC, skfE, skfF, skfG, skfH, spo0E, spoVG, yknW, yknX, yknY, yknZ, yoaW, yvlB, yvlD, yxaB, yxaM, yxbB, yxbC, yxbD, yxnB	9.15E-14
Spo0A	abrB, dltB, dltC, dnaA, fruR, pit, racA, sdpA, sdpB, sdpC, sinI, sipW, skfA, skfB, skfC, skfE, skfF, skfG, skfH, spo0A, spo0F, spoIIE, spoIIGA, tasA, yfmI, yfmJ, ykaA, yneF, yqcF, yqxI, yqxJ, yqxM, yqzC, yqzD, yrrL, yuxH, yvhJ, yvyE, yxbC, yxbD	4.31E-09
PhoP	phoD, pstA, pstBA, pstBB, pstS, skfA, skfB, skfC, skfE, skfF, skfG, skfH, tuaA, tuaB, vpr, yjdB	0.009716
Btr	feuA, feuB, feuC, ybbA	0.026852
SinR	epsA, epsB, epsD, epsE, epsF, epsH, epsI, sipW, tasA, yqxM	0.029519
YvrH	bdbA, sigX, sunA, sunT, wapA, wprA, yolJ, yxxG	0.031218
Rok	bdbA, sboA, sdpA, sdpB, sdpC, sunA, sunT, yolJ, yybK, yybM, yybN	0.056811
SdpR	sdpI, sdpR	0.451124
RghRA	rapD, rapG, rapH	0.526677
DnaA	dnaA, sda	0.95788

Table 4. HPUra response among LexA-dependent, DnaA-independent genes

Gene	*ΔdnaA*	q-value	*dnaA+*	q-value	*ΔdnaA* vs. *dnaA+*	q-value
aprX	1.402	0	1.192	1.00E-06	0.21	0.999496
dinB	3.287	0	3.578	0	-0.291	0.999496
lexA	1.392	5.10E-05	1.623	1.00E-06	-0.231	0.999496
ligA	1.06	0.000458	1.118	0.000102	-0.059	0.999496
parC	0.794	0.000262	1.23	0	-0.436	0.573358
parE	0.543	0.027759	0.915	4.80E-05	-0.372	0.825475
recA	2.626	0	2.502	0	0.124	0.999496
ruvA	1.209	0.000201	1.041	0.000734	0.168	0.999496
ruvB	0.748	0.032513	0.942	0.003131	-0.194	0.999496
tagC	5.152	0	4.744	0	0.407	0.999496
tgt	0.632	0.00334	0.838	3.90E-05	-0.206	0.986422
uvrA	1.851	0	2.381	0	-0.531	0.296325
uvrB	1.281	2.00E-05	1.587	0	-0.306	0.970233
uvrX	1.871	0	2.025	0	-0.154	0.999496

xkdA	2.565	0.000126	2.301	0.000278	0.264	0.999496
ydiO	0.925	0.001498	1.205	1.50E-05	-0.281	0.986422
yerH	0.636	0.013545	0.848	0.00034	-0.212	0.999496
yhaO	2.333	0	1.645	6.00E-06	0.687	0.701149
yhaZ	1.907	0	2.199	0	-0.292	0.958928
yhjB	1.339	0.000814	0.778	0.05642	0.561	0.878623
yhjD	3.051	0	3.247	0	-0.196	0.999496
yneA	3.708	0	3.906	0	-0.199	0.999496
yneB	3.693	0	3.72	0	-0.027	0.999496
ynzC	1.37	0.004401	1.45	0.001347	-0.079	0.999496
yokF	-1.33	4.00E-06	-0.447	0.154539	-0.883	0.14125
yolD	1.584	0	1.819	0	-0.235	0.999496
yopT	0.78	0.002581	1.146	4.00E-06	-0.366	0.867513
yopV	0.604	0.415662	1.707	0.001565	-1.104	0.620891
yopW	0.408	0.536837	2.249	1.00E-06	-1.841	0.032665
yopY	0.523	0.129822	0.917	0.001671	-0.394	0.921982
yoqC	0.483	0.484571	2.037	2.70E-05	-1.555	0.149113
yoqH	0.47	0.446835	1.515	0.000609	-1.045	0.452532
yorB	3.927	0	4.136	0	-0.209	0.999496
yorC	2.277	0	2.861	0	-0.584	0.819362
yorE	0.841	0.235632	2.76	2.00E-06	-1.919	0.115874
yorF	1.303	0.064761	3.384	0	-2.081	0.121456
yorH	-0.385	0.699451	1.96	0.001453	-2.344	0.054556
yorI	0.39	0.674256	2.238	0.00012	-1.848	0.158523
yozL	1.211	0.000485	1.458	1.30E-05	-0.246	0.999496
yqjW	1.855	0	2.345	0	-0.49	0.450084
yqjX	2.023	0	2.086	0	-0.063	0.999496
yqjZ	0.153	0.440007	0.586	4.30E-05	-0.433	0.204258

Table 5. Comparison of the HPUra response between *ΔdnaA* and *dnaA*+ strains

Gene	*ΔdnaA*	q-value	*dnaA*+	q-value	*ΔdnaA* vs. *dnaA*+	q-value	Potential Regulator
abrB	**1.661**	**0**	**-0.355**	**0.233895**	**2.016**	**1e-06**	**Spo0A, AbrB**
alaR	-0.518	0.052331	0.567	0.02071	-1.085	0.011525	Unknown
blyA	-0.638	0.048088	0.723	0.013988	-1.361	0.007582	*SPβ*
dhbE	-4.088	0	-1.546	0.012283	-2.542	0.025684	Fur
i_pucM_pucE	-0.442	0.245752	1.049	0.000525	-1.491	0.004613	Unknown
i_yfmG_yfmF	**-2.267**	**0**	**0.045**	**0.96685**	**-2.312**	**0.000381**	
i_yonN_yonK	1.035	0.056482	3.911	0	-2.876	0.000284	*SPβ*, LexA
i_yydA_yycS	**0.515**	**0.167328**	**-0.809**	**0.010214**	**1.324**	**0.022119**	**DnaA**
nrdE	-0.771	0.003613	0.336	0.280258	-1.107	0.015801	ResD
nrdF	-1.243	0	0.426	0.028035	-1.669	0	ResD
pyrAA	0.966	3.5e-05	-1.109	1e-06	2.075	0	PyrR, PurR

pyrAB	0.614	0.001477	-0.523	0.004775	1.136	0.00015	PyrR, PurR
pyrB	1.467	0.006592	-1.09	0.040982	2.558	0.003359	PyrR, PurR
pyrC	0.797	0.010999	-1.227	2.6e-05	2.024	1.9e-05	PyrR, PurR
pyrD	1.539	0	-0.507	0.038461	2.046	0	PyrR, PurR
Pyre	1.106	0.000181	-0.719	0.011863	1.825	6.1e-05	PyrR, PurR
pyrF	1.281	1e-06	-0.885	0.000272	2.166	0	PyrR, PurR
pyrK	1.02	0.000893	-0.507	0.12381	1.527	0.002211	PyrR, PurR
pyrP	0.419	0.119367	-0.542	0.022006	0.961	0.02551	PyrR, PurR
ybcP	**-3.217**	**1e-06**	**-0.16**	**0.907686**	**-3.057**	**0.004551**	**AbrB**
ybcS	**-4.081**	**0**	**-0.602**	**0.366013**	**-3.479**	**2.1e-05**	**AbrB**
ybcT	**-3.276**	**0**	**-0.731**	**0.016004**	**-2.545**	**0**	**AbrB**
ybdD	**-3.225**	**0**	**-1.075**	**0.005515**	**-2.15**	**0.000803**	**AbrB**
ybdE	**-3.03**	**0**	**-0.44**	**0.343337**	**-2.589**	**9e-06**	**AbrB**
ybdG	**-2.285**	**0**	**-0.168**	**0.77769**	**-2.118**	**3.2e-05**	**AbrB**
ybeC	**2.101**	**0**	**0.75**	**0.02071**	**1.351**	**0.020634**	
yddG	2.796	0	1.138	0.000697	1.658	0.00456	RapI
yddH	2.39	0	0.918	0.001921	1.472	0.004214	RapI
yetG	-2.16	0	-0.216	0.733854	-1.944	0.001254	Fur
yfmE	**-1.908**	**0**	**-0.074**	**0.917041**	**-1.834**	**0.000381**	**Fur**
yfmF	**-2.325**	**0**	**-0.056**	**0.938683**	**-2.269**	**6e-06**	**Fur**
yfmI	**-2.398**	**0**	**-1.236**	**1e-06**	**-1.163**	**0.004904**	**Spo0A**
ykuP	-3.484	0	-1.329	0.008607	-2.155	0.02021	Fur
ymaA	**-1.11**	**0**	**0.445**	**0.032203**	**-1.555**	**1e-06**	**ResD**
ymaB	-0.574	0.070126	0.624	0.032418	-1.198	0.020634	ResD
yomI	-0.31	0.615156	1.786	1.3e-05	-2.096	0.00237	*SPβ*, LexA
yomM	-0.179	0.804568	1.633	4.8e-05	-1.812	0.01318	*SPβ*, LexA
yomR	0.385	0.54266	2.383	0	-1.998	0.009741	*SPβ*, LexA
yomV	0	0.999787	2.944	0	-2.944	0.001616	*SPβ*, LexA
yonA	-0.286	0.768115	2.45	1.3e-05	-2.736	0.00456	*SPβ*, LexA
Yond	0.586	0.446835	3.031	0	-2.446	0.015801	*SPβ*, LexA
yonE	-0.09	0.881103	1.776	0	-1.866	0.000274	*SPβ*, LexA
yonF	0.807	0.155887	3.265	0	-2.458	0.002474	*SPβ*, LexA
yonG	0.335	0.46577	2.452	0	-2.117	6.1e-05	*SPβ*, LexA
yonI	0.7	0.201184	2.887	0	-2.187	0.004794	*SPβ*, LexA
yonJ	0.798	0.17021	3.378	0	-2.581	0.001634	*SPβ*, LexA
yonK	0.574	0.051291	1.954	0	-1.38	0.002076	*SPβ*, LexA
yonP	-0.078	0.931587	1.732	4.7e-05	-1.81	0.022286	*SPβ*, LexA
yoqM	-0.69	0.317342	1.545	0.003588	-2.235	0.023353	*SPβ*, LexA
yosN	-0.081	0.948136	2.48	2.5e-05	-2.562	0.017803	*SPβ*, LexA
yosO	-0.188	0.817395	1.801	5.5e-05	-1.988	0.015088	*SPβ*, LexA
yosP	-1.144	0	0.71	0.000705	-1.853	0	*SPβ*, LexA
ytzD	0.713	0.017337	-0.497	0.106377	1.21	0.017651	Unknown
yusV	-1.299	0	-0.429	0.036081	-0.87	0.014908	Fur
ywlC	**0.691**	**0.013727**	**-1.09**	**2.7e-05**	**1.78**	**2.1e-05**	**DnaA**

Note: Genes highlighted in blue and pink are previously described direct targets of DnaA. (Breier and Grossman, 2009; Goranov et al., 2005). Expression of those in blue appears to be independent of *sda*; overexpression or deletion of *sda* does not significantly affect their difference in expression between *ΔdnaA* vs. *dnaA+* cells. Expression of those in pink, however, appears to be dependent on *sda*. Their expression is significantly changed in both *ΔdnaA* vs. *dnaA+* and *Δsda* vs. *sda+* comparisons and is therefore likely mediated by regulators downstream of Sda.

Appendix

YabA associates with chromosomal regions bound by Rok and depends on Rok to associate with these regions

Houra Merrikh, Tracy Washington, and Alan D. Grossman

This work was done in collaboration with Houra Merrikh, who performed all ChIP-chip experiments. I analyzed and plotted all ChIP-chip data.

Replication initiation is an essential cellular process. Its timing and frequency must be carefully controlled to coordinate with cell growth. In *E. coli*, regulation of replication initiation is maintained by multiple mechanisms, including RIDA, origin sequestration, and titration of DnaA. In *B. subtilis*, however, much less is known about which factors govern initiation control. Several proteins, such as DnaD, Soj, SirA, and YabA, have been shown to have effects on initiation, and all have been shown to localize to and affect DnaA binding at the origin. Specifically, our interests are focused on YabA, a negative regulator of initiation. YabA interacts directly with DnaA to associate with the origin. Its presence at the origin affects DnaA binding, which inhibits initiation. *In vitro* YabA has been shown to lower the cooperativity of DnaA binding.

Given YabA's localization to the origin and influence on DnaA binding, does YabA associate with other chromosomal regions bound by DnaA? Might it also influence gene expression at these regions? To answer these questions, we determined the genome-wide binding profile of YabA in *B. subtilis* by ChIP-chip and compared its binding to its previously published effects on gene expression. We found that YabA does associate with chromosomal regions outside of the origin; however, it alone does not appear to play a role in regulating the expression of genes at these regions. As expected, YabA associates with some regions bound by DnaA. However, it also associates with several regions not shown to be DnaA-bound. Genes at some of these regions were shown to be regulated by the nucleoid binding protein and transcription factor Rok (Fig 1).

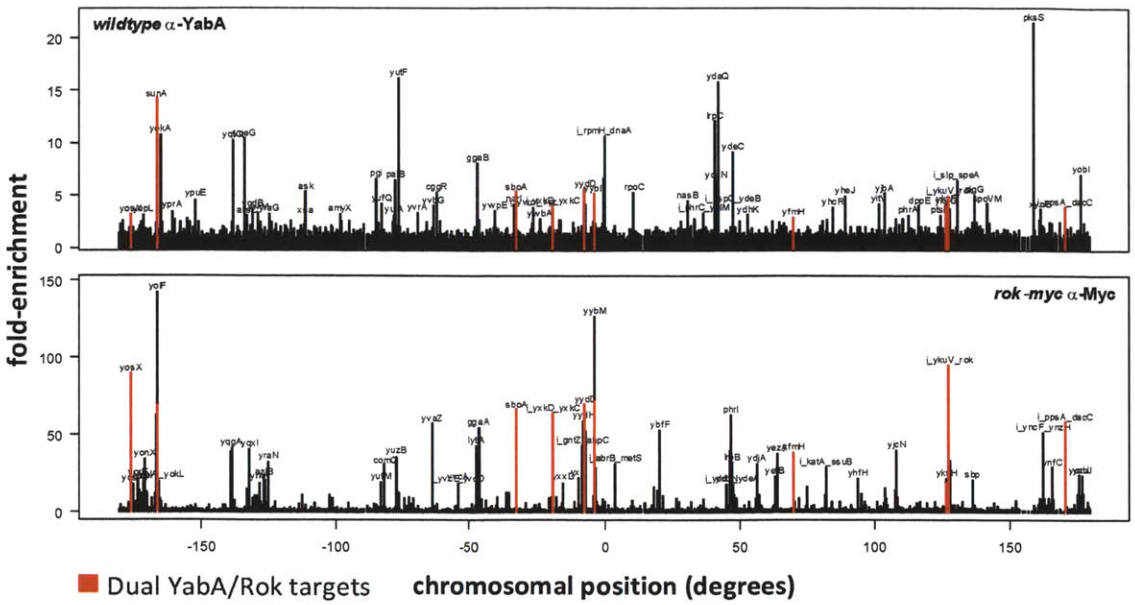

Figure 1. YabA and Rok associate together at specific regions.

Genome-wide association of YabA and Rok was measured by ChIP-chip. YabA was pulled down using an anti-YabA antibody; Rok was Myc-tagged and pulled down using an anti-Myc antibody. YabA association is depicted in the top panel; Rok association is depicted in the bottom panel. Some of the regions to which YabA localizes are also occupied by Rok, and these regions are colored in orange. They include, from left to right, *yosX, sunA, sboA, i_yxkD_yxkC, yydD, yfmH, i_ykuV_rok,* and *i_ppsA_dacC*. Intergenic regions are denoted by the letter "i", followed by the names of the flanking genes, which are separated by an underscore.

Thus, we predicted that association of YabA at these regions may be due to the presence of Rok. To test this hypothesis, we reexamined the genome-wide binding profile of YabA in a *rok* null mutant and showed that *rok* is essential for YabA association at some of these regions (Fig 2).

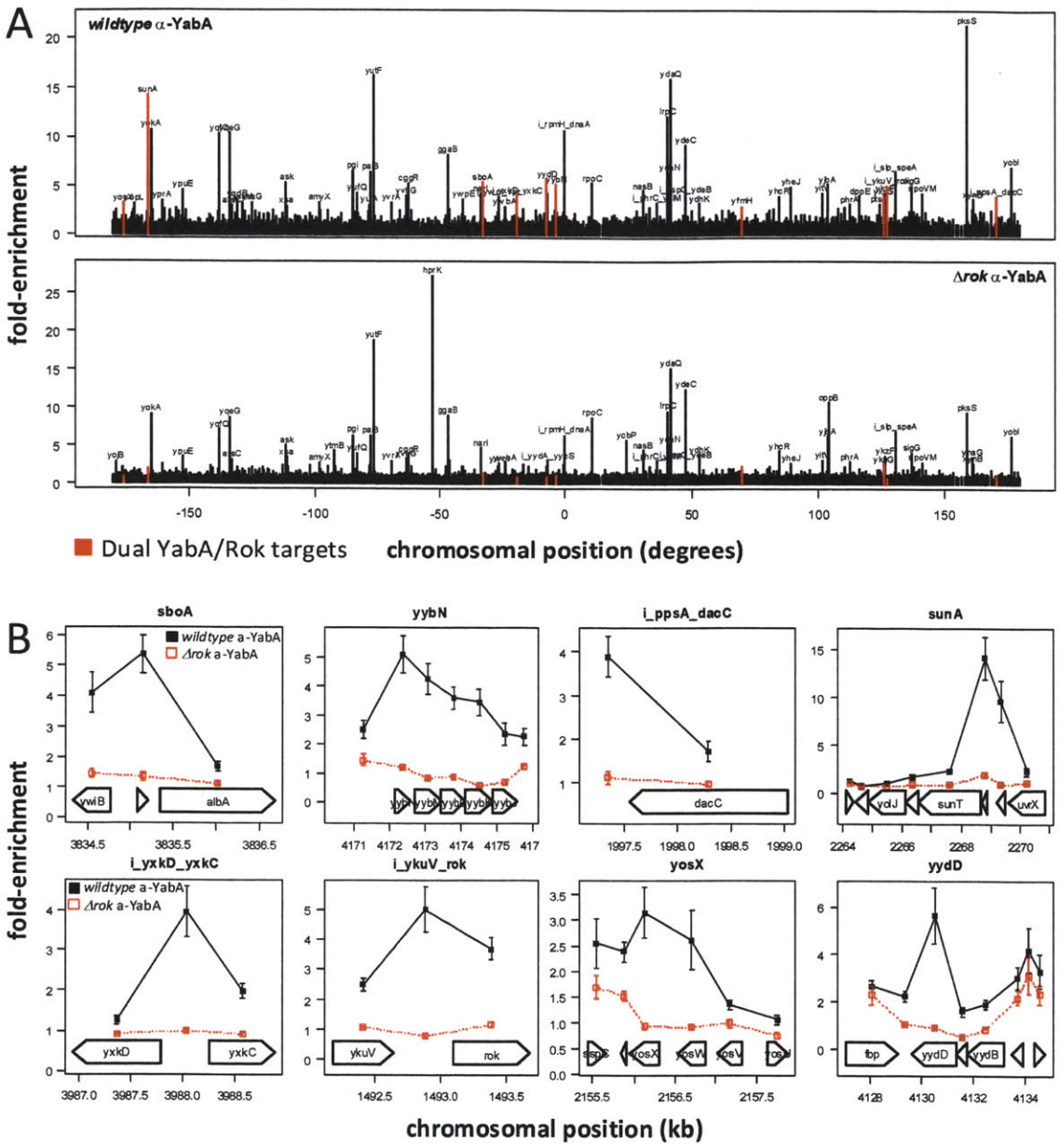

Figure 2. YabA depends on Rok to associate with some regions.

Genome-wide YabA association was determined in the presence versus absence of *rok* (top panels). YabA association at some regions was found to be absolutely dependent on *rok*. These regions are plotted in more detail in the bottom panels. All ChIP-chip measurements were performed at least three times; error bars depicted in the bottom panels represent the standard error of the mean.

Additional work regarding the connections among DnaA, YabA, and Rok is being performed by Charlotte Seid, a current graduate student in the Grossman lab. She is currently investigating

by ChIP-seq the dependencies among the three proteins with respect to their patterns of genome-wide association. She is also exploring their individual effects on replication and gene expression.

Chapter 3

Conclusions and Perspectives

DnaA, the bacterial replication initiator, also functions as a transcription factor to regulate gene expression. In *B. subtilis*, I found that most of DnaA's effects on gene expression are indirect and occur through DnaA activation of *sda*. The gene *sda* encodes an inhibitor of the histidine kinase KinA, which is responsible for downstream phosphorylation and activation of the transcription factor Spo0A. At least 32% of DnaA's effects on gene expression during exponential growth are dependent on the Spo0A regulon and regulons affected by Spo0A, such as the AbrB and SinR regulons. Initially, DnaA's effect on Spo0A through *sda* was discussed solely within the context of sporulation. A temperature-sensitive *dnaA* mutant was found to be inefficient at sporulating, and mutations in *sda* and DnaA boxes within the *sda* promoter restored wildtype sporulation frequencies within this mutant (Burkholder et al., 2001). The *dnaA(ts)* allele, therefore, appeared to be hyperactive with respect to regulation of *sda* expression and entry to sporulation. We now recognize that DnaA activation of *sda* gene expression may have effects on processes other than sporulation. We have shown that DnaA is necessary for normal competence development, and preliminary results indicate that DnaA's effect on competence may also occur through *sda*. Since sporulation and competence development share common upstream regulators, other processes controlled by these same regulators, such as cell motility, chaining, and biofilm formation, are also likely affected by DnaA and *sda*. Furthermore, given the link between DnaA activity, *sda* expression, and the cell cycle, it would be interesting to verify whether or not these processes are cell cycle dependent. To do so, one could synchronize replication of *B. subtilis* cells using temperature-sensitive replication initiation mutants (e.g. *dnaB(ts)*) and measure the expression of various reporter genes, such as those encoding products necessary for flagella or exopolysaccharide synthesis. Establishing a connection between

replication and cell motility and/or biofilm formation may reveal new insight into their particular functions.

Sda is not widely conserved and is present only within the Bacilli. However, despite this lack of conservation, *sda* regulatory activity could represent a common mechanism or at least hint at potential regulatory targets (e.g. two-component systems, signaling networks) for DnaA in other systems. Not much is known about DnaA transcriptional regulatory activity in other organisms, except that DnaA control of other processes seems to be closely related to its role in replication during the cell cycle. For example, in *C. crescentus*, DnaA has been shown to activate expression of *ftsZ*, an essential component of the cell division machinery. It also drives cell cycle progression by activating the cell cycle regulator CtrA indirectly via activation of the gene *gcrA* (Collier et al., 2006; Hottes et al., 2005). DnaA in *B. subtilis* has also previously been shown to repress expression of *ftsL* during replication stress (Goranov et al., 2005). FtsL like FtsZ is also an essential component of the cell division machinery. As mentioned previously, DnaA also activates expression of genes encoding ribonucleotide reductase, the enzyme responsible for synthesizing deoxyribonucleotides from ribonucleotides (Gon et al., 2006). DnaA control of these genes is likely directly related to its role in initiating DNA replication. Lastly, in eukaryotes, depletion of a subunit of the origin recognition complex Orc6 has been shown to result in aberrant cell division (Prasanth et al., 2002; Scholefield et al., 2011).

Given this common theme, it appears that DnaA's transcriptional regulatory activity across many organisms is intricately linked to its role in replication initiation and that perhaps most of the genes DnaA controls are somehow related to cell cycle progression. In *B. subtilis*, sporulation, which includes a chromosome partitioning and cell division-like event during the formation of the endospore and mother cell, must be preceded by DNA replication, and as such

constitutes a process for which DnaA targeting and control could be useful. DnaA's effects on competence development therefore could be mere coincidence due to crosstalk between the sporulation and competence development regulatory networks. Alternatively, competence development could serve a role related to replication, such as DNA repair. Such a function for competence development has been shown in other organisms (Charpentier et al.).

All of the gene expression measurements made in Chapter 2 were made during mid-exponential growth, and many of the genes found to be affected by DnaA are involved in processes related to stationary phase. It would be interesting to determine the effects of DnaA on gene expression during the approach to stationary phase, especially since it has been shown that nucleotide pools, notably ATP, rapidly decrease upon cell entry into stationary phase (Buckstein et al., 2008). Since DnaA binding at the *sda* promoter is especially sensitive to DnaA-ATP versus DnaA-ADP and *sda* expression cycles with replication initiation, DnaA activity at the *sda* promoter may rapidly change during stationary phase due to decreases in ATP levels. Regulators of DnaA activity at the origin may also provide additional regulation of DnaA activity at *sda* during changes in nucleotide levels.

DnaA, a widely conserved bacterial replication initiator, governs the expression of many genes, and within *B. subtilis* and perhaps many of the Bacilli, through the KinA inhibitor Sda. Sda and its control of gene expression may reflect a unique strategy among the Bacilli for linking the cell cycle to not only sporulation but competence development and other cell processes as well.

References

Albano, M., Smits, W. K., Ho, L. T., Kraigher, B., Mandic-Mulec, I., Kuipers, O. P., and Dubnau, D. (2005). The Rok protein of Bacillus subtilis represses genes for cell surface and extracellular functions. J Bacteriol *187*, 2010-2019.

Allenby, N. E., O'Connor, N., Pragai, Z., Ward, A. C., Wipat, A., and Harwood, C. R. (2005). Genome-wide transcriptional analysis of the phosphate starvation stimulon of Bacillus subtilis. J Bacteriol *187*, 8063-8080.

Bai, U., Mandic-Mulec, I., and Smith, I. (1993). SinI modulates the activity of SinR, a developmental switch protein of Bacillus subtilis, by protein-protein interaction. Genes Dev *7*, 139-148.

Banse, A. V., Chastanet, A., Rahn-Lee, L., Hobbs, E. C., and Losick, R. (2008). Parallel pathways of repression and antirepression governing the transition to stationary phase in Bacillus subtilis. Proc Natl Acad Sci U S A *105*, 15547-15552.

Bonilla, C. Y., and Grossman, A. D. (2012). The primosomal protein DnaD inhibits cooperative DNA binding by the replication initiator DnaA in *Bacillus subtilis*. J Bacteriol *194*, 5110-5117.

Bramhill, D., and Kornberg, A. (1988). Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the E. coli chromosome. Cell *52*, 743-755.

Braun, R. E., O'Day, K., and Wright, A. (1985). Autoregulation of the DNA replication gene dnaA in E. coli K-12. Cell *40*, 159-169.

Breier, A. M., and Grossman, A. D. (2009). Dynamic association of the replication initiator and transcription factor DnaA with the Bacillus subtilis chromosome during replication stress. J Bacteriol *191*, 486-493.

Britton, R. A., Eichenberger, P., Gonzalez-Pastor, J. E., Fawcett, P., Monson, R., Losick, R., and Grossman, A. D. (2002). Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. J Bacteriol *184*, 4881-4890.

Brown, N. C. (1970). 6-(p-hydroxyphenylazo)-uracil: a selective inhibitor of host DNA replication in phage-infected *Bacillus subtilis*. Proc Natl Acad Sci U S A *67*, 1454-1461.

Bruand, C., Velten, M., McGovern, S., Marsin, S., Serena, C., Ehrlich, S. D., and Polard, P. (2005). Functional interplay between the *Bacillus subtilis* DnaD and DnaB proteins essential for initiation and re-initiation of DNA replication. Mol Microbiol *55*, 1138-1150.

Buckstein, M. H., He, J., and Rubin, H. (2008). Characterization of nucleotide pools as a function of physiological state in Escherichia coli. J Bacteriol *190*, 718-726.

Burkholder, W. F., Kurtser, I., and Grossman, A. D. (2001). Replication initiation proteins regulate a developmental checkpoint in Bacillus subtilis. Cell *104*, 269-279.

Camara, J. E., Breier, A. M., Brendler, T., Austin, S., Cozzarelli, N. R., and Crooke, E. (2005). Hda inactivation of DnaA is the predominant mechanism preventing hyperinitiation of Escherichia coli DNA replication. EMBO Rep *6*, 736-741.

Caspi, R., Altman, T., Dreher, K., Fulcher, C. A., Subhraveti, P., Keseler, I. M., Kothari, A., Krummenacker, M., Latendresse, M., Mueller, L. A., *et al.* The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res *40*, D742-753.

Chai, Y., Chu, F., Kolter, R., and Losick, R. (2008). Bistability and biofilm formation in Bacillus subtilis. Mol Microbiol *67*, 254-263.

Chai, Y., Norman, T., Kolter, R., and Losick, R. (2010). An epigenetic switch governing daughter cell separation in Bacillus subtilis. Genes Dev *24*, 754-765.

Charpentier, X., Kay, E., Schneider, D., and Shuman, H. A. Antibiotics and UV radiation induce competence for natural transformation in Legionella pneumophila. J Bacteriol *193*, 1114-1121.

Cho, E., Ogasawara, N., and Ishikawa, S. (2008). The functional analysis of YabA, which interacts with DnaA and regulates initiation of chromosome replication in *Bacillus subtils*. Genes Genet Syst *83*, 111-125.

Chu, F., Kearns, D. B., Branda, S. S., Kolter, R., and Losick, R. (2006). Targets of the master regulator of biofilm formation in Bacillus subtilis. Mol Microbiol *59*, 1216-1228.

Chu, F., Kearns, D. B., McLoon, A., Chai, Y., Kolter, R., and Losick, R. (2008). A novel regulatory protein governing biofilm formation in Bacillus subtilis. Mol Microbiol *68*, 1117-1127.

Collier, J., Murray, S. R., and Shapiro, L. (2006). DnaA couples DNA replication and the expression of two cell cycle master regulators. Embo J *25*, 346-356.

Cunningham, K. A., and Burkholder, W. F. (2009). The histidine kinase inhibitor Sda binds near the site of autophosphorylation and may sterically hinder autophosphorylation and phosphotransfer to Spo0F. Mol Microbiol *71*, 659-677.

Duderstadt, K. E., and Berger, J. M. (2008). AAA+ ATPases in the initiation of DNA replication. Crit Rev Biochem Mol Biol *43*, 163-187.

Furbass, R., Gocht, M., Zuber, P., and Marahiel, M. A. (1991). Interaction of AbrB, a transcriptional regulator from Bacillus subtilis with the promoters of the transition state-activated genes tycA and spoVG. Mol Gen Genet *225*, 347-354.

Gaballa, A., and Helmann, J. D. (2007). Substrate induction of siderophore transport in Bacillus subtilis mediated by a novel one-component regulator. Mol Microbiol *66*, 164-173.

Gon, S., Camara, J. E., Klungsoyr, H. K., Crooke, E., Skarstad, K., and Beckwith, J. (2006). A novel regulatory mechanism couples deoxyribonucleotide synthesis and DNA replication in Escherichia coli. Embo J *25*, 1137-1147.

Goranov, A. I., Katz, L., Breier, A. M., Burge, C. B., and Grossman, A. D. (2005). A transcriptional response to replication status mediated by the conserved bacterial replication protein DnaA. Proc Natl Acad Sci U S A *102*, 12932-12937.

Goranov, A. I., Kuester-Schoeck, E., Wang, J. D., and Grossman, A. D. (2006). Characterization of the global transcriptional responses to different types of DNA damage and disruption of replication in Bacillus subtilis. J Bacteriol *188*, 5595-5605.

Hahn, J., Roggiani, M., and Dubnau, D. (1995). The major role of Spo0A in genetic competence is to downregulate abrB, an essential competence gene. J Bacteriol *177*, 3601-3605.

Hassan, A. K., Moriya, S., Ogura, M., Tanaka, T., Kawamura, F., and Ogasawara, N. (1997). Suppression of initiation defects of chromosome replication in *Bacillus subtilis dnaA* and *oriC*-deleted mutants by integration of a plasmid replicon into the chromosomes. J Bacteriol *179*, 2494-2502.

Herrick, J., and Sclavi, B. (2007). Ribonucleotide reductase and the regulation of DNA replication: an old story and an ancient heritage. Mol Microbiol *63*, 22-34.

Hilbert, D. W., and Piggot, P. J. (2004). Compartmentalization of gene expression during Bacillus subtilis spore formation. Microbiol Mol Biol Rev *68*, 234-262.

Hoch, J. A. (1993). The phosphorelay signal transduction pathway in the initiation of Bacillus subtilis sporulation. J Cell Biochem *51*, 55-61.

Hottes, A. K., Shapiro, L., and McAdams, H. H. (2005). DnaA coordinates replication initiation and cell cycle transcription in Caulobacter crescentus. Mol Microbiol *58*, 1340-1353.

Hulett, F. M. (2002). The Pho Regulon, In Bacillus subtilis and its closest relatives: From genes to cells, A. L. Sonenshein, J. A. Hoch, and R. Losick, eds. (Washington, D.C.: ASM Press), pp. 193-201.

Ishikawa, S., Ogura, Y., Yoshimura, M., Okumura, H., Cho, E., Kawai, Y., Kurokawa, K., Oshima, T., and Ogasawara, N. (2007). Distribution of stable DnaA-binding sites on the Bacillus subtilis genome detected using a modified ChIP-chip method. DNA Res *14*, 155-168.

Iyer, L. M., Leipe, D. D., Koonin, E. V., and Aravind, L. (2004). Evolutionary history and higher order classification of AAA+ ATPases. J Struct Biol *146*, 11-31.

Jaacks, K. J., Healy, J., Losick, R., and Grossman, A. D. (1989). Identification and characterization of genes controlled by the sporulation-regulatory gene *spo0H* in *Bacillus subtilis*. J Bacteriol *171*, 4121-4129.

Kaguni, J. M. (2006). DnaA: controlling the initiation of bacterial DNA replication and more. Annu Rev Microbiol *60*, 351-375.

Katayama, T., Ozaki, S., Keyamura, K., and Fujimitsu, K. (2010). Regulation of the replication cycle: conserved and diverse regulatory systems for DnaA and *oriC*. Nat Rev Microbiol *8*, 163-170.

Kaushal, B., Paul, S., and Hulett, F. M. (2010). Direct regulation of Bacillus subtilis phoPR transcription by transition state regulator ScoC. J Bacteriol *192*, 3103-3113.

Kearns, D. B., Chu, F., Branda, S. S., Kolter, R., and Losick, R. (2005). A master regulator for biofilm formation by Bacillus subtilis. Mol Microbiol *55*, 739-749.

Krause, M., and Messer, W. (1999). DnaA proteins of *Escherichia coli* and *Bacillus subtilis*: coordinate actions with single-stranded DNA-binding protein and interspecies inhibition during open complex formation at the replication origins. Gene *228*, 123-132.

Krause, M., Ruckert, B., Lurz, R., and Messer, W. (1997). Complexes at the replication origin of *Bacillus subtilis* with homologous and heterologous DnaA protein. J Mol Biol *274*, 365-380.

Lauhon, C. T. (2012). Mechanism of N6-threonylcarbamoyladenonsine (t(6)A) biosynthesis: isolation and characterization of the intermediate threonylcarbamoyl-AMP. Biochemistry *51*, 8950-8963.

Lobner-Olesen, A., Skovgaard, O., and Marinus, M. G. (2005). Dam methylation: coordinating cellular processes. Curr Opin Microbiol *8*, 154-160.

Magnuson, R., Solomon, J., and Grossman, A. D. (1994). Biochemical and genetic characterization of a competence pheromone from B. subtilis. Cell *77*, 207-216.

Merrikh, H., and Grossman, A. D. (2011). Control of the replication initiator DnaA by an anti-cooperativity factor. Mol Microbiol *82*, 434-446.

Messer, W., Blaesing, F., Majka, J., Nardmann, J., Schaper, S., Schmidt, A., Seitz, H., Speck, C., Tungler, D., Wegrzyn, G., *et al.* (1999). Functional domains of DnaA proteins. Biochimie *81*, 819-825.

Miller, J. H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory).

Molle, V., Fujita, M., Jensen, S. T., Eichenberger, P., Gonzalez-Pastor, J. E., Liu, J. S., and Losick, R. (2003). The Spo0A regulon of Bacillus subtilis. Mol Microbiol *50*, 1683-1701.

Morigen, Lobner-Olesen, A., and Skarstad, K. (2003). Titration of the *Escherichia coli* DnaA protein to excess *datA* sites causes destabilization of replication forks, delayed replication initiation and delayed cell division. Mol Microbiol *50*, 349-362.

Moriya, S., Hassan, A. K., Kadoya, R., and Ogasawara, N. (1997). Mechanism of anucleate cell production in the *oriC*-deleted mutants of *Bacillus subtilis*. DNA Res *4*, 115-126.

Moriya, S., Ogasawara, N., and Yoshikawa, H. (1985). Structure and function of the region of the replication origin of the Bacillus subtilis chromosome. III. Nucleotide sequence of some 10,000 base pairs in the origin region. Nucleic Acids Res *13*, 2251-2265.

Mott, M. L., and Berger, J. M. (2007). DNA replication initiation: mechanisms and regulation in bacteria. Nat Rev Microbiol *5*, 343-354.

Nievera, C., Torgue, J. J., Grimwade, J. E., and Leonard, A. C. (2006). SeqA blocking of DnaA-*oriC* interactions ensures staged assembly of the *E. coli* pre-RC. Mol Cell *24*, 581-592.

Noirot-Gros, M. F., Velten, M., Yoshimura, M., McGovern, S., Morimoto, T., Ehrlich, S. D., Ogasawara, N., Polard, P., and Noirot, P. (2006). Functional dissection of YabA, a negative regulator of DNA replication initiation in *Bacillus subtilis*. Proc Natl Acad Sci U S A *103*, 2368-2373.

Ogawa, T., Yamada, Y., Kuroda, T., Kishi, T., and Moriya, S. (2002). The datA locus predominantly contributes to the initiator titration mechanism in the control of replication initiation in Escherichia coli. Mol Microbiol *44*, 1367-1375.

Ogura, Y., Imai, Y., Ogasawara, N., and Moriya, S. (2001). Autoregulation of the *dnaA-dnaN* operon and effects of DnaA protein levels on replication initiation in *Bacillus subtilis*. J Bacteriol *183*, 3833-3841.

Okumura, H., Yoshimura, M., Ueki, M., Oshima, T., Ogasawara, N., and Ishikawa, S. Regulation of chromosomal replication initiation by oriC-proximal DnaA-box clusters in Bacillus subtilis. Nucleic Acids Res *40*, 220-234.

Okumura, H., Yoshimura, M., Ueki, M., Oshima, T., Ogasawara, N., and Ishikawa, S. (2012). Regulation of chromosomal replication initiation by oriC-proximal DnaA-box clusters in Bacillus subtilis. Nucleic Acids Res *40*, 220-234.

Olliver, A., Saggioro, C., Herrick, J., and Sclavi, B. DnaA-ATP acts as a molecular switch to control levels of ribonucleotide reductase expression in Escherichia coli. Mol Microbiol *76*, 1555-1571.

Perego, M., Spiegelman, G. B., and Hoch, J. A. (1988). Structure of the gene for the transition state regulator, AbrB: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. Mol Microbiol *2*, 689-699.

Prasanth, S. G., Prasanth, K. V., and Stillman, B. (2002). Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. Science *297*, 1026-1031.

Rahn-Lee, L., Gorbatyuk, B., Skovgaard, O., and Losick, R. (2009). The conserved sporulation protein YneE inhibits DNA replication in *Bacillus subtilis*. J Bacteriol *191*, 3736-3739.

Rahn-Lee, L., Merrikh, H., Grossman, A. D., and Losick, R. (2011). The sporulation protein SirA inhibits the binding of DnaA to the origin of eeplication by contacting a patch of clustered amino acids. J Bacteriol *193*, 1302-1307.

Riber, L., Olsson, J. A., Jensen, R. B., Skovgaard, O., Dasgupta, S., Marinus, M. G., and Lobner-Olesen, A. (2006). Hda-mediated inactivation of the DnaA protein and *dnaA* gene autoregulation act in concert to ensure homeostatic maintenance of the *Escherichia coli* chromosome. Genes Dev *20*, 2121-2134.

Rowland, S. L., Burkholder, W. F., Cunningham, K. A., Maciejewski, M. W., Grossman, A. D., and King, G. F. (2004). Structure and mechanism of action of Sda, an inhibitor of the histidine kinases that regulate initiation of sporulation in *Bacillus subtilis*. Mol Cell *13*, 689-701.

Ruvolo, M. V., Mach, K. E., and Burkholder, W. F. (2006). Proteolysis of the replication checkpoint protein Sda is necessary for the efficient initiation of sporulation after transient replication stress in Bacillus subtilis. Mol Microbiol *60*, 1490-1508.

Salzberg, L. I., and Helmann, J. D. (2008). Phenotypic and transcriptomic characterization of Bacillus subtilis mutants with grossly altered membrane composition. J Bacteriol *190*, 7797-7807.

Schaper, S., and Messer, W. (1995). Interaction of the initiator protein DnaA of Escherichia coli with its DNA target. J Biol Chem *270*, 17622-17626.

Scholefield, G., Errington, J., and Murray, H. (2012). Soj/ParA stalls DNA replication by inhibiting helix formation of the initiator protein DnaA. Embo J.

Scholefield, G., Veening, J. W., and Murray, H. (2011). DnaA and ORC: more than DNA replication initiators. Trends Cell Biol *21*, 188-194.

Seitz, H., Weigel, C., and Messer, W. (2000). The interaction domains of the DnaA and DnaB replication proteins of Escherichia coli. Mol Microbiol *37*, 1270-1279.

Sekimizu, K., Bramhill, D., and Kornberg, A. (1987). ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. Cell *50*, 259-265.

Serizawa, M., Kodama, K., Yamamoto, H., Kobayashi, K., Ogasawara, N., and Sekiguchi, J. (2005). Functional analysis of the YvrGHb two-component system of Bacillus subtilis: identification of the regulated genes by DNA microarray and northern blot analyses. Biosci Biotechnol Biochem *69*, 2155-2169.

Sloma, A., Rufo, G. A., Jr., Theriault, K. A., Dwyer, M., Wilson, S. W., and Pero, J. (1991). Cloning and characterization of the gene for an additional extracellular serine protease of Bacillus subtilis. J Bacteriol *173*, 6889-6895.

Smits, W. K., Goranov, A. I., and Grossman, A. D. (2010). Ordered association of helicase loader proteins with the *Bacillus subtilis* origin of replication in vivo. Mol Microbiol *75*, 452-461.

Smits, W. K., and Grossman, A. D. (2010). The transcriptional regulator Rok binds A+T-rich DNA and is involved in repression of a mobile genetic element in Bacillus subtilis. PLoS Genet *6*, e1001207.

Smits, W. K., Merrikh, H., Bonilla, C. Y., and Grossman, A. D. (2011). Primosomal proteins DnaD and DnaB are recruited to chromosomal regions bound by DnaA in *Bacillus subtilis*. J Bacteriol *193*, 640-648.

Smyth, G. K. (2005). Limma: linear models for microarray data, In Bioinformatics and Computational Biology Solutions using R and Bioconductor, V. C. R. Gentleman, S. Dudoit, R. Irizarry, and W. Huber, ed. (New York: Springer), pp. 397-420.

Speck, C., Weigel, C., and Messer, W. (1999). ATP- and ADP-dnaA protein, a molecular switch in gene regulation. EMBO J *18*, 6169-6176.

Spizizen, J. (1958). Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc Natl Acad Sci USA *44*, 407-408.

Strauch, M., Webb, V., Spiegelman, G., and Hoch, J. A. (1990). The SpoOA protein of Bacillus subtilis is a repressor of the abrB gene. Proc Natl Acad Sci U S A *87*, 1801-1805.

Su'etsugu, M., Takata, M., Kubota, T., Matsuda, Y., and Katayama, T. (2004). Molecular mechanism of DNA replication-coupled inactivation of the initiator protein in *Escherichia coli*: interaction of DnaA with the sliding clamp-loaded DNA and the sliding clamp-Hda complex. Genes Cells *9*, 509-522.

Sun, G., Birkey, S. M., and Hulett, F. M. (1996). Three two-component signal-transduction systems interact for Pho regulation in Bacillus subtilis. Mol Microbiol *19*, 941-948.

Sutton, M. D., and Kaguni, J. M. (1997). The Escherichia coli dnaA gene: four functional domains. J Mol Biol *274*, 546-561.

van Sinderen, D., Luttinger, A., Kong, L., Dubnau, D., Venema, G., and Hamoen, L. (1995). comK encodes the competence transcription factor, the key regulatory protein for competence development in Bacillus subtilis. Mol Microbiol *15*, 455-462.

Veening, J. W., Murray, H., and Errington, J. (2009). A mechanism for cell cycle regulation of sporulation initiation in *Bacillus subtilis*. Genes Dev *23*, 1959-1970.

Waldminghaus, T., and Skarstad, K. (2009). The *Escherichia coli* SeqA protein. Plasmid *61*, 141-150.

Whitten, A. E., Jacques, D. A., Hammouda, B., Hanley, T., King, G. F., Guss, J. M., Trewhella, J., and Langley, D. B. (2007). The structure of the KinA-Sda complex suggests an allosteric mechanism of histidine kinase inhibition. J Mol Biol *368*, 407-420.