Regulation of DNA replication and the replication initiator, DnaA, in *Bacillus subtilis*

by

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B.S., Microbiology
University of Wisconsin-Madison, 2012

Submitted to the Microbiology Graduate Program
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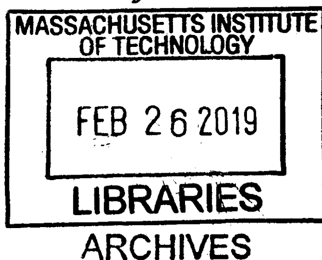
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ABSTRACT

DNA replication is a highly regulated process across all organisms. Improper regulation of DNA replication can be detrimental. I identified an overinitiating, conditional synthetic lethal mutant of *Bacillus subtilis*. I isolated suppressors of this mutant and uncovered novel genes associated with DNA replication. These suppressors acted both at the steps of initiation and elongation to overcome the detrimental replication initiation of the synthetic lethal Δ*yabA dnaA1* mutant. One class of suppressors decreased levels of the replicative helicase, DnaC. I showed that decreased levels of helicase are sufficient to limit replication initiation under fast growth conditions.

I also explored the regulation of DnaA as a transcription factor. The replication initiation inhibitor, YabA, binds to DnaA and prevents its cooperative binding at the origin. In addition to its role in replication initiation, DnaA also directly regulates expression of several genes. YabA has been shown to inhibit DnaA binding at several promoters but its effect on DnaA-mediated gene expression is unclear. I found that YabA inhibits *sda* activation by DnaA but does not significantly affect repression of *ywlC* by DnaA. Lastly, I showed that YabA appears to stimulate autoregulation of *dnaA*. This preliminary data illustrates a role for YabA regulation in DnaA-mediated gene expression.

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Chapter 1

Introduction

DNA replication

DNA replication is an essential process for all domains of life. As cells grow and divide, their genetic material must be duplicated to ensure that each daughter cell receives a complete copy of the genetic information. This requires that cells coordinate their DNA replication with growth rate and environmental cues. During favorable, fast growth conditions, cells must replicate their DNA more quickly. DNA replication is a highly regulated process in all organisms. Under slower growth or nutrient poor conditions, for example, cells must regulate DNA replication to conserve resources and prevent overinitiation. Multiple mechanisms are used to coordinate DNA replication with other cellular processes such as metabolism and cell division, as well as to sense external cues that can impact DNA replication. Failure to properly regulate DNA replication can result in a variety of consequences, such as cell division defects, anucleate daughter cells, DNA damage, and in higher organisms, disease (Fig. 1; O'Donnell, Langston et al. 2013, Magdalou, Lopez et al. 2014).

Figure 1. Effects from improper regulation of the cell cycle.

When cells divide without having completed replication of the chromosome it can result in anucelate cells, where a daughter cell does not inherit the genetic material. If cells are unable to properly segregate their chromosomes before cell division, division septa can form over the chromosomes, resulting in guillotined chromosomes.

Initiation

In all organisms, DNA replication consists of three stages: initiation, elongation, and termination (Fig. 2). DNA replication is primarily controlled at the step of initiation. Some organisms, such as the bacteria *Escherichia coli* and *B. subtilis*, can initiate new rounds of replication before the previous round has finished, resulting in multifork replication (Fig. 3). The increased replication initiation allows faster duplication of the genome even though the rate of elongation remains the same. Multifork replication results in each daughter cell inheriting a chromosome with multiple active replication forks. This allows cells to divide more quickly under favorable conditions while maintaining proper duplication of the genetic material for each daughter cell. When conditions are unfavorable, these bacteria reduce the rate of initiation to match growth rate, ensuring resources are conserved and preventing overinitiation. In other organisms, such as eukaryotes and the Gram-negative aquatic bacterium *Caulobacter crescentus*, the chromosome can only be replicated once per cell cycle. The variations in cell cycle structure between organisms result in different mechanisms for replication control between organisms, although both prokaryotes and eukaryotes focus regulation on the step of initiation.

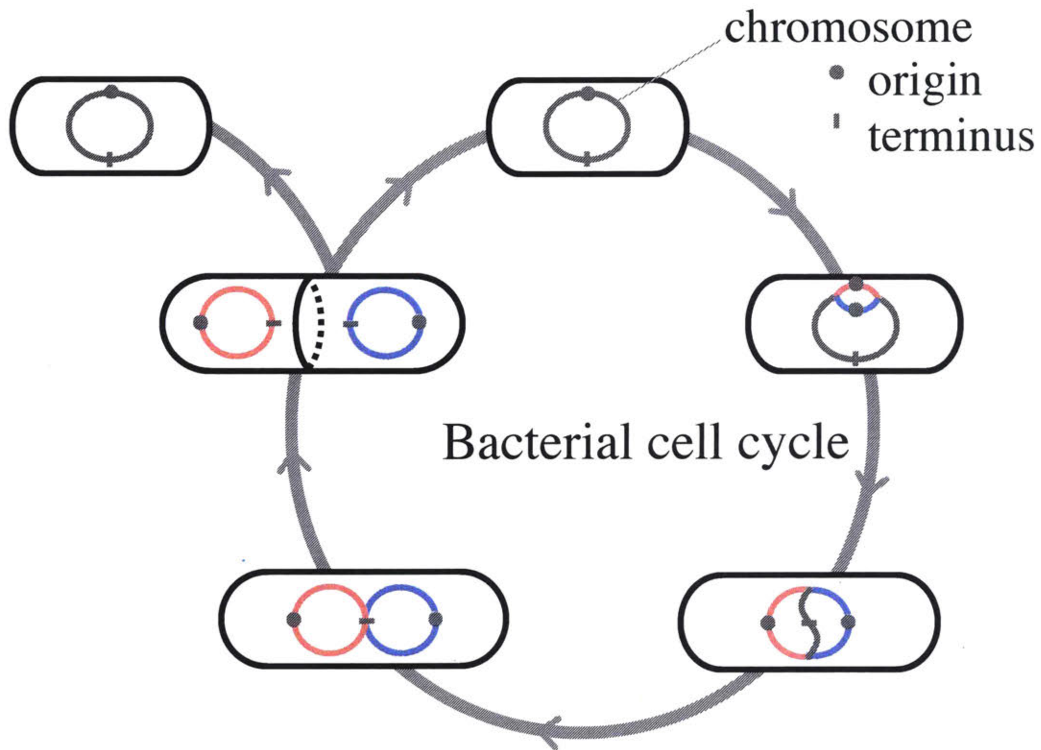


Figure 2. Bacterial cell cycle.

DNA replication initiates at the origin and proceeds bi-directionally to the terminus, situated roughly opposite the origin. The chromosomes are segregated and cells divide, each with a completed copy of the chromosome.

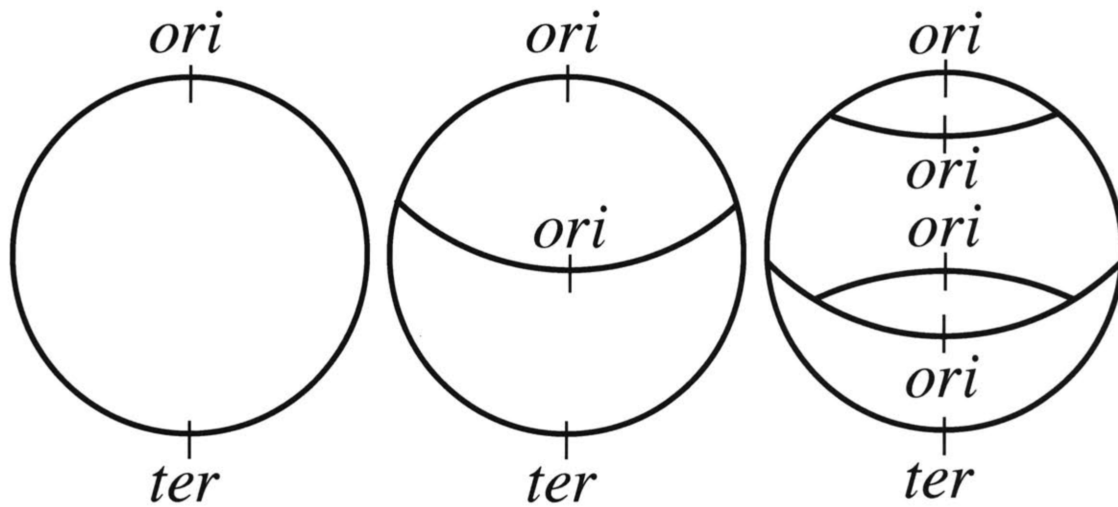


Figure 3. Multifork DNA replication.

Certain bacteria can undergo multifork DNA replication. New rounds of DNA replication can be initiated before the previous round has finished. This results in daughter cells receiving a chromosome with active replication forks.

In both prokaryotes and eukaryotes, a *trans*-acting replication initiator protein binds to *cis*-acting sequences of DNA, that constitutes an origin of replication, to initiate DNA replication. The bacterial genome typically consists of a single circular chromosome with a single origin of replication, although certain species can have multiple or linear chromosomes (reviewed in Hinnebusch and Tilly 1993; Egan, Fogel et al. 2005). The initiator in bacteria is DnaA, a AAA+ ATPase (ATPase associated with various cellular activities), which binds to clusters of DnaA-boxes that constitute the single origin of replication (Fig. 4; Davey, Jeruzalmi et al. 2002, Kaguni 2006). Eukaryotes have multiple linear chromosomes, each with multiple origins of replication. The initiator in eukaryotes is the origin recognition complex (ORC) and is composed of six different subunits, ORC1-6, several of which are also AAA+ ATPases (Davey, Jeruzalmi et al. 2002, Watson, Baker et al. 2014). ORC binds to autonomously replicating sequences (ARS) located at the origins on each chromosome (Watson, Baker et al. 2014).

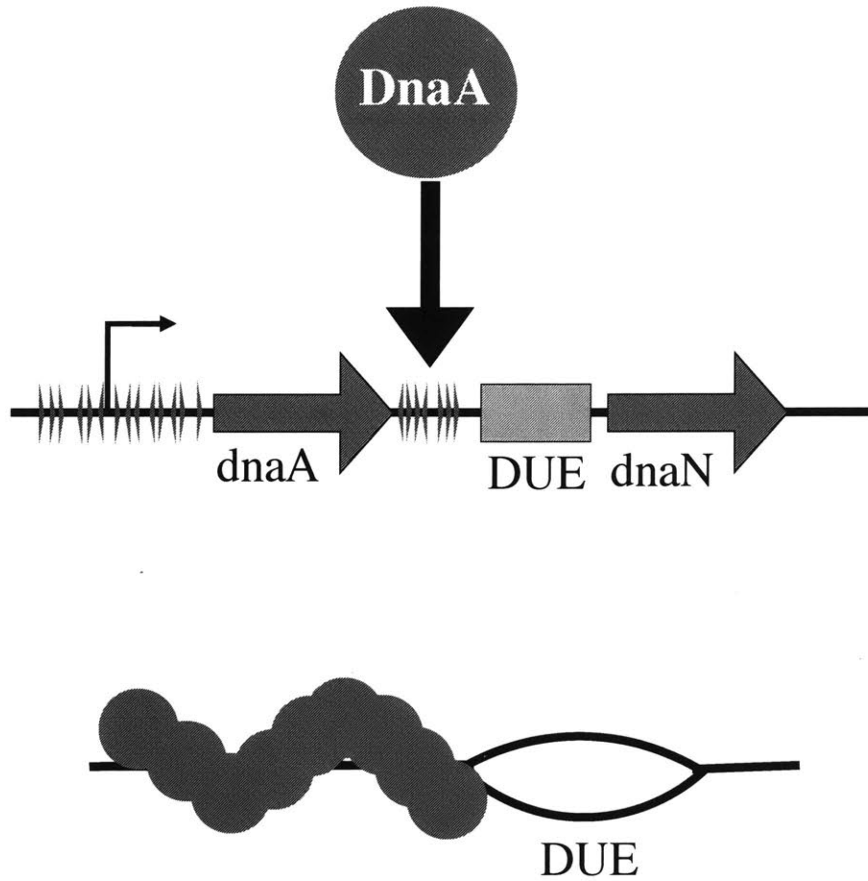


Figure 4. DnaA binding initiates DNA replication at the origin.

DnaA-ATP binds cooperatively to DnaA-boxes that origin in *B. subtilis*. DnaA boxes are located upstream of the AT-rich DNA unwinding element (DUE). Cooperative binding causes formation of a nucleoprotein helical filament, which stimulates melting of the adjacent DUE.

In bacteria, cooperative binding of ATP-bound DnaA at the origin results in the formation of a nucleoprotein helical filament that causes unwinding of the adjacent AT-rich DNA unwinding element (DUE; reviewed in Jameson and Wilkinson 2017). This results in single-stranded DNA onto which the replication machinery can be assembled. DnaA directly recruits helicase loader proteins to the origin for helicase loading. In *Escherichia coli*, DnaA recruits the AAA+ ATPase helicase loader (DnaC in *E. coli*), which is sufficient to load the hexameric helicase (DnaB in *E. coli;* reviewed in Kornberg and Baker 1992). The loader breaks open the helicase around the DNA, hydrolyzes ATP, and dissociates (Reviewed in Davey, Jeruzalmi et al. 2002; Davey and O'Donnell 2003). In *B. subtilis* and other low G+C Gram-positive bacteria, additional proteins, DnaD and DnaB, are required for loading the replicative helicase (Bruand, Ehrlich et al. 1995; Bruand, Farache et al. 2001; Marsin, McGovern et al. 2001; Velten, McGovern et al. 2003; Rokop, Auchtung et al. 2004; Li, Kurokawa et al. 2004; Li, Kurokawa et al. 2007), and also help with chromosomal compaction and remodeling at the origin (reviewed in Briggs, Smits et al. 2012). DnaB is also membrane-bound and believed to be responsible for localizing DNA replication to the cell membrane (reviewed in Briggs, Smits et al. 2012). DnaA first recruits DnaD, followed by DnaB, and then the helicase loader, DnaI, and the helicase, DnaC are recruited last (Smits, Goranov et al. 2010). Unlike *E. coli,* which employs a ring-breaker mechanism to load the helicase around the ssDNA, *B. subtilis* uses a ring-maker mechanism, whereby individual DnaI-DnaC monomers are loaded around the DNA to form the hexameric helicase (reviewed in Davey and O'Donnell 2003). Upon loading of the helicase, the primase DnaG forms an RNA primer on the template DNA, leading to the dissociation of the helicase loader and activation of the helicase. The sliding clamp (DnaN) and DNA polymerase are then loaded, completing the replication machinery (reviewed in Jameson and Wilkinson 2017).

Unlike bacteria, binding of ORC to the eukaryote origin is not sufficient to unwind the adjacent DNA. Instead, ORC recruits helicase loading proteins, Cdc6 and Cdt1, and two copies of the helicase complex, Mcm2-7, which are loaded around double-stranded DNA, releasing Cdc6 and Cdt1. Activation of Mcm2-7 requires the kinases CDK and DDK, along with the helicase-activating proteins Cdc45 and GINS, which form the CMG (Cdc45/Mcm2-7/GINS) complex. During this time, the leading-strand polymerase (ε) is recruited before DNA unwinding, after which the lagging strand polymerase (δ) and DNA polymerase α/primase are finally recruited (reviewed in Watson, Baker et al. 2014).

Elongation

DNA elongation proceeds bi-directionally through the chromosome(s), and uses a conserved strategy although the components vary between organisms. Replication is divided between the leading and lagging strands of DNA. For leading strand synthesis in bacteria, DNA polymerase extends from the RNA primer made by primase (DnaG) and extends along the leading strand, from 5' to 3'. Progression is facilitated by the sliding clamp (DnaN), which is positioned behind DNA polymerase. The sliding clamp makes DNA polymerase much more processive, allowing incorporation of up to 1000 bases/second. The lagging strand synthesizes DNA in short fragments from RNA primers synthesized by primase (DnaG), creating Okazaki fragments. The RNA primers are removed and the fragments connected by DNA ligase (Watson et al. 2014).

As mentioned above for eukaryotes, some organisms employ multiple polymerases for DNA replication, specialized for different roles. *B. subtilis* uses two DNA polymerases, PolC and DnaE. DnaE is specific for lagging strand synthesis, while PolC functions in both leading and lagging strand synthesis (Dervyn, Suski et al. 2001; Sanders, Dallmann et al. 2010). It's believed

that DnaE extends the RNA primer and then PolC takes over and to extend the strand; this is similar to eukaryotes, where ε performs the initial extension from the RNA primer before handing off to the lagging strand polymerase, δ (Sanders, Dallmann et al. 2010; Robinson, J Causer et al. 2012). In contrast, *E. coli* uses a single DNA polymerase enzyme, Pol III, which is composed of three subunits: the catalytic subunit (α), a proofreading subunit (ε), and a non-essential subunit (θ), that may stimulate the activity of ε (reviewed in Beattie and Reyes-Lamothe 2015).

As replication forks progress through the chromosome, they are susceptible to inactivation when encountering roadblocks such as strand breaks or unrepaired DNA lesions (Fig. 5; Cox, Goodman et al. 2000). Inactivation can lead to the disassembly of the replisome components and collapse of the replication fork. DNA damage and collapsed forks activate the bacterial SOS response, a transcriptional response that affects genes involved in DNA repair, DNA replication, and cell division to preserve genome integrity and cell viability (Lenhart, Schroeder et al. 2012). When DNA damage occurs, DNA repair or processing of the fork by specialized nucleases generate ssDNA, upon which RecA is loaded (Fig. 6; Lenhart, Schroeder et al. 2012). RecA is a highly conserved protein that forms a nucleoprotein filament with the ssDNA. In this context, RecA stimulates self-cleavage of LexA, the transcriptional repressor responsible for control of the SOS response. LexA cleavage relieves repression of these promoters, inducing the transcriptional SOS response.

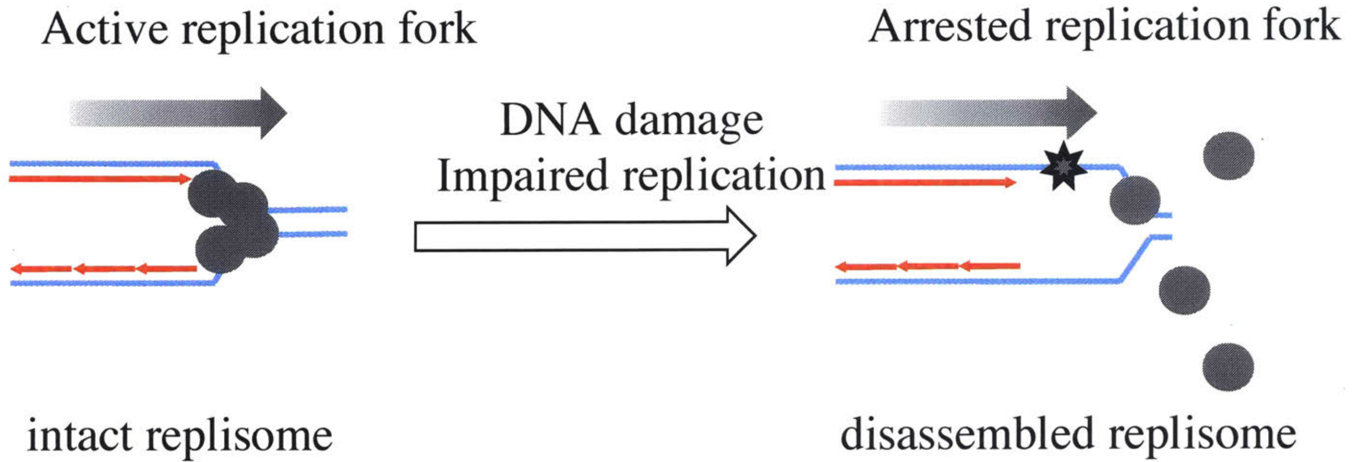


Figure 5. Encountering DNA damage can cause disassembly of the replisome.

Active replication forks will encounter DNA damage during elongation. This can cause collapse of the DNA replication forks, dissembling the replisome.

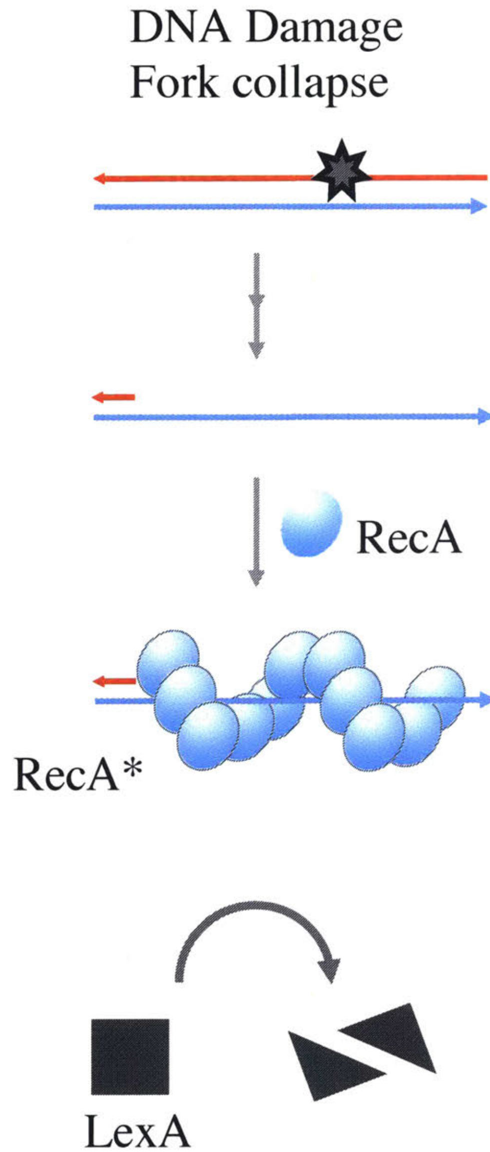


Figure 6. DNA damage response.

When DNA damage occurs, RecA is loaded onto ssDNA. RecA binding stimulates self-cleavage of LexA, the transcriptional repressor responsible for control of the SOS response. LexA cleavage relieves repression of these promoters, inducing the transcriptional SOS response.

Replication forks are also frequently inactivated under normal growth conditions when they encounter DNA damage that does not evoke the SOS response (Cox, Goodman et al. 2000). Particularly, *B. subtilis* has a high tolerance to DNA damage before the SOS response is activated and can efficiently repair damage in the absence of the SOS response in comparison to other bacteria, such as *E. coli* (Simmons, Goranov et al. 2009; Bernard, Marquis et al. 2010).

After DNA damage is repaired, the replisome must be reassembled on the DNA. Primosome assembly at sites of replication restart differs from the assembly at the origin in the mechanism of helicase loading. During replication restart, the protein PriA is required for loading the replicative helicase back onto DNA. PriA is a highly conserved 3'-5' helicase that is a member of the ϕX174-type primosomal protein family, which also includes other replication proteins such as the replicative helicase and primase (Marians 1992). PriA recognizes and binds to the collapsed replication fork, recruiting DnaD and starting the stepwise reassembly process (Marsin, McGovern et al. 2001). DnaD directly interacts with DnaB, which in turn recruits DnaI. These proteins reload the helicase (DnaC), which is again followed by primase (DnaG), at which point replication elongation can continue (reviewed in Lenhart, Schroeder et al. 2012).

As the DNA is unwound at the replisome, positive DNA supercoils are introduced in front of the replication fork. If left unbroken, the DNA strands will be unable to unwind due to the torsion created by the double helix structure of the chromosome and supercoiling. Topoisomerases are enzymes that help unwinding of the DNA by cutting either one or both strands, passing them through the break to create negative supercoils, thereby releasing the tension caused by DNA unwinding (Watson et al. 2014). In bacteria, this is accomplished by DNA gyrase, which is a type II topoisomerase that cuts both strands of the DNA, generating

negative supercoiling to release the tension caused by unwinding of the DNA (Gellert, Mizuuchi et al. 1976).

Termination

In bacteria, replication elongation proceeds to the site of termination, which is situated roughly opposite (~180°) of the site of initiation (0°) on the circular chromosome. Replication elongation is blocked by *ter* (termination) sites that allow fork progression from only a single direction. This ensures complete replication of the chromosome after the forks reach a specific chromosomal region (terminus). In *B. subtilis*, a replication termination protein Rtp (Tus in *E. coli*) binds to ter sites, blocking progression of helicase and terminating DNA replication (reviewed in Jameson and Wilkinson 2017). In organisms that initiate from multiple origins, replication typically terminates in a non-sequence specific manner. Replication forks progress until they meet another replication fork, where termination occurs.

DnaA: Structure and function

DnaA is the highly conserved replication initiator and is present and essential in almost all bacteria. The only known exceptions are in obligate insect endosymbionts, such as *Wigglesworthia glossinidia, Blochmannia floridanus, Blochmannia pennsylvanicus, and Baumannia cicadellinicola*, which have lost the *dnaA* gene (reviewed in Zakrzewska-Czerwińska, Jakimowicz et al. 2007).

Along with other replication initiator proteins, DnaA is a member of the AAA+ ATPase family of proteins. AAA+ ATPases have a conserved domain consisting of 200-250 amino acids termed the 'AAA+ module' which contains the core nucleotide binding domain consisting of the Walker A and Walker B motifs (reviewed in Snider and Houry 2008). DnaA belongs to the

helicase and clamp loaders (HEC) group, which also includes several other proteins involved in DNA replication, such as the clamp loaders, the replicative helicase in eukaryotes (Mcm2-7) and one of the proteins required for its assembly (Cdc6), and the helicase loader in *E. coli* (DnaC) (Davey, Jeruzalmi et al. 2002; Snider and Houry 2008).

DnaA can bind both ATP and ADP, and both forms are able to bind DnaA-boxes at the origin of replication (Sekimizu, Bramhill et al. 1987; Messer 2002; Smith and Grossman 2015). However, only ATP-bound DnaA is capable of cooperative binding and DNA unwinding at the origin (Sekimizu, Bramhill et al. 1987). DnaA binds to clusters of DnaA-boxes at the origin of replication as well as throughout the genome (consensus sequence 5'- TTATNCACA; (Fuller, Funnell et al. 1984; Fukuoka, Moriya et al. 1990; Smith and Grossman 2015). DnaA can tolerate mismatches from the consensus sequence but with lower affinity (Fujikawa, Kurumizaka et al. 2003; Ishikawa, Ogura et al. 2007; Leonard and Grimwade 2011). Allowing one mismatch in the 9-bp consensus sequence results in a possible 11,983 occurrences in the genome (for *B. subtilis;* Breier and Grossman 2009), indicating other factors affecting specificity are likely relevant.

DnaA is composed of 4 domains (Fig. 7). The N-terminal domain I is used in oligomerization of DnaA molecules (Weigel, Schmidt et al. 1999; Simmons, Felczak et al. 2003), and has been shown to interact with helicase in *E. coli* (Sutton, Carr et al. 1998; Seitz, Weigel et al. 2000). Domain II is composed of a poorly conserved flexible linker. Domain III contains the Walker A and Walker B motifs required for ATP binding and hydrolysis (Messer 2002; Kawakami and Katayama 2010). It is also used for ssDNA binding, and contributing to oligomerization of DnaA (Messer, Blaesing et al. 1999; Jameson and Wilkinson 2017). Finally,

the C-terminal domain IV is necessary and sufficient for dsDNA binding (Roth and Messer 1995).

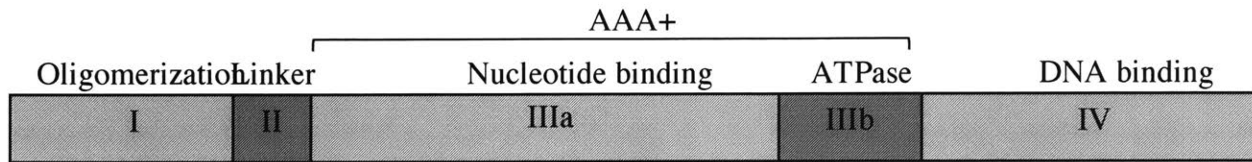


Figure 7. DnaA domains.

Domain I of DnaA is involved in oligomerization. Domain II is a poorly conserved flexible linker. Domain III is composed of the AAA+ ATPase that binds and hydrolyzes ATP, and is also involved in oligomerization. The C-terminal domain IV is required for DNA binding.

Regulation of replication initiation

It is essential for cells to regulate DNA replication initiation with the cell cycle to ensure successful propagation. Failure to properly regulate replication initiation is often detrimental to cell growth and is therefore tightly controlled. However, the mechanisms of regulation vary between different organisms. In eukaryotes, this is accomplished by having distinct phases for loading and activation of the Mcm2-7 helicase. Bacteria control replication initiation by regulating the activity and binding of DnaA in different ways.

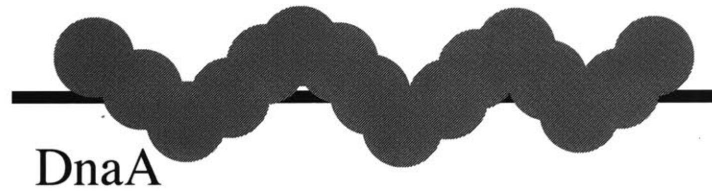
Regulation in *B. subtilis*

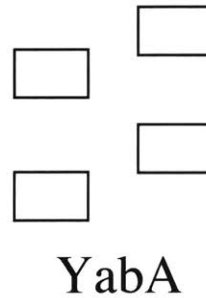
The primary mechanism for regulating DNA replication initiation in *B. subtilis* involves limiting cooperative binding of DnaA the origin of replication (*oriC*). Four known proteins regulate DnaA using this mechanism: YabA, DnaD, Soj, and SirA.

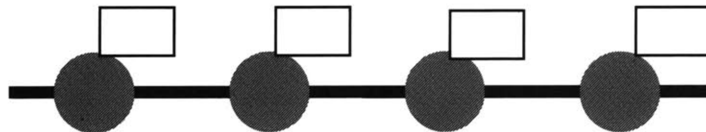
YabA. YabA is a small (119 amino acids) protein that has two predicted domains: an N-terminal leucine zipper motif proposed to facilitate protein-protein interactions, and a potential C-terminal zinc cluster motif (Noirot-Gros, Velten et al. 2006). YabA is not homologous to other known regulators of DnaA but is conserved among other low G+C Gram-positive bacteria (Noirot-Gros, Dervyn et al. 2002; Hayashi, Ogura et al. 2005). YabA is a negative regulator of DnaA and a null mutation in *yabA* results in increased and asynchronous initiation, whereas overexpression of *yabA* decreases replication initiation (Noirot-Gros, Dervyn et al. 2002; Hayashi, Ogura et al. 2005; Goranov, Breier et al. 2009). *In vitro*, YabA inhibits cooperative binding of DnaA at the origin and prevents helix formation required for origin melting, independent of the ATPase activity of DnaA (Merrikh and Grossman 2011; Scholefield and Murray 2013). *In vivo,* YabA also limits binding of DnaA at the origin as well as other DnaA

binding sites throughout the cell (Fig. 8; Merrikh and Grossman 2011). In addition to interacting with DnaA, YabA was found to interact with the sliding clamp DnaN. YabA can interact with both DnaA and DnaN simultaneously, forming a ternary DnaA-YabA-DnaN complex (Noirot-Gros, Dervyn et al. 2002; Noirot-Gros, Velten et al. 2006). Fluorescently-tagged YabA has been shown to localize with the replisome during DNA replication through its interaction with DnaN, recruiting DnaA to the replisome through its interactions with YabA (Hayashi, Ogura et al. 2005; Noirot-Gros, Velten et al. 2006; Goranov, Breier et al. 2009). These interactions have been proposed to antagonize YabA inhibition on DnaA, through DnaN titrating YabA away from DnaA. In fact, overexpression of DnaN decreases DnaA association with *oriC* and causes overinitiation dependent on YabA (Merrikh and Grossman 2011).

DnaA binding cooperative







DnaA binding non-cooperative

Figure 8. YabA inhibits cooperative binding of DnaA.

DnaA binds cooperatively to DNA, forming a nucleoprotein helical filament. Inhibitors of DnaA, such as YabA, bind directly to DnaA, inhibiting this cooperative binding to regulate DNA replication initiation (Merrikh and Grossman, 2011).

DnaD. DnaD is recruited by DnaA to the origin and, along with DnaB, is required to recruit the helicase loader DnaI (Smits, Goranov et al. 2010). In addition to its role in initiating replication initiation, DnaD also negatively regulates DnaA binding at the origin. *In vitro,* DnaD inhibits cooperative binding of DnaA at the origin (Bonilla and Grossman 2012) and inhibits DnaA-DNA helix formation (Scholefield and Murray 2013). Like YabA, DnaD does not affect the ATPase activity of DnaA (Bonilla and Grossman 2012). The effects of DnaD on replication initiation have not been studied *in vivo*, as it is an essential protein. In addition to its association with DnaA at *oriC*, DnaD has been shown to associate with DnaA at other binding sites throughout the chromosome (see 'DnaA as a transcription factor').

Soj. Soj is a negative regulator of DnaA that inhibits helix formation at the origin (Scholefield, Errington et al. 2012). Soj is a homolog of the chromosome partitioning protein (ParA) and was isolated as a suppressor of Spo0J (ParB) (Ireton, Gunther et al. 1994). The conserved Par system functions by partitioning chromosomes and low-copy plasmids in bacteria and is composed of two *trans*-acting factors, ParA, which binds ParB, that bind to *cis*-acting *parS* sites in the DNA (Gerdes, Møller-Jensen et al. 2000; Livny, Yamaichi et al. 2007). Spo0J (ParB) binds to *parS* sites in the chromosome that are located near *oriC,* occupying the sites closest to the chromosome most frequently (Lin and Grossman 1998; Breier and Grossman 2007). *parS* sites facilitate spreading of Spo0J to nearby regions of DNA, creating large nucleoprotein complexes around the *parS* sites (Murray, Ferreira et al. 2006; Breier and Grossman 2009). Soj (ParA) is a Walker-type ATPase that binds cooperatively to non-specific DNA when in an ATP-bound dimer form and helps segregate chromosomes through its interaction with Spo0J (Leonard, Butler et al. 2005; Lee and Grossman 2006). Spo0J stimulates

the ATP hydrolysis activity of Soj, causing Soj to dissociate from DNA (Leonard, Butler et al. 2005; Scholefield, Whiting et al. 2011). When unbound to ATP, Soj exists in a monomeric state, which is capable of binding DnaA and inhibiting helix formation at the origin (Scholefield, Whiting et al. 2011; Scholefield, Errington et al. 2012). Dimeric Soj bound to DNA near the origin relieves this inhibition and stimulates replication initiation (Scholefield, Whiting et al. 2011).

SirA. SirA is the sporulation inhibitor of replication and inhibits DNA replication initiation during the transition from vegetative growth to sporulation. Under starvation conditions, cells activate a phosphorelay pathway that results in phosphorylation (activation) of the transcriptional regulator, Spo0A, which is the main determinant for entering sporulation (Burbulys, Trach et al. 1991). Cells entering sporulation undergo asymmetrical division, producing a larger mother cell and a smaller forespore (Wu and Errington 1994). A final round of DNA replication results in one chromosome remaining in the mother cell while the other is transported to the forespore (Setlow, Magill et al. 1991; Wu and Errington 1994). DNA replication must be regulated to ensure the chromosome copy number is at two upon entering sporulation. SirA expression is activated by the master sporulation regulator, Spo0A (Fujita, Gonzalez-Pastor et al. 2005; Rahn-Lee, Gorbatyuk et al. 2009). SirA directly binds to the N-terminus of DnaA and co-localizes with DnaA at the origin (Wagner, Marquis et al. 2009; Jameson, Rostami et al. 2014). SirA negatively inhibits replication initiation in cells entering sporulation by preventing DnaA binding at the origin (Wagner, Marquis et al. 2009; Rahn-Lee, Gorbatyuk et al. 2009). Artificial expression of *sirA* during vegetative growth is conditionally lethal and leads to anucleate cells and guillotined chromosomes (Wagner, Marquis et al. 2009; Rahn-Lee, Gorbatyuk et al. 2009).

Regulation of DNA replication in other bacteria

Regulating cooperative binding of DnaA to the origin has also been demonstrated as a point of replication control in other bacteria. In *E. coli*, DiaA binds to domain I of DnaA and promotes oligomerization at the origin, acting as a positive regulator for initiation, as opposed to negative regulators found in *B. subtilis* (Keyamura, Fujikawa et al. 2007, Natrajan, Hall et al. 2007). This mechanism is also used by *Helicobacter pylori*, where the DiaA ortholog, HobA also functions as a positive regulator of DnaA (Natrajan, Hall et al. 2007, Zawilak-Pawlik, Nowaczyk et al. 2017). Although DiaA and HobA share functional and structural homology, they do not share sequence similarity. Like SirA in *B. subtilis,* DiaA and HobA both interact with domain I of DnaA, although they have different binding sequences and are not interchangeable (Zawilak-Pawlik, Donczew et al. 2011).

Another mechanism used to regulate initiation in bacteria involves sequestration of *oriC* to prevent DnaA oligomer formation. In *E. coli*, SeqA binds to hemimethylated GATC sites at the origin to prevent DnaA binding immediately after replication initiation. The *E. coli* origin contains 11 Dam methylase recognition sites, which are hemimethylated immediately after replication initiation, as the newly synthesized strand has yet to be methylated and the parent strand is methylated (Lu, Campbell et al. 1994, Nievera, Torgue et al. 2006, Jameson and Wilkinson 2017). This mechanism functions to prevent reinitiation immediately after the previous round of initiation has finished. This sequestration mechanism by SeqA is only found in *E. coli* and closely related Gram-negative organisms and is not used in *B. subtilis,* which lacks both Dam and SeqA (Hiraga 2000).

Similar to origin sequestration, some bacteria employ a mechanism of sequestering DnaA to limit its availability. *E. coli* has a locus, *datA*, that contains five high-affinity DnaA boxes that titrate available DnaA away from the origin (Kitagawa, Mitsuki et al. 1996, Kitagawa, Ozaki et al. 1998). Mutating the *datA* locus results in overinitiation, indicating that it functions to limit available DnaA thereby inhibiting replication initiation (Kitagawa, Mitsuki et al. 1996, Kitagawa, Ozaki et al. 1998). DnaA binding to *datA* is stimulated by the DNA-bending protein integration host factor, IHF, which is required for proper *datA* function (Nozaki, Yamada et al. 2009). Additionally, binding to *datA* has been shown to stimulate DnaA-ATP hydrolysis, in an IHF-dependent manner (Kasho and Katayama 2013). Additional DnaA-binding sites are located throughout the *E. coli* chromosome with fewer high-affinity DnaA boxes but do not appear to have the same regulatory role as that of *datA* (Roth and Messer 1998). There are also several DnaA-box cluster (DBCs) regions in *B. subtilis* apart from the origin. These six DBCs have been implicated in regulating DNA replication initiation but appear to have only minor effects (Okumura, Yoshimura et al. 2012). Deleting all DBCs resulted in overinitiation but reintroduction of a single DBC into the chromosome was sufficient to partially suppress this phenotype (Okumura, Yoshimura et al. 2012).

There are two other DnaA-binding loci in *E. coli,* that function to reactivate DnaA by promoting nucleotide exchange to generate the active ATP-bound DnaA. These two DnaA reactivating sites (DARS) are located at 17.5 min (DARS1) and 64 min (DARS2) on the chromosome and mutations in either DARS inhibit DNA replication due to the decrease in DnaA-ATP required for initiation (Fujimitsu, Senriuchi et al. 2009). Deleting either DARS can suppress the overinitaiton of *datA* or *seqA* mutants.

Although regulation of the nucleotide-bound state of DnaA has not been observed in *B. subtilis*, it is a key point of control for regulating DnaA activity in *E. coli*. In addition to *datA* and DARS, another mechanism in *E. coli* is the regulatory inactivation of DnaA (RIDA). This process involves inactivating DnaA-ATP through ATP-hydrolysis, resulting in DnaA-ADP. RIDA requires both Hda and the sliding clamp, DnaN. Hda, termed homologous to *dnaA*, is a AAA+ ATPase and has a 48% sequence similarity to the ATPase domain of *dnaA* (Kato and Katayama 2001). Hda and DnaA interact through their ATPase domains, while Hda binds the N-terminus of DnaN (Kurz, Dalrymple et al. 2004), acting as a bridge for the trimeric DnaA-Hda-DnaN complex. Hda promotes ATP-hydrolysis both *in vitro* and *in vivo* and is dependent on DnaN bound to DNA (Kato and Katayama 2001; Nishida, Fujimitsu et al. 2002; Su'etsugu, Shimuta et al. 2005). A homolog of Hda, HdaA, exists in *C. crescentus* and also acts by stimulating ATP-hydrolysis by DnaA (Collier and Shapiro 2009, Jonas, Chen et al. 2011). The RIDA mechanism is thought to inactivate DnaA after initiation through its interaction with the replication fork through DnaN. Although the mechanism of inhibition is very different, Hda resembles YabA in its ability to bind both DnaA and DnaN, resulting in DnaA to the replisome, linking initiation with elongation.

Regulating levels of DnaA also plays an important part in controlling DNA replication initiation. In addition to the autoregulation of *dnaA* transcription (see below), certain organisms, such as *C. crescentus*, have mechanisms to regulate levels of DnaA through proteolysis. In *C. crescentus,* the DnaA protein is unstable under proteotoxic stress and degraded by the Lon protease (Jonas et al. 2013; Liu, Francis et al. 2016). *Caulobacter* also uses protein degradation to control the levels of the cell-cycle regulator, CtrA, which also functions to negatively regulate DNA replication initiation. CtrA is a transcriptional regulator that controls gene expression

needed for the transition between stalked (active for replication) and swarmer cells (inactive for replication). CtrA is present in swarmer cells but degraded by ClpP for transition into stalked cells (Gorbatyuk and Marczynski 2005; McGrath, Iniesta et al. 2006). There are five binding sites for CtrA in the chromosomal origin (*Cori*), and binding of CtrA blocks DnaA association at the origin, thereby negatively regulating replication initiation in swarmer cells (Quon, Yang et al. 1998).

These examples of regulating DNA replication initiation across different species highlight the varied mechanisms cells have adapted to control initiation. Interestingly, the varied mechanisms of regulating DnaA seem to have adapted to the properties of DnaA in that particular organism. For example, the nucleotide exchange rate for *B. subtilis* DnaA is much higher than that for *E. coli* DnaA (Sekimizu, Bramhill et al. 1987; Bonilla and Grossman 2012). Therefore, regulating the nucleotide exchange rate in *B. subtilis* would have less of an effect on activity than in an organism such as *E. coli,* where exchange is very slow. Indeed, it has been proposed that organisms with a fast rate of nucleotide exchange have mechanisms that regulate DnaA binding, whereas organisms with a slower rate of exchange utilize mechanisms that focus on nucleotide hydrolysis and exchange (Bonilla and Grossman 2012).

DnaA as a transcription factor

In addition to its essential role the replication initiator, DnaA also serves as a transcription factor. In *B. subtilis*, DnaA directly regulates several genes as well as indirectly affecting the expression of many more genes (Goranov, Katz et al. 2005, Breier and Grossman 2009, Washington, Smith et al. 2017). As mentioned above, DnaA binds to chromosomal regions outside of *oriC* (and P*dnaA*) that contain DnaA-box clusters (Okumura, Yoshimura et al. 2012)

and directly regulates expression of several of these genes or operons. DnaA binds to and directly activates expression of *sda* and appears to directly repress the expression of several genes, including *ywlC, ywcI-sacT, yydA,* and *trmE-noc* (Table 1; Burkholder, Kurtser et al. 2001; Goranov, Katz et al. 2005; Ishikawa, Ogura et al. 2007; Breier and Grossman 2009; Hoover, Xu et al. 2010; Smith and Grossman 2015). Additionally, DnaA autoregulates *dnaA-dnaN* expression by binding to DnaA boxes in P*dnaA* (Ogura, Imai et al. 2001).

Table 1: Transcriptional targets of DnaA in *B. subtilis*

DnaA targets	DnaA: activation or repression	Number of putative DnaA-boxes in promoter[1]
sda	Activates	8
dnaA-dnaN	Represses	12
ywlC	Represses	9
yydA	Represses	7
trmE-noc	Represses	4
ywcI-sacT	Represses	11

[1](Smith and Grossman, 2015)

One circumstance when DnaA-regulated gene expression is of particular importance is during replication stress. Replication stress is caused when replication forks are inactivated, due to DNA damage, chemical or UV inactivation, or simply during regular growth condition (Cox, Goodman et al. 2000). In addition to the transcriptional network regulated by LexA during the SOS response (see above), DnaA has an important role in gene regulation during replication stress. Upon replication arrest, many of the replisome components are released from the fork, including the sliding clamp DnaN and YabA. As a consequence, any DnaA molecules localized to the replisome based on its interactions with YabA and DnaN are also released (Hayashi, Ogura et al. 2005, Noirot-Gros, Velten et al. 2006, Goranov, Breier et al. 2009). This results in free DnaA able to bind to transcriptional targets, thereby coupling DnaA-mediated gene expression with replication stress (Goranov, Katz et al. 2005).

In addition to these directly regulated targets, there are many additional transcriptional changes in response to DnaA activity that are believed to be indirect (Washington, Smith et al. 2017). The only gene activated by DnaA, *sda*, indirectly inhibits activation of the master sporulation regulator, Spo0A (Burkholder, Kurtser et al. 2001). Sda is a small, unstable protein that binds to one of the histidine kinases (KinA) in the phosphotransfer pathway required for phosphorylation of Spo0A, to generate the active form Spo0A~P (Burbulys, Trach et al. 1991; Burkholder, Kurtser et al. 2001). Spo0A regulates a large network of genes required for the transition from vegetative growth to sporulation (Molle, Fujita et al. 2003, Fujita, Gonzalez-Pastor et al. 2005, Chastanet and Losick 2011). This connection between DnaA and Spo0A links DNA replication and DnaA activity to another highly important checkpoint, entry into sporulation.

As well as contributing to the regulation of sporulation transcriptional network, DnaA mediates expression of proteins involved in a range of other cellular activities. *ywlC* is an essential gene involved in the conserved threonylcarbamoyladenosine (t(6)A) tRNA modification (Lauhon 2012) and *trmE* is a GTPase also involved in tRNA modification (Scrima, Vetter et al. 2005). *noc,* in the same operon as *trmE,* is a DNA-binding protein involved in nucleoid occlusion: preventing division sites from forming over the chromosome (Wu, Ishikawa et al. 2009, Wu and Errington 2011). The diverse range of DnaA transcriptional targets highlights the coordination between DNA replication and other cellular processes through DnaA-mediated gene expression. Many of the genes directly regulated by DnaA have homologs in other bacteria, and several of these have putative DnaA-boxes in their promoter regions (Goranov, Katz et al. 2005). Given the high conservation of DnaA, it is possible that certain transcriptional targets of DnaA are also conserved among other bacteria.

Similar to *B. subtilis, E. coli* DnaA also serves as a transcription factor and autoregulates its own expression (Atlung, Clausen et al. 1985, Braun, O'Day et al. 1985, Kucherer, Lother et al. 1986, Wang and Kaguni 1987). DnaA also directly represses several other genes in *E. coli,* such as *mioC, rpoH, uvrB,* and *proS* (Messer and Weigel 1997). These genes all have DnaA-boxes in, or immediately upstream of their promoters, except for *proS*, where the DnaA-binding site is 220 base pairs upstream of the promoter. DnaA is also an activator in *E. coli,* directly binding to and regulating *fliC* and *glpD* expression. Both promoters have two DnaA-boxes between -20 and -70 upstream of the transcriptional start sites.*E. coli* DnaA also regulates expression of *nrdAB*, which encodes ribonucleotide reductase, required for dNTP synthesis. ATP-bound DnaA represses *nrdAB* expression, whereas ADP-bound DnaA causes increased expression (Olliver, Saggioro et al. 2010).

The activity and regulation of DnaA as a transcription factor is poorly understood. The number and organization of DnaA-boxes varies greatly between the DnaA-regulated promoters (Goranov, Katz et al. 2005, Breier and Grossman 2009, Smith and Grossman 2015). Several known regulators of DnaA in *B. subtilis* are also associated with transcriptional targets of DnaA, such as YabA and DnaD (Merrikh and Grossman 2011, Smits, Merrikh et al. 2011). It is possible that they have a regulatory role concerning gene expression, although it is also possible that they are simply bound at these sites due to their interaction with DnaA and do not affect its activity as a transcription factor. YabA has been shown to affect DnaA binding at several target promoters *in vivo* although the effects on gene expression remain unclear (Merrikh and Grossman 2011). During replication stress, YabA does not influence the activity of DnaA as a transcription factor (Goranov, Breier et al. 2009), but effects from DnaN-YabA interaction may relieve any possible effects of YabA on gene expression.

Additionally, it is unclear how the nucleotide-bound state of DnaA may affect gene expression. *In vitro* studies have shown that the majority of DnaA binding sites have a preference for ATP-bound DnaA over ADP-bound DnaA (Smith and Grossman 2015). This is presumably due to DnaA-ATP's ability to cooperatively bind at these promoters, although it is unknown whether cooperative binding is essential for gene regulation by DnaA. Some of the lower affinity binding sites show a slight preference for DnaA-ADP, altough these sites have not been observed to bind DnaA *in vivo* (Smith and Grossman 2015).

Thesis summary

I have isolated a conditional synthetic lethal mutant that overinitates DNA replication in *B. subtilis*. By isolating suppressors of this mutant, I have identified several new genes that are involved in regulating DNA replication. These mutants suppressed overinitiation by either directly decreasing initiation or stimulating elongation to keep up with initiation. I have found that decreased levels of the replicative helicase (DnaC) are sufficient to limit replication initiation during fast growth or under the conditions of high replication initiation.

I also looked at the regulation of DnaA activity as a transcription factor in *B. subtilis*. I found that YabA directly regulates DnaA activation at P*sda* but does not appear to affect expression from the repressed P*ywlC*. YabA appears to have the opposite effect at P*dnaA*, where DnaA activity is enhanced in the absence of *yabA*. Further characterization of the regulation of DnaA as a transcription factor is in progress.

Chapter 2

Suppressors of a conditional synthetic lethal mutant that overinitiates DNA replication in *Bacillus subtilis*

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This chapter is being prepared for publication.

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Abstract

DNA replication is a highly regulated process that is primarily controlled at the step of initiation. In the Gram-positive bacterium, *Bacillus subtilis*, the replication initiator, DnaA, is regulated by in part by YabA, which prevents cooperative binding at the origin. Strains lacking YabA have increase and asynchronous initiation. We found an overinitiating *dnaA1* mutant was synthetic lethal with a deletion of *yabA* in rich medium and the Δ*yabA dnaA1* had an additive effect on DNA replication initiation. We isolated suppressors of Δ*yabA dnaA1* and identified several classes of suppressors that acted to either decrease replication initiation or stimulate replication elongation. One class of suppressors decreased levels of the replicative helicase, DnaC, limiting replication initiation. We found that decreased levels of helicase were sufficient to decrease replication initiation under fast growth conditions. Our results identified novel genes involved in DNA replication control and highlight the multiple mechanisms used to regulate replication in the cell.

Introduction

DNA replication is a highly regulated, essential process across all domains of life. All organisms need to control DNA replication based on environmental cues and growth rate to ensure each daughter cell has a complete copy of the genome. Bacteria typically have a single circular chromosome and initiate bi-directional DNA replication from an origin, finishing roughly 180° opposite, at the terminus.

Under favorable, nutrient rich conditions, certain bacteria, such as *Escherichia coli* and *Bacillus subtilis*, can undergo multifork replication; that is, initiate a new round of DNA replication before the previous round has finished. Multifork replication results in each daughter cell receiving a chromosome with an active replication fork and multiple origins, enabling cells to divide more quickly, while still ensuring each daughter cell receives a completed chromosome (reviewed in Skarstad and Katayama 2013). Under slow growth conditions bacteria restrict replication initiation to avoid overinitiation. Overinitiation can lead to replication fork collapse and the DNA damage response, as well as problems with cell division and chromosome segregation (Katayama 2001; Bach and Skarstad 2004; O'Donnell, Langston et al. 2013; Magdalou, Lopez et al. 2014). This flexibility in regulating the rate of replication initiation gives bacteria the advantageous ability to reliably adjust growth and division based on environmental and internal cues. We have identified several new genes that contribute to regulation of DNA replication in *B. subtilis*.

In bacteria, DNA replication initiation is primarily regulated by controlling the levels and activity of the replication initiator, DnaA. DnaA is AAA+ ATPase which binds both ADP and ATP (reviewed in Davey, Jeruzalmi et al. 2002). The ATP-bound form is active for replication initiation and binds cooperatively to DnaA boxes located near the origin of replication (*oriC*).

This cooperative binding causes formation of a nucleoprotein helical filament that melts the AT-rich DNA unwinding element (DUE) (reviewed in Leonard and Grimwade 2005). In *B. subtilis,* DnaA recruits additional proteins required for chromosome organization and helicase loading (DnaD and DnaB) before the helicase loader (DnaI) which loads the hexameric replicative helicase (DnaC) monomer by monomer around the DNA. The remaining replication machinery is recruited to the origin, and DNA replication proceeds bi-directionally (reviewed in Kaguni 2006; Mott and Berger 2007; Leonard and Grimwade 2011).

The protein levels and activity of DnaA directly affect initiation. For example, increased expression of *dnaA* causes overinitiation (Atlung, Løbner-Olesen et al. 1987; Skarstad, Løbner-Olesen et al. 1989) and various DnaA mutants have been characterized that either enhance or inhibit DNA replication initiation (Moriya, Kato et al. 1990; Guo, Katayama et al. 1999; Murray and Errington 2008; Scholefield and Murray 2013). One such mutant, *dnaA1*, was isolated as a temperature sensitive mutant that inhibits replication initiation under non-permissive temperatures where the DnaA1 protein is unstable (Moriya, Kato et al. 1990). We have found however, that under permissive conditions, *dnaA1* exhibits increased replication initiation.

DnaA is also regulated by preventing its cooperative binding at the origin, and therefore preventing initiation of DNA replication. In *Escherichia coli,* this is primarily accomplished by regulating the availability of ATP-bound DnaA, either through sequestration or titration (*seqA/datA*) or regulated inactivation of DnaA (RIDA) by Had (reviewed in Kaguni 2006; Leonard and Grimwade 2011). However, in Gram positive bacteria like *B. subtilis,* DnaA is regulated by preventing cooperative binding at the origin through its direct interactions with several proteins, including YabA (Wagner, Marquis et al. 2009; Merrikh and Grossman 2011; Scholefield, Errington et al. 2012; Bonilla and Grossman 2012; Scholefield and Murray 2013).

YabA binds directly to DnaA and prevents the necessary cooperative binding at DnaA boxes near the origin, thereby inhibiting formation of the nucleoprotein filament and melting of the origin (Merrikh and Grossman 2011; Scholefield and Murray 2013). A null mutation in *yabA* leads to increased and asynchronous DNA replication (Hayashi, Ogura et al. 2005; Goranov, Breier et al. 2009).

When combined, the *dnaA1* mutant and a *yabA* null mutation have an additive effect on DNA replication initiation and a growth defect in defined minimal medium. Interestingly, the double mutant has a conditional synthetic lethal phenotype, unable to grow in rich medium (LB). We aimed to isolate suppressors of the conditional synthetic lethal phenotype of the double mutant to elucidate novel mechanisms of DNA replication regulation in *B. subtilis*.

We isolated 30 independent suppressors of the Δ*yabA dnaA1* double mutant that were able to survive fast growth conditions (LB). We preformed whole-genome sequencing and identified 5 classes of suppressors that had multiple independent mutations in the same gene. We determined that null mutations in *cshA* and *ytmP* suppressed lethality by decreasing replication initiation and a null mutation in *nrdR* and a mutation affecting the synthetase domain of *relA* suppressed lethality by stimulating replication elongation to keep pace with increased initiation. We found that mutations that decrease levels of the replicative helicase, DnaC, were sufficient to limit DNA replication initiation under high initiation conditions, either in the overinitiating Δ*yabA dnaA1* background or under fast growth conditions. In addition to elucidating novel genes that can regulate replication initiation, the results of this screen highlight the multiple ways *B. subtilis* can regulate DNA replication, by employing changes in other cellular processes to compensate for a detrimental overinitiation phenotype.

Results

Mutations in *yabA* and *dnaA* cause a synthetic lethal phenotype in rich medium

We found that the *dnaA1*(S401F) missense mutation caused a synthetic lethal phenotype when combined with loss of function mutations in *yabA*. *dnaA1* is a temperature sensitive mutant of *dnaA* that results in a loss of replication initiation at non-permissive temperatures ((1990)). We measured replication initiation in the *dnaA1* mutant by marker frequency analysis of the origin (*ori*) and terminus (*ter*) regions of chromosomes for cells grown in defined minimal medium with glucose as a carbon source; increased *ori/ter* typically indicates increased initiation (or decreased elongation, see below). The *dnaA1* mutant had a ~40% increase in *ori/ter* (Table 1), consistent with an increase in initiation of DNA replication. The increase in replication initiation is likely due to an increase in the activity of the DnaA1 mutant protein and is consistent with its increased activity as a transcription factor (Burkholder, Kurtser et al. 2001).

YabA is a negative regulator of replication initiation and interacts with DnaA (Noirot-Gros, Dervyn et al. 2002; Hayashi, Ogura et al. 2005; Merrikh and Grossman 2011; Scholefield and Murray 2013). Null mutations in *yabA* cause an increase in replication initiation (Hayashi, Ogura et al. 2005; Goranov, Breier et al. 2009). Consistent with these reports, we found that *yabA* mutant cells had an ~30% increase in *ori/ter* when grown in defined minimal glucose medium (Table 1).

A Δ*yabA dnaA1* double mutant was found to have a significant growth defect in defined minimal medium, making small colonies on agar plates and having a doubling time of 98 minutes compared to 50 minutes for wild type. In addition, the double mutant was unable to grow on LB agar plates or in LB medium. We found that in defined minimal medium, the Δ*yabA dnaA1* mutant had an *ori/ter* ratio that was ~80% greater than that of wild type cells,

which was a bigger effect than that of either single mutant: ~40% for *dnaA1* and ~30% for Δ*yabA* (Table 1).

Table 1: ***ori/ter*** **relative to wild type**

Strain	Relevant genotype	*ori/ter* relative to wild type[1]
AG1866	Wild type	1.00 ± 0.04
KPL2	*dnaA1*	1.28 ± 0.12
MEA64	Δ*yabA*	1.38 ± 0.05
CAL2320	*dnaA1* Δ*yabA*	1.84 ± 0.26
MEA537	*dnaA1* Δ*yabA* Δ*nrdR*	0.84 ± 0.09
MEA102	*dnaA1* Δ*yabA relA102*(Δ264-295)[2]	1.04 ± 0.31
MEA370	*dnaA1* Δ*yabA PdnaC(T7)*	1.11 ± 0.02
MEA303	*dnaA1* Δ*yabA* Δ*cshA*	1.55 ± 0.21
MEA323	*dnaA1* Δ*yabA* Δ*ytmP*	1.36 ± 0.28

[1]Strains were grown at 37˚C in minimal glucose medium, and exponentially growing cells were collected to isolate genomic DNA. qPCR was used to determine marker frequency analysis of the origin/terminus. *ori/ter* ratios are normalized to wild type (wt=1). A minimum of 3 biological replicates were included for average and standard error mean.

[2]MEA102 is the originally isolated suppressor mutation in *relA*. All other strains are reconstructed in a clean mutant background.

Too much replication initiation can lead to replication fork collapse ((Magdalou, Lopez et al. 2014)) and induction of the *recA*-dependent SOS response. We found that the SOS response was induced in the Δ*yabA dnaA1* double mutant growing in defined minimal glucose medium. *dinC* is a "damage inducible" gene that is activated in response to the RecA-dependent DNA damage response (Love, Lyle et al. 1985; Gillespie and Yasbin 1987). We used RT-qPCR to measure expression of *dinC,* a gene whose expression can be used as an indicator of the SOS response (Cheo, Bayles et al. 1991), and compared expression to the housekeeping genes *sigA* and *gyrA*. In the Δ*yabA dnaA1* double mutant, there was an ~4-fold increase in *dinC* expression during growth compared to wild type cells (Fig. 1). These results indicate that there is indeed DNA damage in the Δ*yabA dnaA1* double mutant and we infer that this is likely due to replication fork collapse caused by too much replication initiation, leading to the conditionally synthetic lethal phenotype of the Δ*yabA dnaA1* mutant.

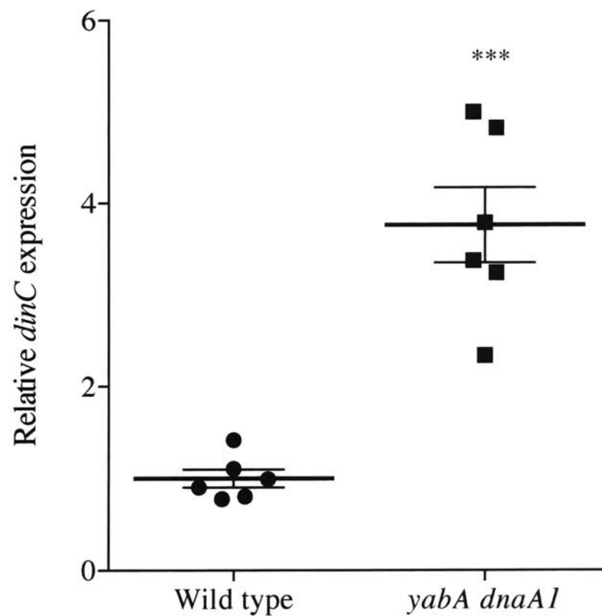

Figure 1: SOS-response is elevated in *ΔyabA dnaA1* compare to wild type. Strains were grown in minimal glucose medium and exponentially growing cells were collected to isolate RNA. cDNA was synthesized using reverse transcriptase and RT-qPCR was used to measure expression of *dinC*, a gene activated during the DNA damage response. *dinC* expression was normalized to housekeeping genes, *sigA* and *gyrA*. Error bars reflect standard error mean of 6 biological replicates. Wild type (AG1866); Δ*yabA dnaA1* (CAL2320).

Isolation of suppressors of the synthetic lethal phenotype caused of the Δ*yabA dnaA1* double mutant

We isolated 45 independent suppressors of Δ*yabA dnaA1* that restored the ability to grow on LB agar plates. Briefly, 45 independent cultures of the Δ*yabA dnaA1* double mutant (strain CAL2320) were grown in defined minimal medium and then an aliquot from each was plated on LB agar. The frequency of suppressors was ~1 per 10^5 cells. To ensure independent suppressor mutations, a single colony was chosen from each plate for further analyses. We were most interested in pseudo-revertants that were not in *dnaA*. To eliminate suppressors with mutations in *dnaA*, we amplified by PCR and sequenced *dnaA* from each of the suppressor strains. Five of the suppressors had at least one additional mutation in *dnaA* and these were not analyzed further.

We selected 30 independent suppressors for whole genome sequencing to determine the gene altered in each suppressor. Most strains had multiple mutations, but comparing them enabled us to focus on a handful of extragenic suppressors. There were several classes of suppressors that had multiple hits in the same gene independently (Table 2). For example, we isolated 10 independent mutants that had the same mutation in the promoter region of *dnaC*, the replicative helicase. These results indicated that mutations in these genes were likely responsible for enabling the Δ*yabA dnaA1* double mutant to grow on LB agar plates.

Table 2: Mutations that suppress the conditional synthetic lethal phenotype of Δ*yabA dnaA1*

Gene	Occurrences	Nature of mutation
nrdR	1	Δ329 bp
	1	R12L
relA	1	Nonsense mutation at S600
	1	Δ264-295 AA in the synthetase domain of *relA*
dnaC	10	Mutation in the promoter region of *dnaC*
cshA	1	T27I; first recA-like domain
	2	H312Y; second recA-like domain
	1	Δ169 bp in c-terminal domain
ytmP	1	R61P
	1	A17V

To confirm that these mutations (Table 2) were responsible for suppression of the Δ*yabA dnaA1* double mutant, we constructed defined mutations in each gene and introduced each allele into the Δ*yabA dnaA1* mutant (Table 3). As the Δ*yabA* dnaA1 mutant is substantially less competent and easily develops suppressor mutations, these defined alleles were first moved into the *dnaA1* background. The *yabA* deletion was moved in last, and these transformations were plated on defined minimal medium selection to prevent acquisition of more suppressor mutations. These reconstructed mutants were then tested for growth in rich medium (LB) to confirm the mutations were sufficient for suppression. Unsurprisingly, each of the different classes of suppressors had a decreased *ori/ter* ratio in minimal medium compared to the Δ*yabA dnaA1* parent (Table 1).

Table 3: *B. subtilis* strains used

Strain	Genotype
AG174	*trpC2 pheA1* (Perego *et al.*, 1988)
AG1866	*trpC2 pheA1* Tn917 Ω HU163 (mls)[2]
KPL2	*trpC2 pheA1 dnaA1*-Tn917 Ω HU163 (mls) (Burkholder *et al.*, 2001)
CAL2320	*trpC2 pheA1* Δ*yabA::spec dnaA1*-Tn917 Ω HU163 (mls)
MEA73	*trpC2 pheA1* Δ*yabA::spec dnaA1*-Tn917 Ω HU163 (mls) *PdnaC(T7)*[1]
MEA64	*trpC2 pheA1* Δ*yabA::spec* Tn917 Ω HU163 (mls)
MEA102	*trpC2 pheA1* Δ*yabA::spec dnaA1*-Tn917 Ω HU163 (mls) *relA102*(Δ264-295)[1]
MEA187	*trpC2 pheA1* Δ*nrdR::kan*
MEA250	*trpC2 pheA1* Δ*cshA::kan*
MEA303	*trpC2 pheA1* Δ*yabA::spec dnaA1*-Tn917 Ω HU163 (mls) Δ*cshA::kan*
MEA304	*trpC2 pheA1* Δ*ytmP::kan*
MEA323	*trpC2 pheA1* Δ*yabA::spec dnaA1*-Tn917 Ω HU163 (mls) Δ*ytmP::kan*
MEA359	*trpC2 pheA1* Δ*purA::kan*
MEA360	*trpC2 pheA1 ycgO::*Pspank-*dnaC-cat*
MEA361	*trpC2 pheA1 ycgO::*Pspank-*dnaC-cat* Δ*dnaC::kan*
MEA362	*trpC2 pheA1 PdnaC(T7)*
MEA370	*trpC2 pheA1* Δ*yabA::spec dnaA1*-Tn917 Ω HU163 (mls) *PdnaC(T7)*
MEA415	*trpC2 pheA1 polC::*Pspank-*polC-specR*
MEA537	*trpC2 pheA1* Δ*yabA::spec dnaA1*-Tn917 Ω HU163 (mls) Δ*nrdR::kan*

[1] These were the original strains isolated from the suppressor screen. All other strains were constructed in a clean background.

[2] The Tn917 Ω HU163 is in the *dck* gene and linked to the *dnaA1* mutation.

In initial experiments, we analyzed several of the suppressors to verify their effects on DNA replication (see below). We then focused our analyses on the suppressors found in the promoter of the replicative helicase, *dnaC*.

Mutations in *nrdR* and *relA* stimulate replication elongation

nrdR. Suppressor mutations were isolated in *nrdR* (negative regulator of ribonucleotide reductase genes) one of which was almost a complete deletion of the gene (Table 2), indicating that loss of *nrdR* would suppress the Δ*yabA dnaA1* double mutant. To confirm that *nrdR* was responsible for suppression, a deletion of *nrdR* was constructed in the Δ*yabA dnaA1* background. Δ*nrdR* Δ*yabA dnaA1* was indeed able to grow on LB agar, confirming that Δ*nrdR* is sufficient for suppression. *nrdR* controls levels of the nucleotide pools and a deletion in *nrdR* results in an increase in the overall levels of nucleotides in the cell (Grinberg, Shteinberg et al. 2006; Torrents, Grinberg et al. 2007). Presumably, this effect would help stimulate elongation or initiation in the Δ*yabA dnaA1* strain, which overinitiates and therefore requires higher levels of nucleotide pools.

We measured *ori/ter* ratios in the Δ*nrdR* Δ*yabA dnaA1* strain and observed a significant decrease compared to that of the Δ*yabA dnaA1* double mutant (Table 1). Although *ori/ter* is frequently used as a readout of DNA replication initiation, it is important to note that a decrease in *ori/ter* could be due to a decrease in replication initiation or an increase in replication elongation. An increase in the overall rate of replication elongation would result in an increase in the copy number of the terminus region, thereby causing a reduction in the marker frequency ratio of *ori/ter*.

One way to distinguish a decrease in replication initiation from an increase in replication elongation is to measure incorporation of ^{3}H-thymidine into DNA. The rate of incorporation should decrease if there is a decrease in replication initiation and increase if there is an increase in replication elongation. All measurements of incorporation of ^{3}H-thymidine assume that the specific activity of the labeled nucleoside is the same in all comparison strains. If there are significant changes in the pool sizes inside the cell, then the incorporation into DNA would need to be corrected for these changes.

We observed an apparent decrease in incorporation of ^{3}H-thymidine in the *nrdR* Δ*yabA dnaA1* mutant, indicating a possible decrease in replication initiation (Fig. 2). However, *nrdR* is known to affect nucleotide biosynthesis (Grinberg, Shteinberg et al. 2006; Torrents, Grinberg et al. 2007). Therefore, we measured pool sizes to correct for possible changes in specific activity. We used mass spectrometry to measure levels of dTMP and dUMP in Δ*nrdR* compared to wild type (Table 4). We determined there to be a ~5-6-fold increase in dTMP and dUMP in Δ*nrdR* compared to wild type. Assuming this change is roughly the same in the mutant background, after correcting for altered pool sizes, these data indicate the nrdR mutant has ~2-3-fold increased DNA synthesis compared to Δ*yabA dna1*. Taken with the decrease in *ori/ter*, these data suggest that the Δ*nrdR* primarily suppresses Δ*yabA dnaA1* by stimulating elongation, rather than affecting initiation.

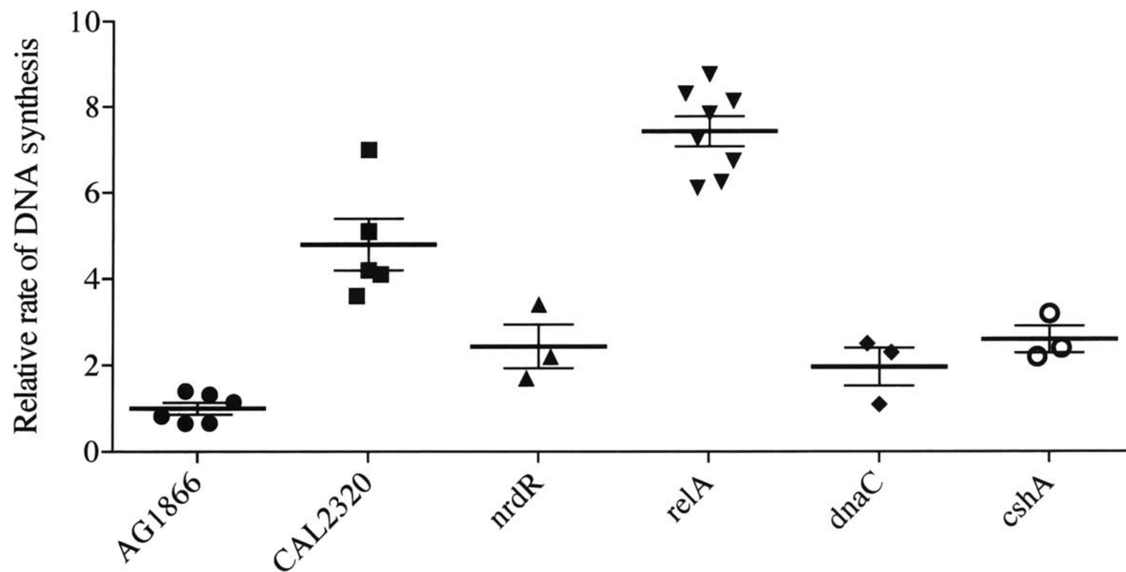

Figure 2: Relative rate of DNA synthesis. Strains were grown at 37°C in minimal glucose medium to mid-exponential phase. Relative rate of DNA synthesis was measured (incorporation of ^{3}H-thymidine into DNA relative to wild type). Wild type (AG1866); Δ*yabA dnaA1* (CAL2320); Δ*yabA dnaA1* Δ*nrdR* (MEA537); Δ*yabA dnaA1 relA102* (MEA102); Δ*yabA dnaA1* P*dnaC(T7)* (MEA370); Δ*yabA dnaA1* Δ*cshA* (MEA303). MEA102 is the originally isolated suppressor mutation in *relA*. All other strains are deletions reconstructed in a clean mutant background. Error bars represent the standard error mean for at least 3 biological replicates. Significant differences compared to the parent strain (CAL2320) are indicated ($P < 0.05$).

Table 4: Δ*nrdR* has increased pools of dTMP and dUMP compared to wild type.

Compound[1]	Δ*nrdR*/wild type
dTMP	5.5
dUMP	6.4

[1] Samples grown at 37˚C in minimal glucose medium to mid-exponential phase were collected for metabolomic analysis using 40% methanol, 40% acetonitrile, 20% water with 0.1M formic acid, and 500 nM each of 17 isotopically-labeled amino acids for extraction buffer (see Methods). Wild type (AG174); Δ*nrdR* (MEA186).

relA. Several mutations were isolated in *relA*, the main protein responsible for (p)ppGpp synthesis and hydrolysis in *B. subtilis*. In *B. subtilis*, *relA* has domains both for synthesis and hydrolysis of (p)ppGpp, as well as a third domain required for interaction with the ribosome (Wendrich and Marahiel 1997). The suppressor mutations affected either the (p)ppGpp synthetase or ribosome binding domains, leaving the hydrolase domain intact (Table 2). One of these mutations includes an in-frame deletion of the conserved synthetase active site residue Asp^{264} (Hogg, Mechold et al. 2004), suggesting this mutation results in a decrease in (p)ppGpp synthesis. Decreased levels of (p)ppGpp have been previously shown to stimulate replication elongation in *B. subtilis* through reduced inhibition of the primase, DnaG (Wang, Sanders et al. 2007). The presumed decrease in (p)ppGpp in the *relA* suppressors would therefore indicate an increase in the processivity of the replication fork, which causes the decrease in *ori/ter* observed in these suppressors.

We attempted to reconstruct a synthetase null *relA(D264G)* (Kriel, Brinsmade et al. 2014) in the Δ*yabA dnaA1* background but were unsuccessful, due to lack of competence in a *relA(D264G)* mutant and the Δ*yabA dnaA1* mutant. We therefore characterized one of the originally isolated suppressors, focusing on the suppressor that affected the synthetase domain of *relA* (Table 1; MEA102). The *relA102* Δ*yabA dnaA1* suppressor mutant had a decrease in the ori/ter ratio in cells growing in defined minimal medium compared to the parent Δ*yabA dnaA1* double mutant (Table 1). Again, this could indicate a decrease in replication initiation or an increase in replication elongation (or both). The apparent rate of DNA synthesis (as measured by pulse labeling with ^{3}H-thymidine) was increased in the suppressor mutant (Fig. 2), consistent with an increase in replication elongation.

Suppressor mutations in the promoter region of *dnaC*, the gene encoding the replicative helicase

Ten of the suppressors had the same mutation, a single base pair deletion in the promoter region of *dnaC*, the gene that encodes the replicative helicase. The mutation *PdnaC(T7)* changes a region of 8 thymines to 7 thymines, changing the spacing between the -10 and -35 regions of the promoter driving expression of *dnaC* (Fig. 3). This decrease in spacing likely affects promoter activity.

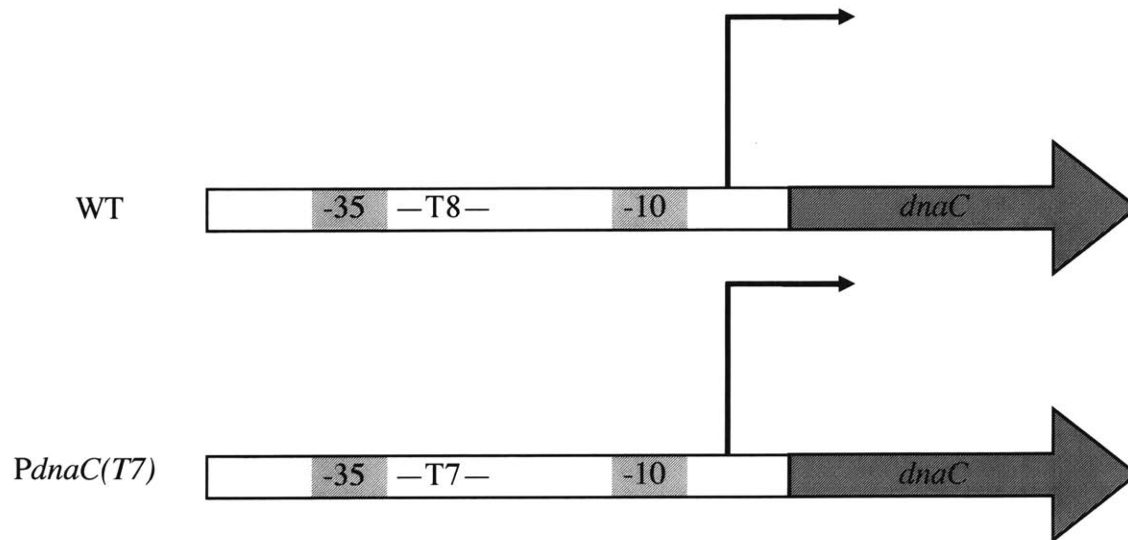

Figure 3: P*dnaC(T7)* changes the spacing between the -10 and -35.

Ten of the suppressors isolated had this specific mutation: a single base pair deletion that changed a string of 8 thymines to 7 thymines. This particular mutation alters the spacing between the -10 and -35 boxes in the promoter region for the replicative helicase, *dnaC*.

In order to confirm that this mutation was responsible for suppression of the Δ*yabA dnaA1* mutant phenotype, the single base pair deletion was reconstructed in the double mutant background. This reconstructed mutant (*PdnaC(T7)* Δ*yabA dnaA1)* grew in rich (LB) medium, confirming that the single base pair deletion in the promoter region of *dnaC* was sufficient to suppress the conditional synthetic lethal phenotype of the Δ*yabA dnaA1* double mutant. Below we describe experiments with the P*dnaC* mutants to determine the mechanism of suppression.

The *PdnaC(T7)* mutation caused a decrease in the amount of the replicative helicase (DnaC)

We hypothesized that the *PdnaC(T7)* mutation likely caused a change in expression of *dnaC*, and consequently a change in the amount of the DnaC protein (helicase). We measured the amount of helicase protein in cells grown in minimal medium with glucose, using Western blots and probing with antibodies to DnaC. Both the wild type and Δ*yabA dnaA1* had roughly the same amount of the replicative helicase, whereas the Δ*yabA dnaA1 PdnaC(T7)* suppressor had ~50% less helicase (Fig. 4A). A similar decrease in DnaC protein was observed for the single mutant, i.e. PdnaC(T7) in a wild-type background (Fig 4B). These data indicate the *PdnaC(T7)* mutation caused a decrease in the amount of the replicative helicase (DnaC).

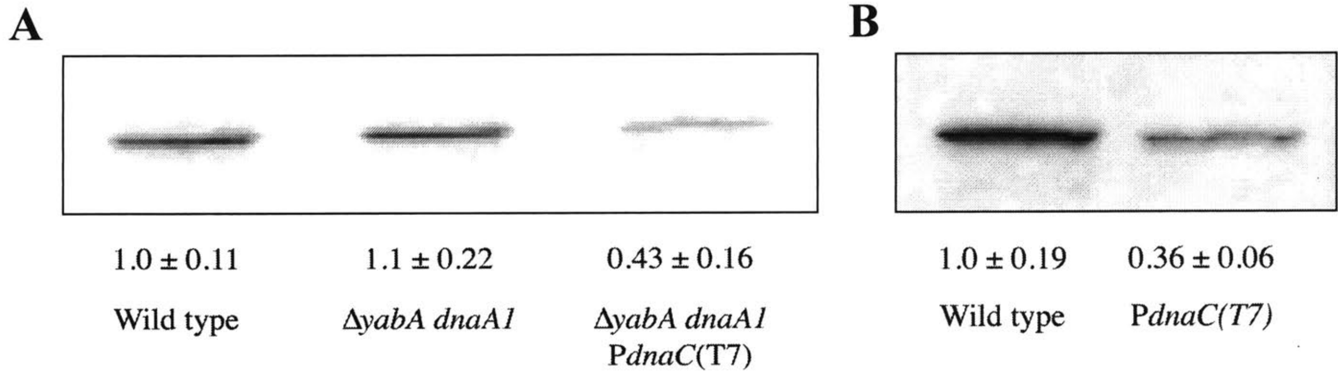

Figure 4: P*dnaC(T7)* causes decreased levels of DnaC protein. Strains were grown at 37°C in minimal glucose medium to mid-exponential phase. Levels of DnaC were determined by quantitative western blot, normalized to optical density (A_{600}; Methods). One representative blot is shown and values represent the average and standard error mean of at least 3 biological replicates.

A. Wild type (AG1866); Δ*yabA dnaA1* (CAL2320); Δ*yabA dnaA1* P*dnaC(T7)* (MEA370).

B. Wild type (AG174); P*dnaC(T7)* (MEA362).

Decreased levels of the replicative helicase are sufficient to lower DNA replication initiation under fast growth conditions

Because the replicative helicase is needed for replication initiation (and elongation) we hypothesized that the decreased levels of helicase in the *PdnaC(T7)* mutants might reduce replication initiation and thereby be responsible for suppressing the overinitiation observed in the Δ*yabA dnaA1* double mutant. We measured *ori/ter* ratios in the P*dnaC*(T7) suppressor and found that the P*dnaC*(T7) mutation caused a significant decrease in *ori/ter* close to the levels of wild type (minimal glucose; Table 1). To determine whether the decrease in *ori/ter* was due to decreased initiation or increased elongation, the relative rate of DNA synthesis was measured. Similar to *ori/ter* results, the P*dnaC*(T7) mutation caused a decrease in total DNA synthesis compared to the Δ*yabA dnaA1* parent (minimal glucose; Fig. 2). Taken together, these data indicate that the P*dnaC*(T7) mutation causes a decrease in DNA replication initiation in a Δ*yabA dnaA1* background, presumably due to the decrease in helicase protein.

We found that the P*dnaC*(T7) mutation also caused a decrease in *ori/ter* ratio in otherwise wild type cells under conditions of rapid growth in rich medium (LB). During rapid growth in LB medium, the *ori/ter* ratio of the P*dnaC*(T7) mutant had a ~20% decrease compared to the isogenic wild type strain (Fig. 5A. 6). During slower growth in minimal glucose medium, the P*dnaC*(T7) mutant had no detectable change in the *ori/ter* ratio (Fig. 6), despite the fact that it contained decreased amounts of the replicative helicase (Fig. 4B). These results indicate that the decrease in the amount of the replicative helicase only has an effect on initiation when the cells are experiencing high rates of DNA replication initiation, such as in fast growth (LB) or in the Δ*yabA dnaA1* mutant.

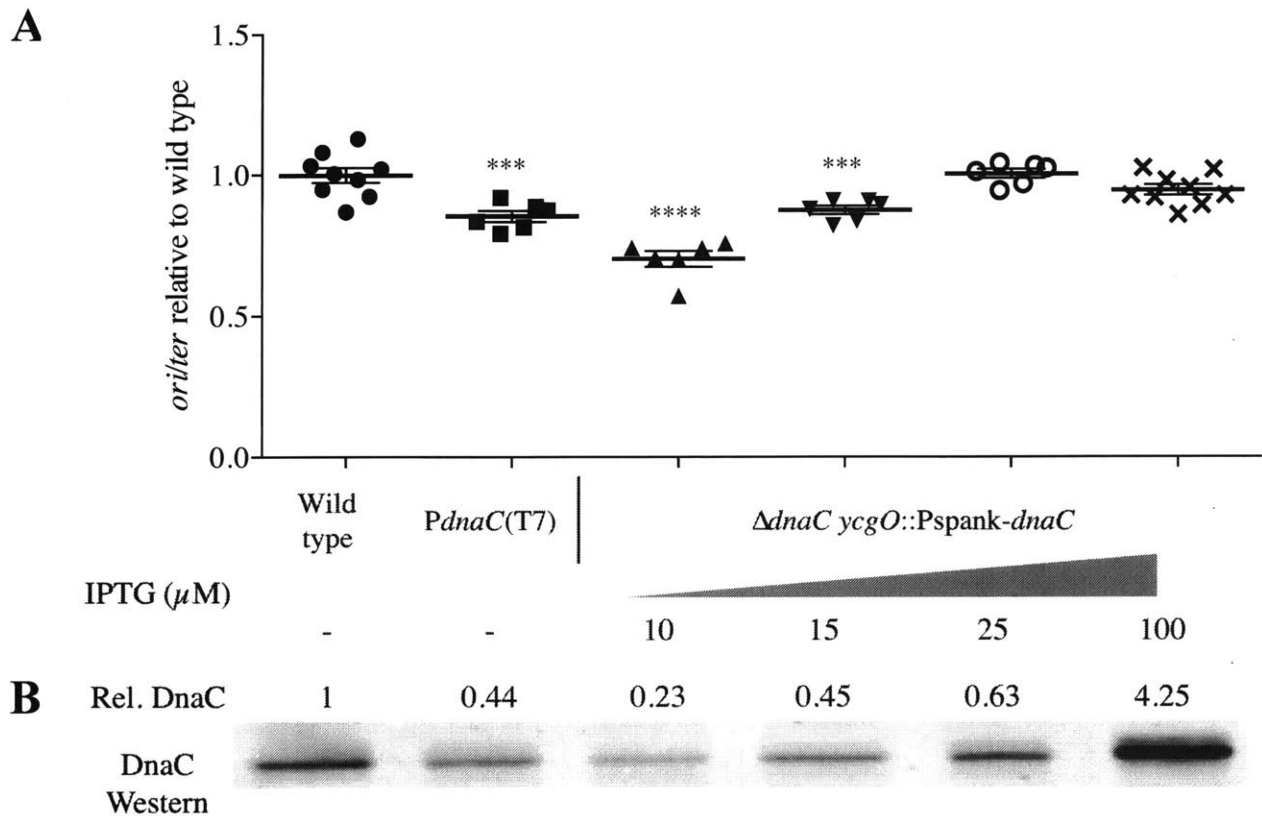

Figure 5: Decreased levels of DnaC are sufficient to decrease *ori/ter*. Strains were grow at 37°C in LB to mid-exponential phase. Expression from Pspank-*dnaC* was induced by addition of varied levels of IPTG (10 μM-100μM). Wild type (AG174); P*dnaC(T7)* (MEA362); Δ*dnaC ycgO*::Pspank-*dnaC* (MEA361).

A. Samples were taken for genomic DNA isolation and used for qPCR to measure *ori/ter* as in Table 1. Error bars represent standard error mean for at least 6 biological replicates. Significant differences compared to the wild type (AG174) are indicated ($P < 0.05$).

B. At the same time, samples were taken to measure DnaC protein with quantitative western as in Figure 3. This is a representative experiment but was repeated with at least 3 biological replicates.

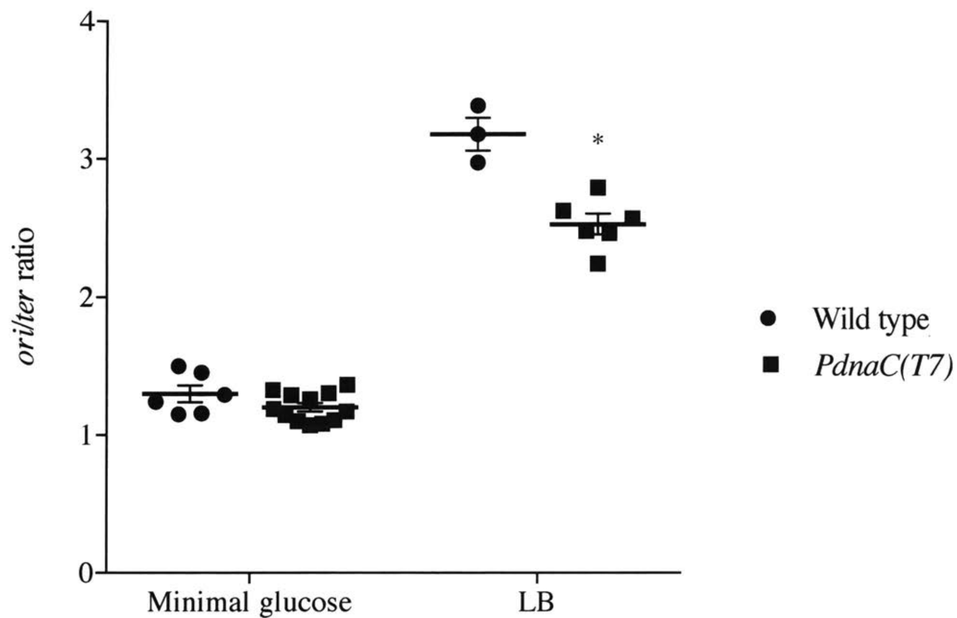

Figure 6: P*dnaC(T7)* only decreases *ori/ter* in fast growth (LB) conditions. Strains were grown at 37°C in either minimal glucose medium or LB, and exponentially growing cells were collected to isolate genomic DNA. qPCR was used to determine marker frequency analysis of the origin/terminus. A minimum of 3 biological replicates were included for average and standard error mean. Wild type (AG174); P*dnaC(T7)* (MEA362). Significant differences compared to the wild type (AG174) are indicated ($P < 0.05$).

Altering expression of *dnaC* from a controllable promoter recapitulates the phenotype caused by the *PdnaC(T7)* mutation

If the decreased initiation phenotype of the *PdnaC(T7)* mutation is really due to altered levels of helicase in the mutant, then we should be able to reproduce the phenotype by varying expression of *dnaC* with a controllable promoter. To vary the amount of the replicative helicase in cells, we fused *dnaC* to the LacI-repressible-IPTG-inducible promoter Pspank (Pspank-*dnaC*). Cells required IPTG for growth, and by growing in different amounts of IPTG different amounts of replicative helicase were obtained, ranging from ~25 to 425 percent of levels in wild type cells (Fig. 5B). We found that there was a decrease in the *ori/ter* ratio as the levels of DnaC decreased. There was a significant decrease in the *ori/ter* ratio when levels of DnaC were decreased below roughly 50% that of wild type (Fig. 5A). At higher levels of DnaC the *ori/ter* ratio was essentially unchanged, even when DnaC was overexpressed ~4-fold over wild type levels.

Decreasing levels of PolC does not decrease DNA replication initiation

In order to show that the decrease in DNA replication initiation was specific to decreased levels of the helicase, and not a general trend among other proteins involved in DNA replication initiation, we varied expression levels of DNA polymerase, *polC*, and measured *ori/ter* ratios. We constructed an IPTG-inducible version of *polC* and measured *ori/ter* ratios under a range of induction levels. Unlike the titration of *dnaC*, we did not observe a decrease in *ori/ter* as the levels of *polC* decreased (Fig. 7). In fact, at the lowest level of induction we observed an increase in the *ori/ter* ratio. This agrees with a previous report that observed a decrease in elongation upon depleting levels of *polC* but no effect on initiation (Dervyn, Suski et al. 2001). At all other levels of induction of *polC* the *ori/ter* was indistinguishable from wild type (Fig. 7).

Although the suppressor screen reported here was not saturated, there were no mutations isolated in other genes involved in DNA replication (apart from *dnaA*). These data indicate that the decrease in replication initiation may be specific to the decrease in levels of the helicase, and not a general phenomenon that applies to other proteins involved in DNA replication initiation.

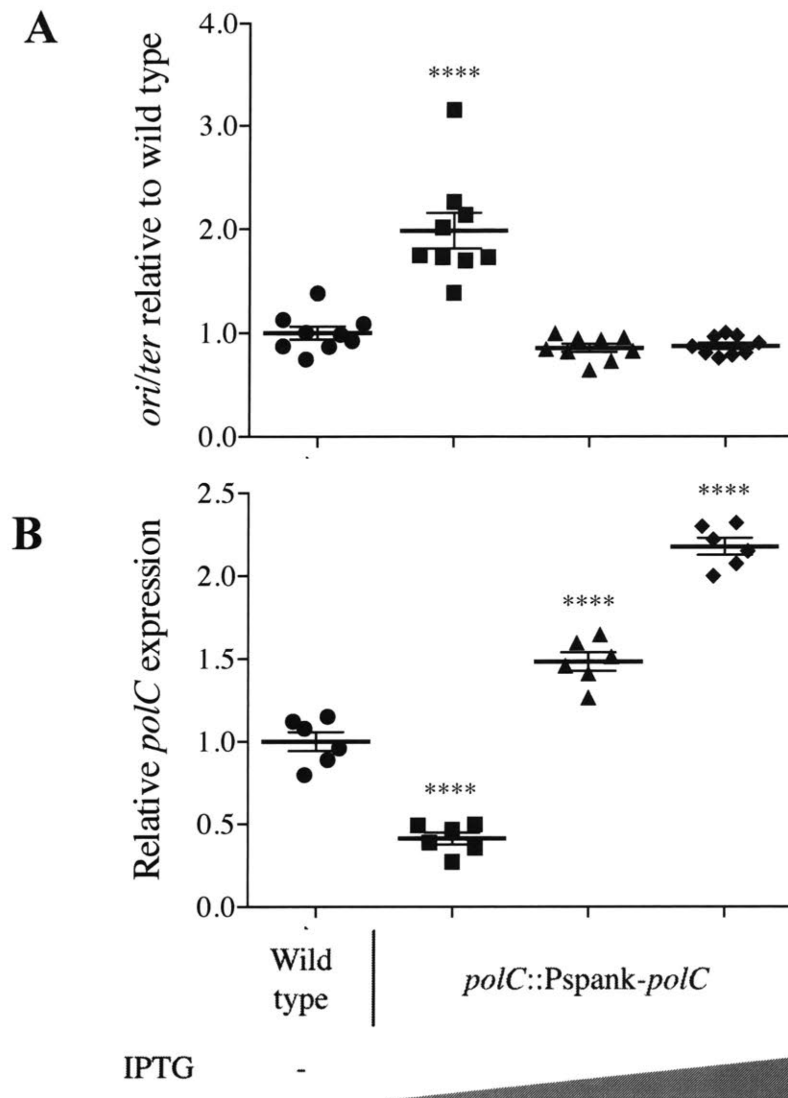

Figure 7: Decreased levels of *polC* does not decrease *ori*/*ter*. Strains were grown at 37°C in minimal glucose medium and exponentially growing cells were collected to isolate RNA and genomic DNA. Expression from Pspank-*polC* was induced by addition of varied levels of IPTG. Error bars reflect standard deviation of 3 biological replicates. Wild type (AG174); *polC*::Pspank-*polC* (MEA415). Significant differences compared to the wild type (AG174) are indicated ($P < 0.05$).

A. gDNA was used for qPCR to measure *ori*/*ter* as in Table 1. Error bars represent standard deviation of 9 biological replicates.

B. RT-qPCR was used to measure expression of *polC* as in Figure 1. *polC* expression was normalized to housekeeping genes, *sigA* and *gyrA*. Error bars show standard deviation of 6 biological replicates.

Suppressors in *cshA* and *ytmP*

cshA. Several mutations were isolated in *cshA*, a cold-shock RNA helicase associated with the RNA degradosome, and a deletion of *cshA* was sufficient to suppress the growth phenotype in the Δ*yabA dnaA1* background. Similar to the other suppressors isolated, Δ*cshA* Δ*yabA dnaA1* had a decrease in both *ori/ter* and the rate of DNA synthesis compared to Δ*yabA dnaA1,* indicating that there was likely a decrease in DNA replication initiation (Table 1). We have not measured nucleotide pools in the *cshA* mutants but believe the effects are largely acting by decreasing initiation.

Given the role of *cshA* in mRNA turnover and ribosome biogenesis, we hypothesized that there may be a change in stability of certain mRNAs or the amount of one or more proteins associated with DNA replication. Given that we determined a decrease in expression of *dnaC*, encoding the replicative helicase, is sufficient to decrease DNA replication initiation above, we measured levels of *dnaC* in Δ*cshA* using RT-qPCR. We found there was a roughly 50% decrease in *dnaC* levels in Δ*cshA* (Fig. 7A). This may contribute to the decrease in *ori/ter* but is likely not the sole factor because there is a greater decrease in *ori/ter* in Δ*cshA* than in the P*dnaC*(T7) mutant in otherwise wild type background when grown in rich medium (Fig. 7B, Fig. 4A). Additionally, Δ*cshA* has a much slower growth rate compared to wild type, with Δ*cshA* having a doubling time of 51 minutes compared to 21 minutes for wild type in LB medium (data not shown). This could contribute to the decrease in *ori/ter* but may also decrease the amount of DnaC needed for DNA replication initiation. Due to the multiple roles of *cshA* and the pleiotropic effect of a *cshA* deletion, it's likely there are multiple effects of Δ*cshA* that contribute to suppression of Δ*yabA dnaA1*.

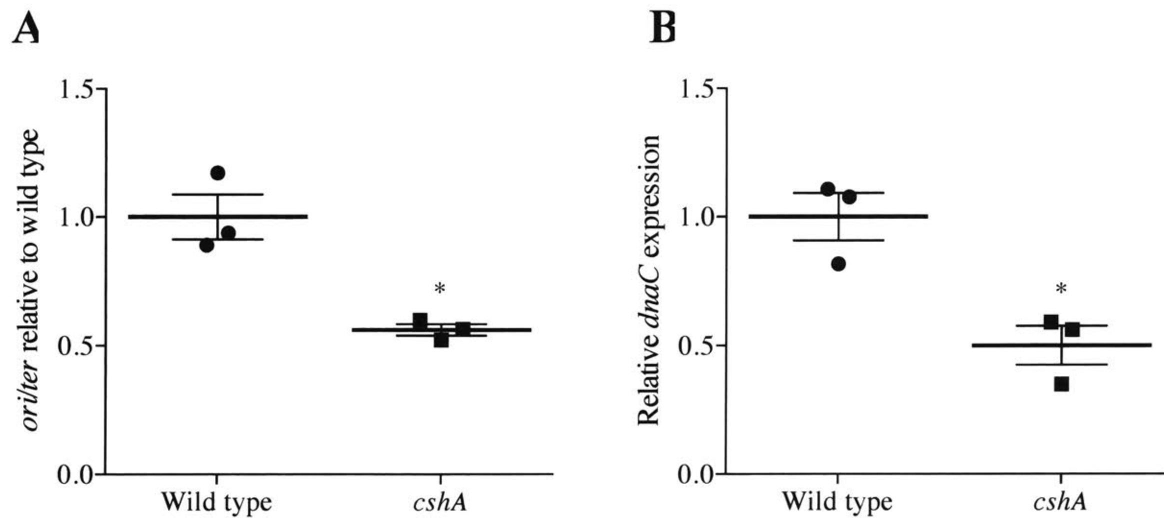

Figure 8: ***dnaC*** **expression and** ***ori/ter*** **are decreased in Δ*cshA*.**

Strains were grown at 37°C in LB and exponentially growing cells were collected to isolate RNA and genomic DNA. Error bars reflect standard error mean of 3 biological replicates. Wild type (AG174); Δ*cshA* (MEA250). Significant differences compared to the wild type (AG174) are indicated ($P < 0.05$).

A. gDNA was used for qPCR to measure *ori/ter* as in Table 1. Error bars represent standard deviation of 3 biological replicates

B. RT-qPCR was used to measure expression of *dnaC* as in Figure 1. *dnaC* expression was normalized to housekeeping genes, *sigA* and *gyrA*.

ytmP. We isolated several mutations in a previously uncharacterized gene, *ytmP*. *ytmP* encodes a protein of unknown function but has homology to the choline kinase family of proteins refs]. A null mutation in *ytmP* was sufficient to suppress the conditional synthetic lethal phenotype of Δ*yabA dnaA1* and resulted in a decrease in *ori/ter* (Table 1). We have yet to characterize this suppressor further.

Discussion

Decreased levels of helicase as a novel mechanism of suppression for DNA replication overinitiation in *B. subtilis*

The majority of the suppressors we identified were in the promoter region of the replicative helicase, *dnaC*, and caused a decrease in protein levels of the helicase. We were able to show that under high initiation conditions, either in the overinitiating double mutant (Δ*yabA dnaA1*) or fast growth conditions (LB), decreased levels of helicase were sufficient to limit DNA replication initiation. This is a novel mechanism for regulating DNA replication in *B. subtilis*.

Altered levels of helicase have previously been shown to affect DNA replication in other organisms. For example, in *E. coli*, perturbing the relative levels of the helicase (*dnaB*) or helicase loader (*dnaC*) inhibits DNA replication initiation and elongation (Allen and Kornberg 1991; Skarstad and Wold 1995; Bruning, Myka et al. 2016). This is believed to be the result of an imbalance between the helicase and the loader proteins, which are normally at a ~1:1 ratio. To load the helicase ring onto the DNA, *E. coli* employs a ring breaker mechanism where the hexameric helicase loader breaks an already formed hexameric helicase ring and loads it around the DNA (reviewed in Davey and O'Donnell 2003). An imbalance between the two proteins causes inhibitory associations between the helicase and the loader: excess loader causes continual reassociation between the helicase and loader, preventing replisome progression (Allen and Kornberg 1991; Skarstad and Wold 1995), whereas excess helicase reduces the probability of the helicase-loader complex formation (Bruning, Myka et al. 2016).

B. subtilis and some other Gram-positive bacteria use a ring maker mechanism where a monomeric helicase loader loads individual monomers around the DNA to create the hexameric ring required for DNA replication (reviewed in Davey and O'Donnell 2003). Any inhibitory

interactions would only affect monomer-monomer interactions, rather than inhibiting an already assembled hexameric helicase, prevent its progression. As shown above, overproduction of DnaC had no effect on *ori/ter*, indicating that a different mechanism for decreasing replication initiation is occurring in *B. subtilis*.

Our hypothesis is that levels of DnaC are limiting under high initiation conditions, causing the decrease in replication initiation. We only observed an effect under conditions with high DNA replication initiation, such as rich medium (LB) or in the overinitiating mutant background of Δ*yabA dnaA1*. During slower growth, where lower concentrations of helicase protein are required, we observed no change in DNA replication initiation. This makes sense given that higher levels of helicase are required when more DNA replication is taking place.

Decreased replication initiation due to lower levels of a replication protein appears to be specific to the helicase, DnaC

We did not isolate suppressor mutations in other genes known to be involved in replication initiation (apart from *dnaA)*. Our screen was not able to isolate suppressors associated with loss of function of most proteins involved in DNA replication because these genes are essential. Loss of function alleles are the most frequent type of mutation isolated from this type of screen. Our screen was able to isolate mutations that affect protein levels or activity, which are much less likely to occur. The P*dnaC* mutation we isolated, which occurred in the promoter region of dnaC, is one example of this type of mutation. The mutation that caused the decreased levels of helicase was a single basepair deletion in a run of thymines. However, we did not isolate any similar mutations in other essential DNA replication genes. This is not surprising, as these types of mutations occur very rarely and our screen was not performed to saturation. It is possible that

the specific mutation in P*dnaC* (Fig. 3) occurs at higher frequency than promoter mutations in other DNA replication genes, due to the tendency of slippage by the replication machinery at poly-N tracts (reviewed in Strauss 1999). It is unique that P*dnaC* is so easy to mutate. Other genes involved with replication initiation, such as *dnaB-dnaI* and *dnaD*, have shorter poly-N tracts in their promoters (≤5) and not in the -10 to -35 region.

It is also possible that we did not isolate mutations affecting the levels of other DNA replication proteins because these proteins are present in excess and a small decrease in levels would not affect initiation. If this is true, helicase is unique in being synthesized to roughly the required amount for fast growth and not in excess. Decreased expression of another protein involved in DNA replication, the replicative polymerase (*polC*), does not decrease DNA replication, although this does not rule out other proteins required for replication initiation.

Mutations that increase elongation can suppress overinitiation mutant

The conditional synthetic lethal phenotype is caused by severe overinitiation, leading to replication fork collapse. We isolated several suppressors that suppress the overinitiation mutant by decreasing replication initiation. Somewhat surprisingly, we also isolated classes of mutations that overcame the severe overinitiation by stimulating replication elongation. Increased replication elongation could help overcome the overinitiation defect by either preventing replication fork collapse, or stimulating repair of collapsed forks.

We determined that mutations in *relA,* likely resulting in a decrease in (p)ppGpp, appear to stimulate replication elongation. (p)ppGpp has previously been shown to affect elongation, specifically by inhibiting primase (DnaG) (Wang, Sanders et al. 2007). DNA primase synthesizes an RNA primer required for lagging strand DNA synthesis. Decreased inhibition on

primase would help increase replication elongation. This could be by either stimulating the rate of elongation in the mutant background, preventing replication fork collapse, or by enhancing replication restart from already collapsed forks, however distinguishing between these two mechanisms is difficult.

Similarly, we determined a null mutation in *nrdR* also suppressed the overinitiation mutant by stimulating replication elongation. Changes in expression of *nrd* genes, which encode nucleotide reductase needed for deoxyribonucleotide biosynthesis (reviewed in Nordlund and Reichard 2006), in relation to perturbations in DNA replication have been well documented (Augustin, Jacobson et al. 1994; Huang, Zhou et al. 1998; Goranov, Katz et al. 2005), establishing a link between DNA replication and nucleotide biosynthesis. In *E. coli*, DnaA itself directly activates the *nrdAB* operon (Augustin, Jacobson et al. 1994), indicating a positive correlation between increased DNA replication initiation and dNTPs, which is also the case for the *nrdR* suppressors isolated in this screen. In *B. subtilis*, expression of the *nrdEF* operon (regulated by *nrdR*) increases upon replication fork arrest induced by blocking replication through HPUra (Goranov, Katz et al. 2005). Increased dNTPs have been proposed to promote the RecA-dependent repair of stalled replication forks in *E. coli* (Robu, Inman et al. 2001). This mechanism supports our model, in which enhanced replication elongation helps overcome the replication fork collapse caused by the severe overinitiation of Δ*yabA dnaA1*.

The diverse mutants isolated during this suppressor screen highlight the varied means that can be used to control detrimental DNA replication in a cell. This study emphasizes how the cell can act at different steps in DNA replication using multiple mechanisms to overcome a replication defect.

Methods

Media and growth conditions

Cells were grown with shaking at 37°C in Luria-Bertani (LB) medium (Miller 1972) or S7 defined minimal medium with MOPS (3-(N-morpholino) propanesulfonic acid) buffer at a concentration of 50 mM rather than 100 mM supplemented with 1% glucose, 0.1% glutamate, trace metals, 40 µg/ml phenylalanine, and 40 ug/ml tryptophan (Jaacks, Healy et al. 1989). Standard concentrations of antibiotics were used when appropriate (Harwood and Cutting 1990). To induce expression of *dnaC* from the LacI-repressible, IPTG-inducible Pspank, varied concentrations of IPTG were used as specified.

Strains and alleles

E. coli strains used for plasmid construction were AG1111 (MC1061 F' *lacI^q lacZ*M15 Tn*10*) (Glaser, Kunst et al. 1993). *B. subtilis* strains were derived from JH642 (*pheA1 trpC2*) (Perego, Spiegelman et al. 1988), are listed in Table 3, and were constructed by natural transformation using genomic DNA.

AG1866 was constructed by transforming AG174 with KPL2 genomic DNA and selecting for mls (macrolide-lincosamide-streptogramin B) resistance and screening for temperature resistance at 50°C, resulting in a wild type isogenic to KPL2 (Tn917 Ω HU163 (mls)).

Δ*yabA::spec* (CAL2055) was constructed by replacing the open reading frame with a spectinomycin resistance cassette using long-flanking homology PCR. MEA64 is the result of backcrossing CAL2055 into AG1866 to generate a Δ*yabA::spec* isogenic to KPL2 and AG1866.

Deletions in *nrdR* (MEA187), *cshA* (MEA250), *purA* (MEA359) and *ytmP* (MEA587) were constructed by replacing the open reading frames with a kanamycin resistance cassette (kan) by

using linear Gibson isothermal assembly (Gibson, Young et al. 2009) fragments containing ~1KB of flanking homology for the indicated gene.

P*dnaC(T7)* was reconstructed in a wild type background as follows: DNA from one of the originally isolated suppressors with the P*dnaC(T7)* mutation (MEA73) was transformed into a strain with Δ*purA::kan* (MEA359; linked to *dnaC* and an adenine auxotroph) selecting for adenine prototrophy on minimal medium plates. Candidates were sequenced to find isolates with the P*dnaC(T7)* mutation, resulting in strain MEA362.

CAL2320 was constructed by transforming CAL2055 into KPL2 and selecting on minimal medium plates with spectinomycin and confirming the presence of *dnaA1* by screening for t.s. at 50˚C. All derivatives of CAL2320 (except MEA370) were constructed by introducing the desired alleles into KPL2 (selecting on rich medium) and then moving Δ*yabA::spec* (CAL2055) in last, selecting on minimal medium plates to avoid generating suppressors. MEA370 was constructed by moving *dnaA1*-Tn917 Ω HU163 (mls) into MEA362 selecting on rich medium. Δ*yabA::spec* (CAL2055) was then moved in last, selecting on minimal medium.

MEA360 was constructed by transforming linearized pMEA358 (cut with DraIII) into AG174. pMEA358 is a derivative of pBOSE1404 that places the open reading frame (and ribosome binding site) of *dnaC* under the IPTG inducible promoter Pspank. pBOSE1404 is a plasmid that introduces a chloramphenicol resistant Pspank at the *ycgO* locus (*ycgO-cat-lacI*-Pspank-*ycgO*) by double crossover. oMEA260 (5'-TTGTGAGCGGATAACAATTAAATGAGAGGACGGTGCTTAGC-3') and oMEA261 (5'-GAATTAGCTTGCATGCGGATCTTTCGAATGAAAAAAACCCCAAGAG-3') were used to amplify a 1420 bp fragment (AG174 as the template) that includes the open reading frame of *dnaC*. Using isothermal assembly (Gibson, Young et al. 2009), this fragment was introduced

into pBOSE1404 cut with NheI and HindIII, generating pMEA358. MEA361 was constructed by replacing the open reading frame of *dnaC* with a kanamycin resistance cassette (kan) by using linear Gibson isothermal assembly (Gibson, Young et al. 2009) fragments containing ~1KB of flanking homology transformed into MEA360, selecting on kanamycin + 1mM IPTG to induce expression of the essential *dnaC*.

Suppressor screen

Independent cultures of CAL2320 (Δ*yabA dnaA1*) were grown in defined minimal medium with 1% glucose (see above) at 37°C to mid exponential phase. Dilutions of each independent culture were plated on LB agar at 37°C. A single colony was chosen from each of the original independent cultures and streaked 2x on LB agar. Colonies with different growth rates and morphologies were chosen deliberately with the aim to diversify the mutants isolated.

Genome sequencing

Each suppressor mutant and CAL2320 were grown in minimal medium with 1% glucose to mid exponential phase. Cells were harvested by centrifugation and DNA was isolated using a Qiagen 100 G tips purification kit. DNA was sheared using a Covaris ultrasonicator. Sample preparation, including incorporation of a 3' barcode, selection of 300–600 bp fragments (after addition of adaptors and amplification), and paired-end read sequencing (150-150 nt) on an Illumina MiSeq were performed by the MIT BioMicro Center. Reads were mapped to the *B. subtilis* strain JH642 (GenBank: CP007800.1; Smith, Goldberg et al. 2014) as described (Deatherage and Barrick 2014).

qPCR to determine *ori/ter* ratio

Cultures were grown to mid-exponential phase and diluted back to OD 0.05 and grown to mid-exponential phase (OD 0.2-0.4) in defined minimal medium at 37°C. Cells were harvested

in ice-cold methanol (1:1 ratio) and pelleted. Genomic DNA was isolated using Qiagen DNeasy kit with 40 μg/ml lysozyme. The copy number of the origin (*ori*) and terminus (*ter*) were quantified by qPCR to generate an *ori/ter* ratio. qPCR was done using SSoAdvanced SYBR master mix and CFX96 Touch Real-Time PCR system (Bio-Rad). Primers used to quantify the origin region were oMEA316 (5'-TTGCCGCAGATTGAAGAG-3') and oMEA317 (5'-AGGTGGACACTGCAAATAC-3'). Primers used to quantify the terminus region were oMEA318 (5'-CGCGCTGACTCTGATATTATG-3') and oMEA319 (5'-CAAAGAGGAGCTGCTGTAAC-3'). Origin-to-terminus ratios were determined by dividing the number of copies (as indicated by the Cp values measured through qPCRs) of the origin by the number of copies quantified at the terminus. Ratios were normalized to the origin-to-terminus ratio of a temperature sensitive mutant, *dnaB134* (KPL69), that was grown to have synchronized replication initiation, resulting in 1:1 ratio of the origin:terminus.

RT-qPCR

Cultures were grown to mid-exponential phase and diluted back to OD 0.05 and grown to mid-exponential phase (OD 0.2-0.4) in defined minimal medium at 37°C. Cells were harvested in ice-cold methanol (1:1 ratio) and pelleted. RNA was isolated using Qiagen RNeasy PLUS kit with 10 mg/ml lysozyme. iScript Supermix (Bio-Rad) was used for reverse transcriptase reactions to generate cDNA. RNA was degraded by adding 75% volume of 0.1 M NaOH and incubating at 70°C 10 minutes, followed by neutralizing the reaction with adding 75% of the original volume 0.1 M HCl. qPCR was done using SSoAdvanced SYBR master mix and CFX96 Touch Real-Time PCR system (Bio-Rad). Primers used to quantify *dnaC* were oMEA126 (5'-AGCTGCAAGTCCCTGTTATC-3') and oMEA127 (5'- CCTGCTCGATACTTCCTGATTC-3'). Primers used to quantify *sigA* were oMEA252 (5'- ATACCGGCTCTTGAGCAATC-3')

and oMEA253 (5'- ACTTAGGCAGAGAACCAACAC-3'). Primeres used to quantify *gryA* were oMEA128 (5'- TGGAGCATTACCTTGACCATC-3') and oMEA129 (5'- AGCTCTCGCTTCTGCTTTAC-3').

Western blots to measure protein levels

Cultures were grown to mid-exponential phase and diluted back to OD 0.05 and grown to mid-exponential phase (OD 0.2-0.4) in defined minimal medium or LB (as indicated) at 37˚C. Exponentially growing cells were lysed with lysozyme and a protease inhibitor cocktail (Sigma-Aldrich, P8849) at 37˚C. Lysates were run on 12% polyacrylamide gel and transferred to a nitrocellulose membrane using the Trans-blot SD semi-dry transfer cell (Biorad). To represent the same number of cells in each lane, the amounts loaded in each lane were normalized to culture OD. All steps were performed at room temperature. According to the manufacturer's instructions, blots were blocked with Odyssey Blocking Buffer for 1 hr and incubated with primary antibody (rabbit anti-DnaC antibody 1:10,000 in Odyssey Blocking Buffer + 0.2% Tween) for 1 hr. The primary antibody to DnaC was affinity purified rabbit polyclonal antibody (Covance) made against purified DnaC-His6. Blots were washed with PBST (phosphate-buffered saline + 0.2% Tween) for at least 5 x 5 min and then incubated for 30 min with secondary antibody (LiCor dye 800 goat anti-rabbit 1:10,000 in Odyssey Blocking Buffer + 0.2% Tween). Blots were imaged and quantitated on a LiCor scanner. Dilutions of AG174 lysates were used to generate a standard curve and determine the linear range of the fluorescence signal.

^{3}H-thymidine incorporation to measure DNA synthesis

Cells were grown at 37˚C, and were assayed at 4 or 5 time-points between OD 0.2 and 0.5. Incorporation per OD was found to be linear over this range. A 250 μl aliquot was pulse labeled with 10 μl ^{3}H-thymidine (6.7 Ci/mmol; 1 mCi/ml). After 1 min, a short chase was performed by

adding 50 μl 10 mM unlabeled thymidine and incubating for an additional 1 min. An equal volume ice-cold 20% trichloroacetic acid (TCA) was added, and the samples were incubated on ice for 30-60 min. A 350 μl aliquot was vacuum-filtered on glass-fiber filters (24 mm GF/A, Whatman) and washed with 25 ml of ice-cold 5% TCA, followed by 2 ml 100% ethanol. The amount of radioactivity that had been incorporated into nucleic acid was determined by scintillation counting of the dried filters. A time course was performed to confirm that the 1 min pulse was in the linear range of the assay.

Mass spectrometry to measure nucleotide pools

Preparation of cellular extract for metabolomic analysis. Cultures were grown in minimal glucose media to OD 0.45-0.55, transferred to 50 ml conical tube, and quickly chilled to 10°C by swirling in liquid nitrogen. Cells from 5 ml of the chilled culture were collected onto a 25 mm MilliPore Type HAWP 0.45 μM filter using a filtration apparatus chilled to 4°C. The filter was immediately placed in a tube containing 1 ml ice-cold extraction buffer (40% methanol, 40% acetonitrile, 20% water with 0.1M formic acid, and 500 nM each of 17 isotopically-labeled amino acids), and quickly frozen in liquid nitrogen. After removing from liquid nitrogen, 87μl 15% ammonium bicarbonate was added to each tube, and vortexed and shaken vigorously to disrupt the cells. After 5 min centrifugation at 16100 g at 4°C, the supernatant was removed and dried under vacuum. LC-MS profiling and analysis was performed by the Whitehead Institute Proteomics Core Facility essentially as described (Kanarek, Keys et al. 2018).

Chapter 3

Effects of YabA on DnaA-regulated gene expression in *Bacillus subtilis*

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This chapter is in progress and will be prepared for publication after more experiments have been done

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Leah McKinney helped construct strains and did all β-galactosidase assays and Western blots.

Abstract

YabA is a negative regulator of the replication initiator, DnaA, in *Bacillus subtilis*. YabA directly binds to DnaA, preventing cooperative binding to DnaA-boxes at the origin of replication. In addition to initiating replication, DnaA also serves as a transcription factor, binding to DnaA-boxes in the promoters of several genes. Regulation of DnaA as a transcription factor is not well characterized. We looked at the effects of YabA on DnaA-mediated gene expression using promoter-*lacZ* fusions in a background where DNA replication was not affected. We found that YabA inhibited DnaA activation of *sda* expression but had no detectable effect on DnaA repression of *ywlC*. We also found that YabA appears to enhance repression of P*dnaA* by DnaA. Our results indicate that YabA also functions to regulate DnaA as a transcription factor, although this regulation appears to have varying effects depending on the promoter.

Introduction

The initiation of DNA replication is highly regulated across all domains of life and is coordinated with other cellular processes to ensure correct progression of the cell cycle. In bacteria, replication initiation is primarily regulated by controlling the replication initiator protein, DnaA. DnaA is a AAA+ ATPase that can bind both ADP and ATP, although the ATP-bound form is the active form for DNA replication initiation (Davey, Jeruzalmi et al. 2002). In *B. subtilis* DnaA initiates replication by cooperatively binding to DnaA-boxes (consensus sequence 5'- TTATNCACA; Fuller, Funnell et al. 1984; Fukuoka, Moriya et al. 1990; Smith and Grossman 2015) at the origin (*oriC*), causing unwinding of the DNA unwinding element (DUE) and recruitment of the replication machinery (reviewed in (Kaguni 2006; Mott and Berger 2007; Leonard and Grimwade 2011).

Most of what is known about regulation of DnaA has been studied in *Escherichia coli.* In this and related Gram-negative bacteria, DnaA activity at the origin is regulated by controlling the availability of active, ATP-bound DnaA, either by regulating ATPase activity and nucleotide exchange or sequestration/titration of the available ATP-DnaA (reviewed in Kaguni 2006; Leonard and Grimwade 2011). In contrast, the known regulators of DnaA in *Bacillus subtilis* and other Gram-positive bacteria, appear to function solely by inhibiting cooperative binding of DnaA at *oriC* (Wagner, Marquis et al. 2009; Merrikh and Grossman 2011; Bonilla and Grossman 2012; Scholefield, Errington et al. 2012; Scholefield and Murray 2013).

YabA is one of the negative regulators of DnaA in *B. subtilis* (Hayashi, Ogura et al. 2005; Goranov, Breier et al. 2009). YabA interacts with both DnaA and the sliding clamp, DnaN (Noirot-Gros, Dervyn et al. 2002; Noirot-Gros, Velten et al. 2006). YabA directly binds DnaA and limits its association at *oriC in vivo* and inhibits cooperative binding of DnaA to DNA *in*

vitro, independently of the ATPase activity of DnaA (Merrikh and Grossman 2011; Scholefield and Murray 2013). Null mutations in *yabA* result in increased DNA replication initiation due to increased and more cooperative binding of DnaA at the origin (Hayashi, Ogura et al. 2005; Goranov, Breier et al. 2009).

In addition to its role in replication initiation, DnaA also functions as a transcription factor by binding to DnaA-boxes throughout the genome, both activating and repressing genes, depending on the location of its binding sites (reviewed in Messer 2002). In *B. subtilis*, DnaA directly activates expression of *sda* and appears to directly repress the expression of several genes, including *ywlC, ywcI-sacT, yydA,* and *trmE-noc,* in addition to its own autorepression (Table 1; Ogura, Imai et al. 2001; Burkholder, Kurtser et al. 2001; Goranov, Katz et al. 2005; Ishikawa, Ogura et al. 2007; Breier and Grossman 2009; Hoover, Xu et al. 2010; Smith and Grossman 2015). DnaA may have effects on several other operons, likely indirectly (Washington, Smith et al. 2017). All of these transcriptional targets of DnaA have clusters of at least four DnaA-boxes located in their promoter regions (Table 1; Fukuoka, Moriya et al. 1990; Burkholder, Kurtser et al. 2001; Ishikawa, Ogura et al. 2007; Breier and Grossman 2009; Smith and Grossman 2015). It is unclear how DnaA binding is regulated at these promoters and how the organization of the DnaA-boxes influences expression.

We aimed to look at the effect of YabA on DnaA as a transcription factor. YabA is an attractive candidate for regulating both the replication initiation and gene regulation activities of DnaA, as it is non-essential and has already been demonstrated to regulate cooperative binding of DnaA at *oriC* and affect expression of *sda* (Merrikh and Grossman 2011; Scholefield and Murray 2013).

The effects on DnaA-regulated gene expression have been previously studied. However,

these studies have investigated DnaA-dependent gene expression in cells initiating from *oriC,* in cells that overexpress *dnaN*, or in cells where *dnaA-dnaN* are still under regulation by DnaA, all of which are likely to affect the levels or activity of DnaA (Goranov, Katz et al. 2005; Ishikawa, Ogura et al. 2007; Goranov, Breier et al. 2009; Breier and Grossman 2009; C. Seid, unpublished results). In an *oriC* background, a null mutation in *yabA* causes an increase in DNA replication initiation, leading to increased gene copy number, especially around the origin. Additionally, due to the autoregulation of *dnaA-dnaN* by DnaA, any changes in the activity of DnaA will affect levels of DnaA. Increased levels of DnaN, which is known to interact with YabA, have been shown to decrease association of YabA at *oriC* and increase cooperative binding of DnaA at the origin (Goranov, Breier et al. 2009; Merrikh and Grossman 2011). The interaction of YabA with DnaN is thought to help relieve YabA inhibition on DnaA activity at *oriC,* but may also affect DnaA activity as a transcription factor. All of these secondary effects make any results very hard to interpret.

In order to bypass any effects changes in replication initiation will have on gene expression, we constructed strains that use a heterologous origin and initiator isolated from a plasmid found in *B. natto*, *oriN/repN* (Hassan, Moriya et al. 1997), which are not affected by *yabA* (Goranov, Breier et al. 2009). In this background, we introduced *dnaA* under an inducible promoter to avoid any effects of autoregulation, and put *dnaN* under a constitutive promoter that resulted in approximately wild type levels of DnaN. Under these conditions, we were able to determine that YabA does have a direct effect on DnaA as a transcription factor at P*sda* (activated by DnaA), but does not appear to affect at P*ywlC* (repressed by DnaA). When combined with what we observed under conditions where cells are initiating from *oriC*, we believe the majority of the effect of YabA on transcriptional targets of DnaA is due to altered levels of DnaA, rather than a

direct interaction of YabA on DnaA at target promoters. These are just preliminary results and we aim to test more promoters and additional conditions.

Results

In an *oriC* background, *yabA* has an apparent activating effect on DnaA as a transcription factor

We measured the effect of a *yabA* null mutation on the expression of known direct transcriptional targets of DnaA. We made promoter-*lacZ* fusions to targets of interest and used β-galactosidase specific activity as a readout of expression. In all experiments below, strains were grown in minimal defined medium to mid-exponential phase before being collected to assay for β-galactosidase specific activity. We found that there was an apparent decrease in DnaA activity at promoters both activated and repressed by DnaA. P*sda*, the only promoter activated by DnaA, showed a decrease in expression in Δ*yabA* compared to wild type (Fig. 1). P*ywlC*, which is repressed by DnaA, showed an increase in expression in Δ*yabA* compared to wild type (Fig. 2). This was the opposite result we expected if YabA were acting to inhibit DnaA activity as a transcription factor, as it does for DnaA as the replication initiator.

Table 1: Transcriptional targets of DnaA in *B. subtilis*

DnaA targets	DnaA: activation or repression	Number of putative DnaA-boxes in promoter[1]
sda	Activates	8
dnaA-dnaN	Represses	12
ywlC	Represses	9
yydA	Represses	7
trmE-noc	Represses	4
ywcI-sacT	Represses	11

[1] (Smith and Grossman, 2015).

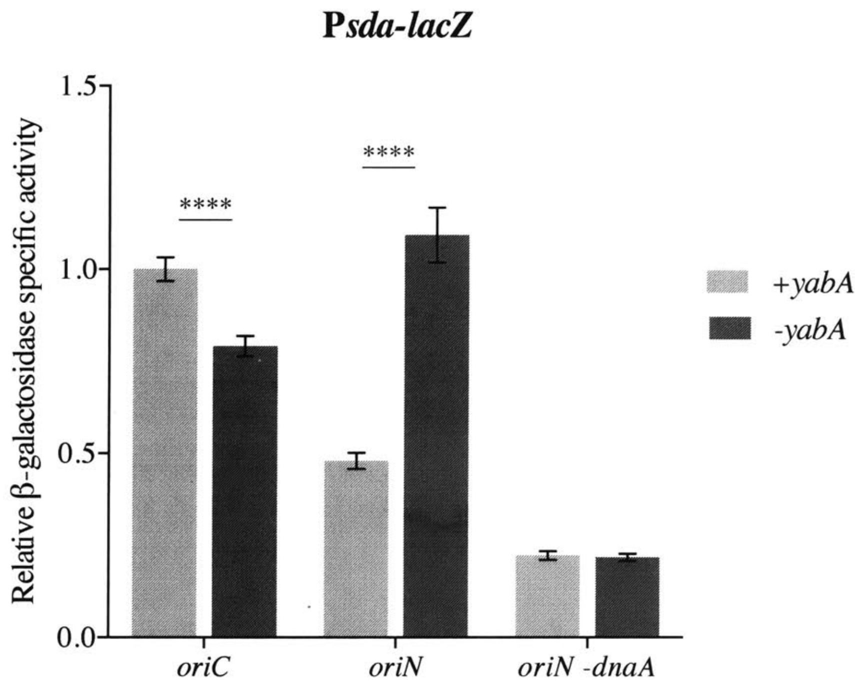


Figure 1. P*sda* expression ±*yabA*. Strains were grown in minimal medium at 37˚C to mid-exponential phase. Samples were collected and β-galactosidase specific activity was measured (Methods). Light grey bars represent *yabA+* strains and dark grey bars represent *yabA-* strains. Error bars represent the standard error mean of at least 3 biological replicates. P*sda-lacZ* was introduced at the ectopic locus, *amyE*. *dnaA* expression was induced in *oriN* strains by 25μM IPTG induction of Pspank-*dnaA*. Strains: *oriC yabA+* (MEA446); *oriC yabA-* (MEA450); *oriN +yabA +dnaA* (MEA463); *oriN +yabA +dnaA* (MEA467); *oriN +yabA -dnaA* (MEA479); *oriN +yabA -dnaA* (MEA489).

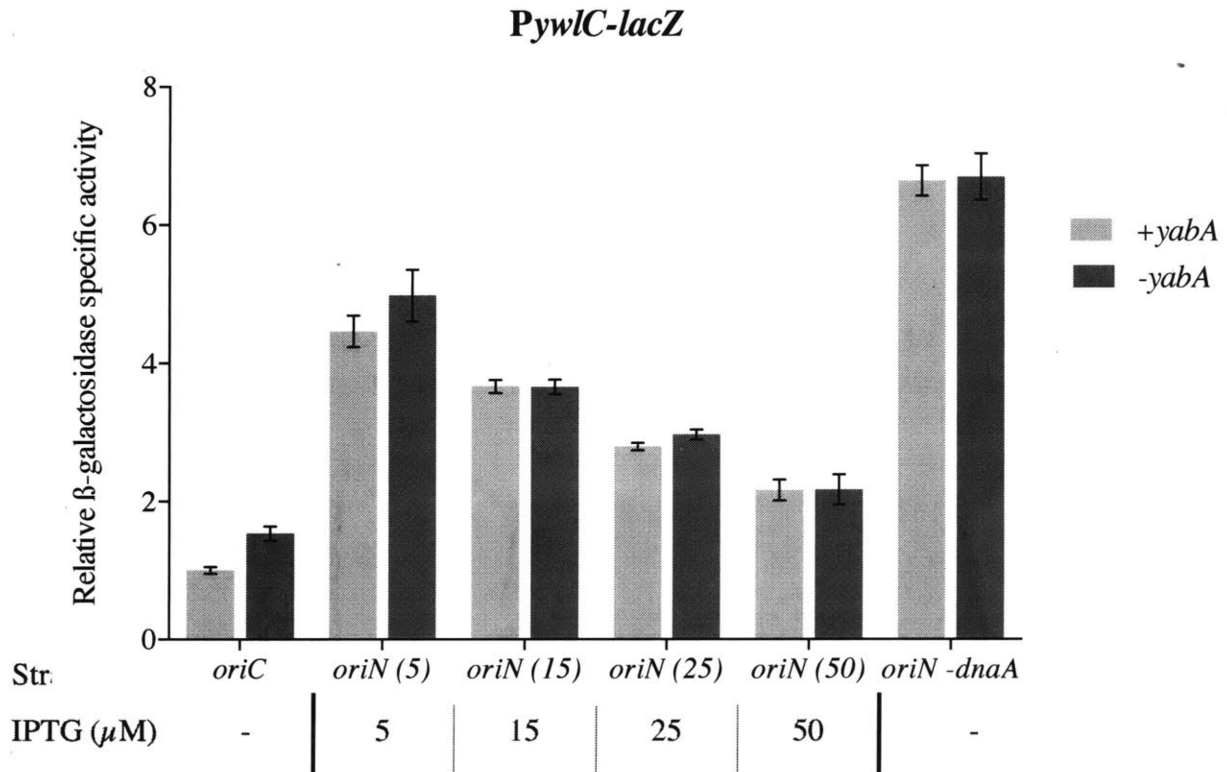


Figure 2. P*ywlC* expression ±*yabA*. Strains were grown in minimal medium at 37°C to mid-exponential phase. Samples were collected and β-galactosidase specific activity was measured (Methods). Light grey bars represent *yabA+* strains and dark grey bars represent *yabA-* strains. Error bars represent the standard error mean of at least 3 biological replicates. P*ywlC-lacZ* was introduced at the ectopic locus, *amyE*. *dnaA* expression was induced in *oriN* strains by varied levels of IPTG induction of Pspank-*dnaA* as indicated. Strains: *oriC yabA+* (MEA448); *oriC yabA-* (MEA452); *oriN +yabA +dnaA* (MEA465); *oriN +yabA +dnaA (*MEA469); *oriN +yabA -dnaA* (MEA473); *oriN +yabA - dnaA* (MEA490).

It seems unlikely that YabA has an opposite effect on DnaA on its role as a transcription factor compared to its role as the replication initiator. The increased replication initiation observed in the absence of *yabA* is likely affecting levels of DnaA, gene copy number of transcriptional targets, or both, contributing to an observed effect of increased DnaA activity at promoters. Due to these possible confounding effects, we decided to look at the impact of Δ*yabA* on DnaA transcriptional targets in a background that uses a heterologous origin, *oriN* ((Hassan, Moriya et al. 1997)), where initiation is unaffected by YabA ((Goranov, Breier et al. 2009)).

Constructing an *oriN* background to measure effects of *yabA* on transcriptional targets of DnaA

oriN has previously been used to look at effects of YabA on transcriptional targets of DnaA (Goranov, Breier et al. 2009). However, *dnaA-dnaN* were under their endogenous promoter, which is autoregulated by DnaA. If there are effects of YabA on transcriptional targets, this could affect levels of DnaA, and therefore complicate any changes, or lack thereof, in expression of other targets. Additionally, we observed unexpected results in regards to the effect of YabA on P*dnaA* (see below), that might further affect levels of DnaA.

To overcome these issues, we constructed an *oriN* background where *dnaA* and *dnaN* are no longer regulated by DnaA. We introduced *oriN,* and its cognate initiator, *repN,* in place of *oriC/dnaA*, and introduced a constitutive promoter to drive *dnaN* expression. *dnaA* was placed under an IPTG-inducible promoter at an ectopic locus, *lacA* (Fig. 3). We mutated a constitutive promoter, Ppen (Yansura and Henner 1984), and tested different versions to find one that produced levels of DnaN protein similar to wild type (data not shown). We also tested various levels of IPTG induction to determine levels of DnaA protein compared to a wild type

background (Fig. 4). The various promoter-*lacZ* fusions were introduced into this *oriN* background ± *yabA*. To confirm *yabA* had no effect on expression of *dnaA* in this *oriN* background, we measured DnaA protein levels at both high and low expression of *dnaA* from Pspank ±*yabA*. As expected there was no difference in DnaA levels ±*yabA* in the *oriN* background with Pspank-*dnaA* (Table 2; *oriN*).

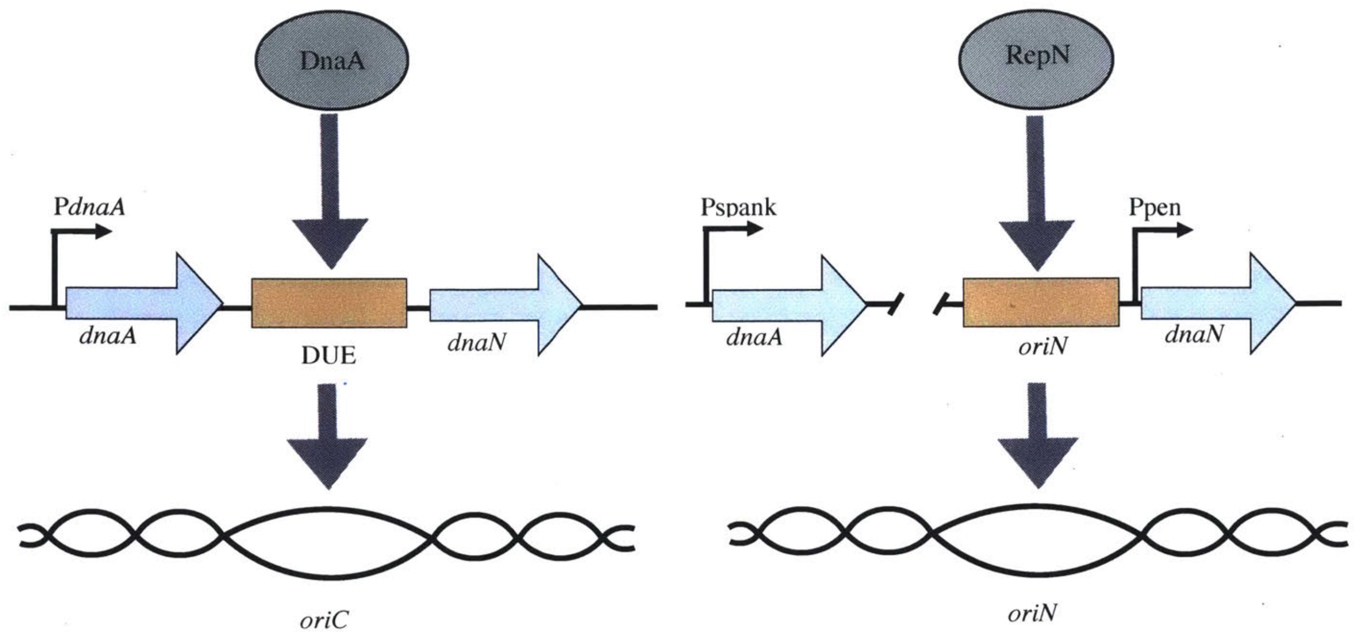
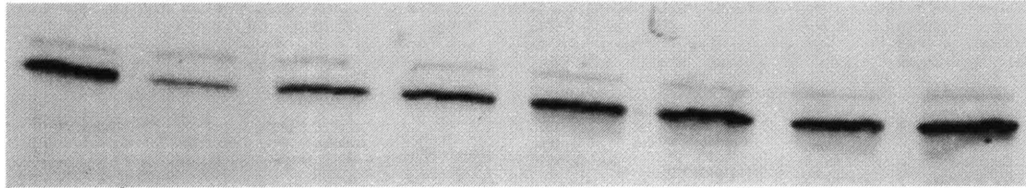


Figure 3. A heterologous origin and initiator.

In the normal *oriC* background, DnaA initiates replication at the DNA unwinding element (DUE) located in the middle of the *dnaA-dnaN* operon (AG174). In the *oriN* background, *dnaA* and the DUE have been replaced with *oriN/repN*. A constitutive promoter (Ppen) drives expression of *dnaN*. *dnaA* has been placed under an IPTG-inducible promoter (Pspank) at an ectopic locus (*lacA*).



Strain background	Wild type	*oriN* Pspank-*dnaA*						
IPTG	-	5 μM	15 μM	20 μM	25 μM	30 μM	40 μM	50 μM
Rel. DnaA	1.00	0.19	0.49	0.61	1.05	1.34	1.66	2.60

Figure 4. Titrating levels of DnaA protein.

Strains were grown at 37˚C in minimal glucose medium with IPTG where indicated to mid-exponential phase. Levels of DnaA protein were determined by quantitative western blot, normalized to total protein (Methods). The lower band represents DnaA on the Western blot. A representative experiment is shown but was repeated with 3 biological replicates. Strains: wild type (MEA446); *oriN* Pspank-*dnaA* (MEA463).

Table 2: Effects of *yabA* on DnaA levels in *oriC* vs. *oriN*

Background	DnaA protein levels[1]: *yabA-/yabA+*
oriC[2]	1.56 ± 0.28
oriN Pspank-*dnaA*[3]	1.11 ± 0.09

[1] Strains were grown at 37°C in minimal glucose medium with IPTG where indicated to mid-exponential phase. Levels of DnaA protein were determined by quantitative western blot, normalized to total protein (Methods). Values indicate averages and standard error mean of at least two biological replicates.

[2] Strains: *yabA+* (AG174); *yabA-* (CAL2055)

[3] *dnaA* expression was induced with 10 μM IPTG. Other expression levels were tested and there was no difference ±*yabA* in any conditions tested. Strains: *yabA+*(MEA463); *yabA-* (MEA467).

YabA has varying effects on the activity of DnaA depending on the target promoter

YabA inhibits DnaA's ability to activate transcription at P*sda*. We measured expression of P*sda*, activated by DnaA, in the *oriN* background ±*yabA* under conditions of ~wild type levels of DnaA. A null mutation in *yabA* caused an increase in expression of P*sda*, indicating that YabA does inhibit DnaA activity as a transcription factor at P*sda* (Fig. 1). This effect of YabA on P*sda* expression was dependent on the presence of DnaA: in a strain background lacking *dnaA* there was no change in expression regardless of the presence of *yabA* (Fig. 1, *oriN -dnaA*). These data indicate that YabA directly inhibits DnaA activity as an activator of P*sda*.

DnaA-mediated repression of transcription at P*ywlC* is unaffected by YabA. We also measured expression of P*ywlC*, a promoter directly inhibited by DnaA, in the *oriN* background ±*yabA*. At ~wild type levels of DnaA, there was no significant difference in P*ywlC* expression ±*yabA* (Fig. 3, 25 μM IPTG). In these experiments, production of the DnaA protein was comparable to a wild type, *oriC* strain (Fig. 4). However, this *oriN* background is an artificial system where growth rate is altered, DNA replication initiation is unregulated, and *dnaA-dnaN* is not autoregulated. Therefore, it is quite possible that these conditions might mask any effect YabA may have on DnaA-regulated gene expression.

To explore this possibility, we titrated levels of DnaA both above and below that of wild type and measured P*ywlC-lacZ* expression ±*yabA*. At varied levels below that of wild type (*oriC*) DnaA (from ~20% to ~50% of wild type; Fig. 4, 5 μM and 20 μM IPTG), we observed the expected increase in expression from P*ywlC,* but no YabA-dependent change (Fig. 2, 5 μM and 20 μM IPTG). We also measured expression from P*ywlC* under conditions with ~2.6-fold higher DnaA compared to wild type (Fig. 4, 50 μM IPTG). Again, we saw the expected decrease in expression but no detectable difference between *yabA*+ and *yabA*- (Fig. 2, 50 μM IPTG).

Unsurprisingly, in the absence of DnaA, we observed an increase in expression from P*ywlC*, but no significant difference in expression ±*yabA* (Fig. 2, *oriN -dnaA*). These data indicate that, although P*ywlC* responds as expected to varied levels of DnaA (lower DnaA, less repression; higher DnaA, greater repression), there is no significant difference in expression from P*ywlC* ±*yabA* under the conditions tested.

P*dnaA* autoregulation ±*yabA*. In addition to the promoters mentioned above, we also looked at the effect of YabA on the autoregulation of P*dnaA*. We constructed a P*dnaA-lacZ* fusion and measured β-galactosidase specific activity as a readout for expression from P*dnaA*. In an *oriC* background we measured expression from P*dnaA* ±*yabA* in exponentially growing cells. As with P*ywlC*, we saw an increase in expression from P*dnaA* in the absence of *yabA* (Fig. 6). Again, this is the opposite expected result for a promoter repressed by DnaA, but the confounding effects of an *oriC* background make this result difficult to interpret. In agreement with this observation, DnaA protein levels are increased ~50% in the absence of *yabA* in an *oriC* background (Table 2; *oriC*).

We again used the *oriN* background (Fig. 4) to look at the direct effects of YabA on DnaA at P*dnaA*. Unexpectedly, we observed an increase in expression from P*dnaA* in the absence of *yabA* in the *oriN* background at wild type levels of DnaA (Fig. 5). In the absence of DnaA, expression from P*dnaA* increased, indicating it is indeed repressed by DnaA (Fig. 5, *oriN -dnaA*). As with the other promoters tested, we observed no difference in expression from P*dnaA* in the absence of DnaA ±*yabA*, indicating that any observed effects of YabA are dependent on DnaA (Fig. 5, *oriN -dnaA*).

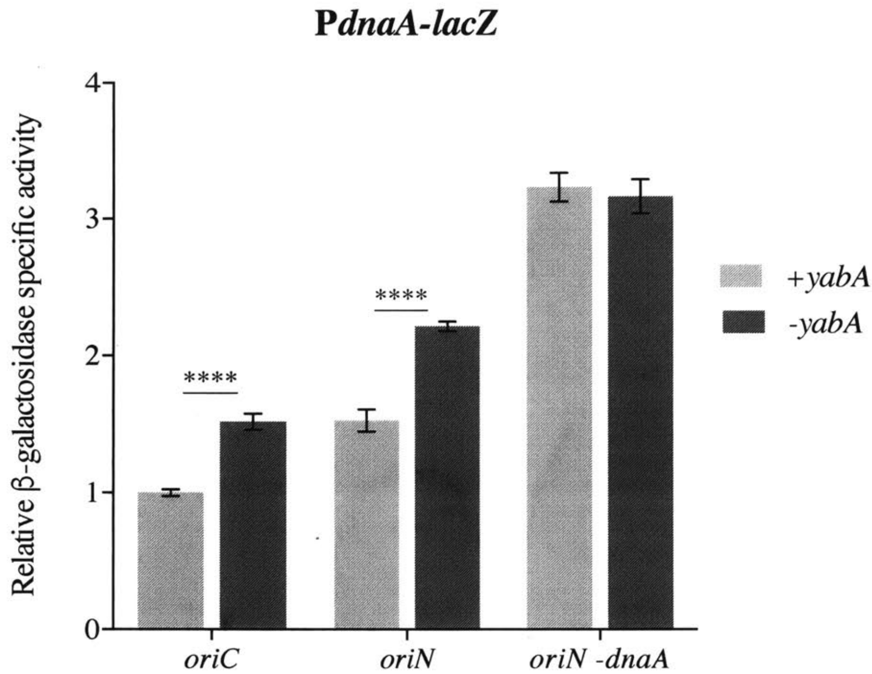


Figure 5. P*dnaA* expression ±*yabA*. Strains were grown in minimal medium at 37°C to mid-exponential phase. Samples were collected and β-galactosidase specific activity was measured (Methods). Light grey bars represent *yabA+* strains and dark grey bars represent *yabA-* strains. Error bars represent the standard error mean of at least 3 biological replicates. P*dnaA-lacZ* was introduced at the ectopic locus, *amyE*. *dnaA* expression was induced in *oriN* strains by 25μM IPTG induction of Pspank-*dnaA*. Strains: *oriC yabA+* (MEA449); *oriC yabA-* (MEA453); *oriN +yabA +dnaA* (MEA468); *oriN +yabA +dnaA* (MEA470); *oriN +yabA -dnaA* (MEA474); *oriN +yabA -dnaA* (MEA478).

Table 3: ***B. subtilis*** **strains used**

Strain	Genotype
AG174	*trpC2 pheA1* (Perego *et al*., 1988)
AMB89	*trpC2 pheA1 lacA::*Pspank-*dnaA-tet*
CAL2027	*trpC2 pheA1 thrC::*Ppen2027-*lacZ-spec*
CAL2055	*trpC2 pheA1 ΔyabA::spec*
MEA183	*trpC2 pheA1 Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN*
MEA446	*trpC2 pheA1 amyE::*P*sda-lacZ-camR*
MEA448	*trpC2 pheA1 amyE::*P*ywlC-lacZ-camR*
MEA449	*trpC2 pheA1 amyE::*P*dnaA-lacZ-camR*
MEA450	*trpC2 pheA1 amyE::*P*sda-lacZ-camR ΔyabA::spec*
MEA452	*trpC2 pheA1 amyE::*P*ywlC-lacZ-camR ΔyabA::spec*
MEA453	*trpC2 pheA1 amyE::*P*dnaA-lacZ-camR ΔyabA::spec*
MEA463	*trpC2 pheA1 amyE::*P*sda-lacZ-camR lacA::*Pspank-*dnaA-tet Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN*
MEA465	*trpC2 pheA1 amyE::*P*ywlC-lacZ-camR lacA::*Pspank-*dnaA-tet Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN*
MEA467	*trpC2 pheA1 amyE::*P*sda-lacZ-camR lacA::*Pspank-*dnaA-tet Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN ΔyabA::spec*
MEA468	*trpC2 pheA1 amyE::*P*dnaA-lacZ-camR lacA::*Pspank-*dnaA-tet Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN*
MEA469	*trpC2 pheA1 amyE::*P*ywlC-lacZ-camR lacA::*Pspank-*dnaA-tet Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN ΔyabA::spec*
MEA470	*trpC2 pheA1 amyE::*P*dnaA-lacZ-camR lacA::*Pspank-*dnaA-tet Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN ΔyabA::spec*
MEA473	*trpC2 pheA1 amyE::*P*ywlC-lacZ-camR Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN*
MEA474	*trpC2 pheA1 amyE::*P*dnaA-lacZ-camR Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN*
MEA478	*trpC2 pheA1 amyE::*P*dnaA-lacZ-camR Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN ΔyabA::spec*
MEA479	*trpC2 pheA1 amyE::*P*sda-lacZ-camR Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN*
MEA489	*trpC2 pheA1 amyE::*P*sda-lacZ-camR Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN ΔyabA::spec*
MEA490	*trpC2 pheA1 amyE::*P*ywlC-lacZ-camR Δ(oriC-L-dnaN)::kanR-oriN-repN*-Ppen2027-*dnaN ΔyabA::spec*

Discussion

YabA appears to directly affect DnaA as a transcription factor at P*sda*, but not P*ywlC*

Using an *oriN* background where *dnaA* and *dnaN* are not regulated by DnaA, we measured the effects of YabA on DnaA-mediated gene expression. Preliminarily, we have looked at expression from both a promoter activated by DnaA, P*sda*, and a promoter repressed by DnaA, P*ywlC,* using promoter-*lacZ* fusions. We found that at approximately wild type levels of DnaA, YabA has a negative effect on expression from P*sda*, and this effect is dependent on DnaA (Fig. 2). Conversely, we were unable to detect any effect of YabA on expression from P*ywlC* under a range of DnaA levels (Fig. 3).

YabA inhibits DnaA as the initiator by preventing cooperative binding of ATP-DnaA at the origin (Merrikh and Grossman 2011; Scholefield and Murray 2013). Likewise, YabA can impact the activity of DnaA in its target promoters. Several factors could affect the interplay between YabA and DnaA in these promoters, discussed below: (i) different mechanisms of DnaA acting as a transcription factor, (ii) effects of the nucleotide-bound state of DnaA, (iii) differences in spacing and orientation of the DnaA boxes, (iv) amount of DnaA bound to each of the controlled promoters all likely influence expression from these promoters.

The mechanisms for activation and repression by DnaA are unknown, although given the location of the DnaA-boxes in the promoters, it's likely that DnaA represses transcription by blocking RNA polymerase binding to the promoter (Fig. 1). P*sda* is unique in that it is the only known promoter to be directly activated by DnaA in *B. subtilis* (Burkholder, Kurtser et al. 2001; Goranov, Katz et al. 2005). Sda inhibits the histidine kinase pathway that is required for phosphorylating the master sporulation regulator, Spo0A, and results in the active form, Spo0A~P (Burkholder, Kurtser et al. 2001). In addition to being regulated by DnaA, P*sda* is also

directly activated by Spo0A~P and repressed by LexA (Fujita, Gonzalez-Pastor et al. 2005; Au, Kuester-Schoeck et al. 2005; Veening, Murray et al. 2009). These other regulators might contribute to effects of YabA on DnaA at P*sda*. Additionally, it's possible that DnaA binding at P*sda* activates expression by helping to stimulate open-complex formation. In this case, the role of DnaA at P*sda* would more closely mimic its role as the replication initiator than its role as a repressor. This might explain the influence of YabA at P*sda* that is not observed at P*ywlC*.

Although we know ATP-DnaA is required for replication initiation, the effects caused by the different nucleotide-bound states of DnaA on its activity as a transcription factor are less concrete. Using IDAP-seq (*in vitro* DNA affinity purification and deep sequencing), DnaA binding at target promoters has been reported with the apparent binding constants (K_d) for ATP-DnaA and ADP-DnaA (Smith and Grossman 2015). Although almost all targets had a preference for ATP-DnaA *in vitro*, the differences between the K_d for ATP vs. ADP-bound DnaA varied substantially between different promoters. For example, when comparing ADP/ATP-DnaA binding at P*sda,* the K_d for ADP-DnaA was 3.4-fold higher than that of ATP-DnaA. Less distinction between the nucleotides was observed for P*ywlC* where ADP-DnaA had a K_d only 1.8-fold higher than that of ATP-DnaA (Smith and Grossman 2015). Since ATP-DnaA is the form required for cooperative binding, this may indicate that cooperative binding is of greater importance for gene expression at P*sda* compared to P*ywlC*, and would therefore explain the different effects of YabA at these promoters.

The organization of DnaA-boxes may similarly influence the effects of YabA at different promoters. The number, spacing, and orientation of DnaA-boxes varies greatly between different promoters and it is unclear how this contributes to DnaA activity at these promoters (Fig. 1; (Goranov, Katz et al. 2005; Ishikawa, Ogura et al. 2007; Breier and Grossman 2009;

Smith and Grossman 2015). YabA could have a greater impact in promoters with DnaA boxes configurations that favor more cooperative binding of DnaA.

Additionally, the amount of DnaA able to bind to a single promoter might also influence the results above. If the levels used here were simply high enough to saturate the promoter, even in the presence of YabA, we would not observe an effect on transcription ±*yabA*. With all of these factors in play, the effects of DnaA and YabA on gene expression can vary substantially between different targets. We aim to test more transcriptional targets of DnaA to get a more complete picture of the role of YabA on DnaA-mediated gene expression.

P*dnaA* behaves differently in response to Δ*yabA*.

Expression from P*dnaA-lacZ* (repressed by DnaA) was measured in both an *oriC* and *oriN* background ±*yabA*. Similar to P*ywlC*, expression from P*dnaA* increased in the absence of *yabA* in an *oriC* background (Fig. 7). Interestingly, in the *oriN* background (controlled expression of *dnaA* and *dnaN*), expression from P*dnaA* was also increased in Δ*yabA* (Fig. 7). In the absence of *dnaA*, P*dnaA* expression increased, confirming the P*dnaA-lacZ* fusion is indeed repressed by DnaA, as expected. The increase in expression in the *oriN* Δ*yabA* background was surprising given the fact that levels of *dnaA* and *dnaN* are under controlled expression in this background (Fig. 4).

One possible explanation for these results is that non-cooperative binding at P*dnaA* is sufficient to regulate expression and in the presence of YabA, DnaA binding is enhanced though not cooperative. We previously looked at the effects of DnaA binding at P*dnaA* *in vitro* and found that at lower concentrations of DnaA, YabA increased DnaA binding, though it is not cooperative (Merrikh and Grossman 2011). If this holds true *in vivo*, and non-cooperative binding is sufficient to regulate expression, it is possible that YabA could stimulate DnaA

binding to promoter, and cause enhanced repression as is seen here for P*dnaA* (Fig. 7). This would argue that YabA actually enhances repression of DnaA targets, thus having the opposite effect on DnaA activity as a transcription factor compared to DnaA activity as a replication initiator.

Another possible explanation for the effects on P*dnaA*, is that there is some other factor influencing expression from P*dnaA*. The most likely explanations are that either an unknown element is acting on P*dnaA* expression, or the location of the fusion at an ectopic locus is affecting regulation of expression of P*dnaA-lacZ* that would not be present at the endogenous locus. DnaD, Soj, and SirA are also known to regulate DnaA as the replication initiator using the same mechanism as YabA (Wagner, Marquis et al. 2009; Bonilla and Grossman 2012; Scholefield, Errington et al. 2012; Scholefield and Murray 2013). None of these proteins have yet been found to interact with YabA (Noirot-Gros, Velten et al. 2006). It is unlikely that expression or activity of these regulators has changed in this background, although we have not ruled out the possibility that they impact the effect of YabA on P*dnaA* expression. Apart from DnaA, Spo0A has also been shown to bind to the DnaA promoter, although not in the context of gene regulation, but rather to inhibit replication initiation during sporulation. Given that Spo0A is indirectly regulated by DnaA (through *sda*, see above), and we have controlled for DnaA levels, it seems unlikely that Spo0A is affecting P*dnaA* through YabA. At this stage, we do not know of any other proteins likely to affect YabA activity at P*dnaA*.

The *dnaA-dnaN* operon is unique, in that the origin is positioned between the two genes (Fig. 4). There are 2 clusters of DnaA-boxes in the promoter region, one before the transcription start site and one after. At the origin, there is another cluster of DnaA-boxes situated before the DNA unwinding element. In a plasmid-based replication system using the *B. subtilis oriC*, DnaA-

boxes both upstream and downstream *dnaA* were required for *in vitro* and *in vivo* replication (Moriya, Firshein et al. 1994). It's possible that, similar to this system, both upstream and downstream clusters of DnaA-boxes influence expression of P*dnaA*, which would not be captured in the P*dnaA-lacZ* fusions. We know that YabA influences DnaA binding at these sites (Merrikh and Grossman 2011; Scholefield and Murray 2013) and it's possible that omission of the downstream DnaA-boxes influences the effect YabA has on P*dnaA* expression.

Broader effects of YabA in an *oriC* background.

Using the *oriN* background allows us to look at the direct effects of YabA on transcriptional targets of DnaA. However, it does not reflect the effects that YabA has on gene expression in a normal *oriC* background. We measured effects on gene expression from P*sda* and P*ywlC* ±*yabA* in an *oriC* background and were surprised to find that YabA had an apparent activating effect towards DnaA as a transcription factor (Fig. 3). Given the results in the *oriN* background, the apparent effects of YabA on DnaA-mediated gene expression in an *oriC* background are likely the result of changes in either expression of *dnaA,* replication initiation, or both. The different apparent effects of YabA highlight the confounding effects changes in DNA replication and gene expression can have on the cell. Although the *oriC* background represents the overall impact YabA has on gene expression, it is clear the individual roles it plays have far reaching effects throughout the cell. We have performed a preliminary analysis of the effect of YabA on gene expression, but plan to expand this study to more targets of DnaA, as well as to look at more conditions and backgrounds, to pin down the precise mechanism and effects of YabA on gene expression.

Methods

Media and growth conditions

Cells were grown with shaking at 37°C in S7 defined minimal medium with MOPS (3-(N-morpholino) propanesulfonic acid) buffer at a concentration of 50 mM rather than 100 mM supplemented with 1% glucose, 0.1% glutamate, trace metals, 40 μg/ml phenylalanine, and 40 ug/ml tryptophan (Jaacks, Healy et al. 1989). Standard concentrations of antibiotics were used when appropriate (Harwood and Cutting 1990). To induce expression of *dnaA* from the LacI-repressible, IPTG-inducible Pspank, varied concentrations of IPTG were used as specified.

Strains and alleles

B. subtilis strains were derived from JH642 (*pheA1 trpC2*) (Perego, Spiegelman et al. 1988), are listed in Table 2, and were constructed by natural transformation using genomic DNA.

lacZ fusions. The promoter-*lacZ* fusions were constructed using Gibson isothermal assembly (Gibson, Young et al. 2009), and transformed into AG174 competent cells to facilitate double crossover at *amyE*. The isothermal assemblies were constructed using pDG268 as a template for *lacZ*, homology to *amyE* for double crossover, and a chloramphenicol resistance cassette (*cat*) (Antoniewski, Savelli et al. 1990). Promoters were amplified using AG174 genomic DNA and assembled with pDG268. Primers used to amplify the promoter regions with overlap for isothermal assembly are as follows: oLKM54 (5'-CGTCAGTAACATTAATGGGAGAGGCACCTCC-3') and oLKM11 (5'-TTGCCGATGATTTTTTAACAAAACCCGCACCCTTC-3') for MEA446 (P*sda-lacZ*); oLKM60 (5'- GTCAGTAACTGCTTGTGCAATTTGTGGATC-3') and oLKM20 (5'-GCCGATGATCCGGAGGAGCTGTTTTCTTC-3') for MEA448 (P*ywlC-lacZ*); oLKM52 (5'-CGTCAGTAACTTCCATCTTCCGGCACGTC-3') and oLKM8 (5'-

TAAGGGTAACTATTGCCGATGATGGAATAGCTGTTAAAGACAGTCTTAC-3') for MEA449 (P*dnaA-lacZ*).

oriN. MEA183 was constructed by introducing the heterologous origin and initiator (*oriN/repN;* Hassan, Moriya et al. 1997) in place of *oriC* and introducing a constitutive promoter to drive expression of *dnaN*. The constitutive promoter was Ppen (Yansura and Henner 1984) with the following sequence replacing the -10 to -35 box (5'-GTTGCATTTATTCTTAGATAGTGTAATACT-3'). The various fragments were amplified by PCR and assembled using isothermal assembly (Gibson, Young et al. 2009). The fragments were amplified using the following primers and templates: upstream *dnaA* was amplified using CLO390 (5'- TACGCCAACCATACTTAATAGCA-3') and oMEA79 (5'-CGCAACTGTCCATACTCTGATGTCTATTATGGTTGCAAGAAATAAAAG-3') a kanamycin cassette was amplified from pGK67 (Lemon, Kurtser et al. 2001) using oMEA80 (5'-CTTTTATTTCTTGCAACCATAATAGACATCAGAGTATGGACAGTTGCG-3') and oMEA81 (5'-GGAAAAGATTTAGGAGGAAGCTGAACCATTTGAGGTGATAGGTAAG-3'); *oriN/repN* was amplified from pDL110 (Hassan, Moriya et al. 1997) using CLO578 (5'-GTTGAACTAATGGGTGCAGCTTCCTCCTAAAATCTTTTCCCAT-3') and CLO579 (5'-CGTTTTTTCGGAAAGAAGAATATGTAGAAGAAGTTATTGATG-3'); Ppen2027 was amplified from CAL2072 using CLO580 (5'-CTTCTACATATTCTTCTTTCCGAAAAAACGGTTGCATTTA-3') and oMEA73 (5'-TCCTCCTAACGGATAATGTATGCAGCCGACTCAAACATCAAATC-3'); *dnaN* was oMEA96 (5'-GCTGCATACATTATCCGTTAGGAGGATAAAAATGAAATTCACGATTCAAAAAGATCGTC-3') and CLO569 (5'- CTGTATCAATTGAAATCGGATTTGC-3').

Pspank-*dnaA.* AMB89 was made by transforming pAMB82 into AG174 competent cells. pAMB82 was constructed by introducing the *dnaA* open reading frame into pMMB752 cut with EcoRI and SphI. pAMB82 was linearized with BsaI and introduced on the chromosome at the ectopic locus *lacA* with a tetracycline resistance marker.

β-galactosidase assays

Cultures were grown to mid-exponential phase and diluted back to OD 0.05 and grown to mid-exponential phase (OD 0.2-0.4) in defined minimal medium at 37°C. Growth was stopped by addition of toluene (1.5% final concentration). β-galactosidase specific activity ({ΔA_{420} per min per ml of culture per OD_{600}} × 1000) was measured as described (Miller 1972) after pelleting cell debris.

Western blots to measure DnaA protein

Cultures were grown to mid-exponential phase and diluted back to OD 0.05 and grown to mid-exponential phase (OD 0.2-0.4) in defined minimal medium at 37°C. Exponentially growing cells were lysed with lysozyme and a protease inhibitor cocktail (Sigma-Aldrich, P8849) at 37°C. Lysates were run on 12% polyacrylamide gel and transferred to a nitrocellulose membrane using the Trans-blot SD semi-dry transfer cell (Biorad). To represent the same number of cells in each lane, the amounts loaded in each lane were normalized to culture OD. All steps were performed at room temperature. According to the manufacturer's instructions, blots were blocked with Odyssey Blocking Buffer for 1 hr and incubated with primary antibody (rabbit anti-DnaA antibody 1:10,000 in Odyssey Blocking Buffer + 0.2% Tween) for 1 hr. The primary antibody to DnaA was affinity purified rabbit polyclonal antibody (Covance) made against purified DnaA. Blots were washed with PBST (phosphate-buffered saline + 0.2% Tween) for at least 5 x 5 min and then incubated for 30 min with secondary antibody (LiCor dye 800

goat anti-rabbit 1:10,000 in Odyssey Blocking Buffer + 0.2% Tween). Blots were imaged and quantitated on a LiCor scanner. Dilutions of AG174 lysates were used to generate a standard curve and determine the linear range of the fluorescence signal. Another identical gel was run and stained for total protein using Pierce Imperial Protein Stain (PI24615). DnaA signal was normalized to total protein as determined by Coomassie staining quantified on a LiCor scanner.

Chapter 4

Conclusions and Perspectives

DNA replication initiation is highly regulated in all domains of life. In *Bacillus subtilis*, as in other bacteria, initiation of DNA replication is primarily controlled by regulating the activity of DnaA. YabA is one of several proteins in *B. subtilis* that regulates the binding of DnaA at the origin, and deletion of *yabA* results in overinitiation of DNA replication (Merrikh and Grossman, 2011; Noirot-Gros, *et al.*, 2006; Hayashi, *et al.*, 2005). A temperature-sensitive point mutation in *dnaA, dnaA1*, also causes overinitiation under permissive conditions (Moriya, *et al.*, 1990). Interestingly, Δ*yabA* and *dnaA1* are synthetically lethal in rich media (LB) and cause severe overinitiation of replication, leading to replication fork collapse and the SOS response. I isolated suppressors of the synthetic lethal phenotype of Δ*yabA dnaA1*, hoping to elucidate additional mechanisms of DNA replication control. My results indicate that some of these suppressors are acting by directly limiting replication initiation, and others appear to be acting at the step of replication elongation. I also explored the effects of YabA on DnaA transcriptional targets, concluding that YabA has varied effects at different promoters.

I isolated suppressor mutations of a conditional synthetic lethal mutant, Δ*yabA dnaA1,* that overcome the severe overinitiation either by directly decreasing replication initiation or by increasing the rate of elongation. I isolated suppressors in the promoter region of *dnaC,* the gene for the replicative helicase. This mutation causes decreased levels of DnaC, which is sufficient to limit DNA replication initiation under high initiation conditions. Ten out of the 30 independently isolated suppressors had the same mutation, a single base pair deletion in the promoter region of *dnaC*. In *B. subtilis, dnaC* encodes the replicative helicase and is required for replication initiation and elongation. During initiation, DnaA recruits DnaD and DnaB, which recruit the helicase loader, DnaI (Smits, *et al.,* 2010). DnaI then loads DnaC monomer by monomer, forming a hexameric ring around the DNA, and the remaining replication machinery is then

recruited (Velten, *et al.,* 2003; Ioannou, *et al.,* 2006; Soultanas, 2012).

The suppressor mutation caused a ~50% decrease in levels of the helicase. We showed that in an otherwise wild type background, the P*dnaC*(T7) mutation was sufficient to lower *ori/ter* in fast-growing cells (LB) but not in slower growth conditions (minimal glucose). By using an IPTG-inducible version of *dnaC in trans*, we showed that decreased levels (~50% lower) of DnaC were sufficient to decrease replication initiation in an otherwise wild type background. Decreased levels of a specific replisome component, the replicative helicase (*dnaC*), are sufficient to decease replication initiation under fast growth or high initiation conditions. These data indicate that there is likely not an overabundance of helicase protein in the cell and that slight changes can affect replication initiation. This could suggest that there is something unique about the regulation of *dnaC* that contributes to regulating DNA replication, or simply that helicase is synthesized to roughly the required amount needed for full initiation potential at fast growth conditions. This effect on initiation appears to be specific to *dnaC*, although we can't rule out that other proteins might have a similar effect on DNA replication.

I isolated multiple suppressors in several other genes: *nrdR, relA, cshA,* and *ytmP*. These suppressors highlight the ability of *B. subtilis* to regulate DNA replication through multiple mechanisms and demonstrate how the cell can use changes in elongation, instead of just initiation, to overcome the overinitiation phenotype.

Several mutations were found in *nrdR*, the negative regulator for ribonucleotide reductase genes (RNR), and are likely acting on elongation. dNTPs are essential for DNA replication, but imbalances in pools can lead to increased mutagenesis and double-strand breaks due to impaired replication fork progress (Wheeler *et al.,* 2005; Guarino *et al.,* 2007; Gon *et al.,* 2011). *B. subtilis* only has a single class of RNR (class 1b), which is composed of the genes *nrdEF* (Scotti *et al.,*

1996; Härtig *et al.,* 2006). NrdR negatively regulates *nrdEF* in *B. subtilis* and a deletion in *nrdR* results in increased mutagenesis during both exponential growth and stationary phase (Castro-Cerritos *et al.,* 2017). We were able to determine that the *nrdR* suppressors were stimulating elongation, rather than decreasing replication initiation. Enhancing replication elongation could overcome detrimental effects of overinitiation by helping prevent fork collapse, or stimulating restart, to keep up with initiation. These suppressors highlight the link between *nrd* expression and DNA replication, an observation that has also been found in other organisms such as *E. coli* and *Saccharomyces cerevisiae* (Augustin *et al.*, 1994; Huang *et al.,* 1998).

Several suppressors were in *relA*, one of the genes responsible for (p)ppGpp synthesis, and thus the stringent response. We hypothesize that these suppressors are overcoming the hyperinitiation by stimulating replication elongation, as is the case with the *nrdR* suppressors. *B. subtilis* has three proteins involved in (p)ppGpp synthesis: RelA, YjbM, and YwaC. RelA is a bifunctional enzyme that can both synthesize and hydrolyze (p)ppGpp, YjbM and YwaC only synthesize (p)ppGpp (Wendrich, *et al.,* 1997; Srivatsan, *et al.,* 2008; Nanamiya, *et al.,* 2008). RelA is composed of three domains, an N-terminal hydrolase domain followed by a synthetase domain, and a C-terminal domain for binding the ribosome (Hogg *et al.,* 2004). In *E. coli,* (p)ppGpp inhibits replication initiation (Levine, *et al.,* 1991; Schreiber, *et al.,* 1995). However, in *B. subtilis,* (p)ppGpp inhibits replication elongation (Levine, *et al.,* 1991) by inhibiting the activity of the primase, DnaG (Wang, *et al.,* 2008), and this was also demonstrated later in *E. coli* (Maciag *et al.,* 2010).

All of the suppressors are located in either the synthetase domain or affect the entire C-terminal end of the protein (including the synthetase domain), the result of which should be a decrease in (p)ppGppp synthesis. Given our previous knowledge of (p)ppGpp inhibition of DNA

primase, we hypothesize that these suppressors may be acting at the step of elongation by relieving any inhibition on DnaG. Rather than speeding up replication, we suspect that decreased (p)ppGpp could be acting by preventing replication fork collapse or enhancing replication restart. The *nrdR* and *relA* suppressors highlight the fact that cells can overcome complications from overinitiation by acting at the step of elongation and emphasize the coordination between the two phases of DNA synthesis.

Several suppressor mutations were in *cshA*, a cold-shock DEAD-box RNA helicase. CshA is one of four DEAD-box RNA helicases in *B. subtilis*, along with CshB, DeaD, and YfmL (Lehnik-Habrink, *et al.,* 2013). Like other DEAD-box proteins CshA has two RecA-like domains and a C-terminal domain involved in protein-protein interactions that are necessary for its association with the RNA degradosome. The RNA degradosome is involved in RNA turnover and processing. In *B. subtilis,* it consists of a 6-protein complex: RNase Y (endoribonuclease), RNase J1 and J2 (both endoribonucleolytic and 5'-3' exoribonucleolytic activity), PnpA (exoribonuclease), and two glycolytic enzymes, enolase (Eno) and phosphofructokinase (PfkA) (Commichau, *et al.,* 2009). CshA is the major RNA helicase of the RNA degradosome and interacts with RNase Y, Eno, and PfkA through its C-terminal domain (Lehnik-Habrink, *et al.,* 2010). Another important role of DEAD-box helicases is that of ribosome biogenesis. Δ*cshA* results in a sharp decrease in the amount of mature 70s ribosomes as well as a decrease in total ribosomal subunits in the cell (Lehnik-Habrink, *et al.,* 2013).

Given the pleiotropic effects of a *cshA* deletion on ribosome assembly and RNA turnover/processing, there is not an obvious model for the mechanism of suppression involving *cshA*. It is important to note that there is a decrease in *dnaC* expression in a Δ*cshA* mutant. This may have a contribution to the decrease in *ori/ter*. However, it is likely not the sole factor, since

there is a greater decrease in *ori/ter* in Δ*cshA* than in the P*dnaC*(T7) mutant.

These *cshA* suppressors point to role for CshA involving DNA replication, presumably at the step of initiation. This could be due to the pleiotropic effects of *cshA*, both in regards to mRNA processing and ribosome biogenesis, effects on a specific replication factor (either mRNA stability of protein synthesis), or indicate another role of CshA in the cell.

These suppressors highlight the varied levels of control a cell uses to regulate DNA replication and the multiple mechanisms that can be employed to overcome a defect at initiation. The ability for the cell to overcome overinitiation by acting at the step of elongation shows the coordination between the different steps of DNA replication, as well as the importance for proper timing and control of this essential process.

In addition to the suppressor screen, my thesis explored the effect of YabA on DnaA as a transcription factor. DnaA can control gene expression by binding to DnaA-boxes throughout the genome, both activating and repressing genes (reviewed in Messer and Weigel, 1997; Goranov *et al.*, 2005). In addition to its autorepression, DnaA directly activates expression of *sda* and appears to directly repress the expression of *ywlC, ywcI-sacT, yydA,* and *trmE-noc* (Ogura *et al.*, 2001; Burkholder *et al.,* 2001; Hoover *et al.,* 2010; Breier and Grossman, 2009; Ishikawa *et al.,* 2007; Cho *et al.,* 2008; Goranov *et al.*, 2005). DnaA may have effects on several other operons, likely indirectly. All of these targets have clusters of DnaA-boxes located in their promoter regions (Breier and Grossman, 2009; Burkholder *et al.,* 2001; Fukuoka *et al.,* 1990; Ishikawa *et al.,* 2007; Smith and Grossman, 2015). It is unclear how DnaA binding is regulated at these promoters and how the organization of the DnaA-boxes influences expression.

The effects on DnaA-regulated gene expression have been previously studied. However, these studies have investigated DnaA-dependent gene expression in cells initiating from *oriC* or

in cells that overexpress *dnaN*, both of which may affect DnaA activity (Breier and Grossman, 2009; Goranov, *et al.,* 2005; Ishikawa *et al.,* 2007; Goranov, *et al.,* 2009; Washington *et al.,*). Increased levels of DnaN, which is known to interact with YabA, have been shown to decrease association of YabA at *oriC* and increase cooperative binding of DnaA at the origin (Goranov *et al.,* 2009; Merrikh and Grossman, 2011). The interaction of YabA with DnaN is thought to help relieve YabA inhibition on DnaA activity at *oriC,* but may also affect DnaA activity as a transcription factor (Merrikh and Grossman, 2011). The interaction of YabA and DnaN points to a mechanism of regulation that could coordinate the activities of DnaA to the replication cycle.

I used an *oriN* background to look at effects of YabA on gene expression, where *dnaA-dnaN* were no longer regulated by DnaA, thereby eliminating indirect effects of YabA on DnaA-mediated gene expression. I have shown that YabA negatively affects DnaA activation of *sda* but does not appear to influence DnaA repression of *ywlC*. Additionally, YabA appears to enhance DnaA repression at P*dnaA*. The full effects of YabA on DnaA are yet uncharacterized and may present broader connections between replication and gene expression. Further experiments are underway to develop a better understanding of YabA's role in gene expression.

Additionally, there are at least three other regulators of DnaA in *B. subtilis*. These regulators, SirA, Soj, and DnaD, each inhibit cooperative binding of DnaA to *oriC*, like YabA (Bonilla and Grossman, 2012; Scholefield *et al.*, 2012; Wagner *et al.*, 2009; Scholefield and Murray, 2013; Murray and Errington, 2008). They may prove to be other potential regulators of DnaA transcriptional activity and can be explored in addition to YabA. Uncovering regulators that affect DnaA as a transcription factor may highlight new mechanisms that the cell uses to couple DNA replication to other cellular processes through DnaA-mediated gene expression.

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