# Bacterial regulation of DNA replication elongation

by

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**Abstract**

Regulation of DNA replication is essential for faithful transmission of genetic material to a cell's progeny. In bacteria, DNA replication was mainly known to be regulated at the initiation phase. In 2007, a novel regulatory mechanism of DNA replication was identified, inhibiting the elongation phase. This regulation is mediated by a direct interaction between the stress-signaling nucleotide (p)ppGpp, and an essential replication enzyme primase (DnaG). (p)ppGpp is a ubiquitous nucleotide in bacteria and is best known for signaling during starvation, reprogramming the cellular machinery to inhibit cell. As part of this growth inhibition, (p)ppGpp rapidly halts replication forks in a reversible and nondisruptive manner upon starvation.

My work looks at the diverse effects of (p)ppGpp regulation of DNA primase, observing its effects on primer synthesis, primase processivity, and priming readthrough. I observe (p)ppGpp decreased primer synthesis, potentially through slowing primase down as well as limiting primase reassociating to the ssDNA template. I then saw reduced processivity of primase in the presence of (p)ppGpp. I then looked at (p)ppGpp inhibition of primase in conditions that more closely mimicked *B. subtilis* during periods of stress. Using low levels of GTP I examined primer synthesis when a pause site was introduced by starving the reaction for a necessary NTP. In the absence of (p)ppGpp I observed that DNA primase readthrough the pause. As (p)ppGpp was titrated into the reaction I observed a decrease in the priming readthrough, indicating (p)ppGpp regulation of DNA primase prevents DNA primase from continuing to synthesize even in the absence of essential substrates. Characterizing the diverse effects of (p)ppGpp regulation of DNA primase has provided insights into how, through inhibiting primase activity, (p)ppGpp may be halting the entire replication fork.

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## Chapter 1: Introduction

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**Importance of Replication**

DNA replication is essential for faithful transmission of genetic material to a cell's progeny. In both the enteric Gram-negative *Escherichia coli* and the soil-dwelling Gram-positive *Bacillus subtilis*, the essential proteins involved in DNA replication have been identified through classical genetics and biochemistry (Kornberg and Baker, 1992; Marians, 1992; Bruand et al., 1995; Dervyn et al., 2001; Barnes et al., 2002; Velten et al., 2003; Sanders et al., 2010). Replication requires the coordinated effort of a group of proteins, termed the replisome, which are functionally conserved across diverse organisms. The replisomes of *E. coli* and *B. subtilis* are comprised of more than 10 individual proteins, all of which are important for growth and, together, are capable of sustaining DNA replication *in vitro* (Table 1, Figure 1) (Beattie and Reyes-Lamothe, 2015).

Over the decades, the biochemical processes involved in DNA replication have been heavily investigated while the regulation of replication remains poorly understood. *E. coli* and *B. subtilis* have been subjected to many studies on their responses to environmental stresses. As such, these organisms are ideal systems in which to examine the coordination of DNA replication and environmental conditions which require regulation of replication. In this chapter I will delve into the components of DNA replication and their mechanisms, the existing knowledge surrounding production of the stress signaling nucleotide (p)ppGpp, and (p)ppGpp's regulation of cellular processes. I will then discuss what is known about (p)ppGpp's role in regulating DNA replication in *B. subtilis*. Lastly, I will discuss in detail the replication protein DNA primase, which is regulated by (p)ppGpp, and the importance of this regulation.

**Overview of replication**

The process of DNA replication can be broadly separated into three main stages: initiation, elongation, and termination. In both *E. coli* and *B. subtilis*, DNA replication commences at a single origin (*oriC*) on a single circular chromosome. Replication proceeds bidirectionally around the chromosome until the termini opposite the *oriC* are reached. Below I will discuss initiation and elongation of replication and the main players involved.

***Initiation***

Initiation of DNA replication has been best studied in *E. coli*. To initiate DNA replication, the duplex DNA at the origin (*oriC*) needs to be melted for replisome loading and assembly. Origin melting is achieved, in most bacteria, via the conserved AAA+ ATPase DnaA. DnaA recognizes and binds short, highly conserved, repetitive sequences within *oriC*, known as DnaA boxes (Cleary et al., 1982; Fuller et al., 1984; Bramhill and Kornberg, 1988; Schaper and Messer, 1995). There are eight DnaA boxes within the *oriC* region of the chromosome with varying affinities for DnaA, and when replication is about to commence, all eight boxes are bound by DnaA (Matsui et al., 1985; Mott and Berger, 2007). When DnaA is recruited to these boxes, protein-protein interactions between protomers allow DnaA to assemble into a helical filament (Erzberger et al., 2006). This filament is capable of opening double-stranded DNA just upstream of the DnaA box at a 13-mer AT-rich sequence known as the DNA unwinding element (DUE) (Speck et al., 1999; McGarry et al., 2004).

Once DnaA has opened the replication origin, two copies of the replicative helicase are then recruited onto the ssDNA with the assistance of the helicase loader (Kobori and Kornberg, 1982; Koboris and Kornberg, 1982; Funnell et al., 1987; Soultanas, 2002). Loading of the helicase occurs in the absence of DNA through six helicase loader protomers latching onto, and

cracking open, a helicase hexamer using an extended N-terminal domain (Arias-Palomo et al., 2019). The interaction between the helicase and helicase loader is stabilized through nucleotide-dependent ATPase interactions and when the complex binds DNA, the helicase loader hydrolyzes the ATP which allows the replicative helicase to isomerize into a closed state that can then bind the DNA primase (Wahle et al., 1989a, 1989b; Arias-Palomo et al., 2019). Once the helicase has been properly situated on the ssDNA, it migrates from the origin.

While the overall process described above is well conserved, there are some minor mechanistic differences in low G-C firmicutes like *B. subtilis*. For initiation, the primary difference is that the replicative helicase, DnaC, requires the proteins DnaD, DnaB, and DnaI (Karamata and Gross, 1970; Bruand et al., 1995; Ogura et al., 2001; Soultanas, 2002). It is thought that DnaD works together with DnaA to melt the dsDNA at the origin (Martin et al., 2019), while DnaB appears to have a role in helicase loading (Bruand et al., 2005). The mechanism and function of these proteins still remains unknown, however it is certain that during initiation in *B. subtilis*, DnaD, DnaB, and DnaI-DnaC assemble at the origin of replication in a sequential manner, followed shortly by the DNA primase DnaG, to complete loading of the primosome complex (Smits et al., 2010).

***Elongation***

The first step to DNA elongation is the assembly of the primosome. After the helicase is loaded, the DNA primase associates to complete assembly of what is known as the primosome (McMacken et al., 1977; Bruand et al., 1995; Fang et al., 1999; Liu et al., 2013). The primary function of the primosome is to lay down RNA primers from which the main DNA polymerase,

unable to replicate *de novo*, can begin duplication (Stukenberg et al., 1991; Kornberg and Baker, 1992).

Upon finishing priming at the origin, the DNA polymerase needs to be loaded at the replication fork. In bacteria, the primary DNA polymerase is comprised of a variety of subassemblies (Table 1). There is a catalytic core responsible for the polymerizing and exonuclease functions, made up of three subunits: α (polymerization), ϵ (exonuclease), and θ (proofreading). DNA polymerase is tethered to the DNA by the β clamp, and is loaded on via the clamp loader complex (γ) comprised of γχδδ’Ψ (Kornberg and Baker, 1992).

The DNA polymerase is recruited by the β processivity clamp (DnaN), which is loaded onto the DNA by the pre-initiation complex comprised of two β subunits loaded onto the DNA by the γ subunit via ATP hydrolysis (Stukenberg et al., 1991; Kornberg and Baker, 1992). The clamp loader makes several protein-protein contacts at the replisome to facilitate clamp loading (via an interaction with single-stranded binding proteins) and physically couple DNA polymerases to each other and the replicative helicase (Shereda et al., 2008).

Replication then proceeds in a semi-continuous manner, due to the antiparallel nature of DNA (Kornberg and Baker, 1992). The leading strand is synthesized continuously, while the lagging strand is synthesized discontinuously. The lagging strand is synthesized in 1 – 2 kb segments known as Okazaki fragments and requires continual primer synthesis (laid down by DnaG), clamp loading, and polymerase loading at the beginning of each fragment (Kornberg and Baker, 1992). Every RNA primer that is laid down then needs to be removed through the coordination of RNaseH I (degrades the RNA primer), DNA polymerase I (synthesizes the DNA

where the RNA primer once was), and ligase to glue the fragments together, completing the lagging strand (Kornberg and Baker, 1992).

During DNA replication, the lagging strand is susceptible to damage due to exposure of the single-stranded DNA. Tetrameric SSB coat the lagging strand, acting both as a protector of the vulnerable DNA and as a recruitment hub through its C-terminal domain, binding many replisome components including DNA primase and the DNA polymerase III subunit χ (Shereda et al., 2008). In some bacteria such as *B. subtilis*, lagging strand synthesis is further complicated by requiring an additional DNA polymerase, DnaE, that synthesizes a short DNA fragment off the RNA primer before hand-off to the major, high-fidelity polymerase, PolC (Sanders et al., 2010; Rannou et al., 2013). This RNA/DNA hybrid 'initiation primer' is then extended upon hand off to PolC, thought to be stimulated by DnaG and SSB (Sanders et al., 2010).

Though more is known than before, the dynamics and signaling to control Okazaki fragment length and when to start the next Okazaki fragment have not been completely worked out. It has been shown that the presence of the primer is a critical signal (Spiering et al., 2017). In phage it has been shown that the primer-primase complex has a long residence time on the DNA, and it is suspected that the DNA polymerase will collide with this complex, signaling for the DNA polymerase to finish synthesis of the Okazaki fragment and then be recycled for synthesis of another Okazaki fragment (Spiering et al., 2017). This triggers the polymerase to move off the DNA template to a newly loaded β clamp at the template-primer junction of the next Okazaki fragment (Stukenberg et al., 1994; Su 'etsugu and Errington, 2011), since in *E. coli* the unloading of the clamp is coupled to initiation of a new Okazaki fragment .

The next sections will cover how the nucleotide alarmone, (p)ppGpp, regulates replication when cells are starved for nutrients, and the importance of this regulation.

**The alarmone (p)ppGpp**

The life of bacteria is feast or famine, requiring bacterial cells to rapidly adapt to changing environments in order to survive. In sudden nutrient downshifts bacteria need to be able to rapidly adapt so they do not consume limited resources and starve. They survive through the production of the nucleotide alarmones guanosine tetraphosphate and guanosine pentaphosphate, collectively known as (p)ppGpp. (p)ppGpp is a pleiotropic stress alarmone that globally reprograms cellular processes in bacteria. Originally found to rapidly accumulate in cells starved for amino acids, (p)ppGpp was shown to inhibit cell growth (Cashel and Gallant, 1969). Subsequent work has also shown (p)ppGpp to accumulate in other stresses, including nitrogen and carbon starvation, and shifts in oxygen, light, temperature, and pH (Gallant et al., 1977; Glass et al., 1979; Flardh et al., 1994; Wells and Gaynor, 2006; Brown et al., 2014; Hood et al., 2016; Irving and Corrigan, 2018).

At a broad level, (p)ppGpp regulates the same major global processes in a cell, such as transcription, translation, replication, and metabolism. Subsequent studies on the function of (p)ppGpp have shown its importance as a pleiotropic regulator to rapidly modify numerous cellular processes in order to promote cell survival. While my work focuses on DNA replication, it is also important to understand how (p)ppGpp promotes adaptation and survival in bacteria more generally. In the following sections I will briefly summarize the current knowledge about (p)ppGpp physiology.

**(p)ppGpp synthesis**

The synthesis and hydrolysis of (p)ppGpp is dependent on (p)ppGpp synthetases and hydrolases sensing environmental changes. pppGpp and ppGpp are synthesized from one molecule of ATP and one molecule of GTP or GDP, respectively (Haseltine et al., 1972; Haseltine and Block, 1973; Hara and Sy, 1983). Synthetases transfer a pyrophosphate from ATP to the 3'-hydroxyl of GDP/GTP, releasing ppGpp/pppGpp and AMP as products. Conversely, (p)ppGpp hydrolases remove the pyrophosphate from the 3'-moiety of the ribose (Hogg et al., 2004; Steinchen and Bange, 2016; Ronneau and Hallez, 2019). Synthetases and hydrolases are classified into two main classes: RelA/SpoT-like homologs (RSHs) and small alarmone synthetases (SASs) (Figure 2). RSHs are multidomain proteins that contain both synthetase and hydrolase domains as well as additional regulatory domains. SASs, as suggested by the name, contain only a single synthetase domain and can be regulated at both expression and activity levels (Steinchen and Bange, 2016). In Firmicutes like *B. subtlis*, pppGpp is the predominant nucleotide produced during nutrient starvation, though depending on the stress ppGpp can be predominant. In Gram-negative proteobacteria, there is an enzyme that converts pppGpp to ppGpp, resulting in ppGpp prevailing over pppGpp (Hara and Sy, 1983).

***RelA***

Bacteria encode either one or two copies of the multidomain RSHs (Atkinson et al., 2011). In *B. subtilis* and other Firmicutes, RelA is the only RSH and is a bi-functional (p)ppGpp synthetase and hydrolase (Ronneau and Hallez, 2019). *E. coli* and other Proteobacteria have a RelA with (p)ppGpp synthetase activity only. However, they also have another RSH, SpoT, which is a bi-functional synthetase and hydrolase. Rel enzymes contain six domains: i) hydrolase, ii)

synthetase, iii) TGS (ThrRS, GTPase, and SpoT), iv) AH (α-helical domain), v) ZFD or RIS (CCHC zinc-finger domain or ribosome inter-subunit domain), and vi) ACT or RRM (aspartate kinase, chorismate mutase and TyrA or RNA recognition motif) (Atkinson et al., 2011; Brown et al., 2016; Steinchen and Bange, 2016).

Previous work in *E. coli* has supported a model where RelA will move between ribosomes to sense deacylated tRNAs and synthesize (p)ppGpp while bound to a stalled ribosome (Li et al., 2016; Gratani et al., 2018). Details of this model have been unveiled through a series of cryo-EM studies of the *E. coli* RelA-ribosome complex (Arenz et al., 2016; Brown et al., 2016; Loveland et al., 2016). Typically, RelA adopts an elongated and open conformation on the ribosome, and is capable of binding without a deacylated tRNA, though this ribosome-RelA interaction is stabilized in the presence of an uncharged tRNA. RelA discriminates between charged and uncharged tRNAs through interactions with the 3'-hydroxyl of the tRNA acceptor end, which interacts with the TGS domain when it is not bound to an amino acid. If the tRNA was aminoacylated there would be a steric clash with the TGS domain of RelA, preventing proper establishment of the A/R-tRNA site (Haseltine and Block, 1973; Brown et al., 2016). Together, these structures provide evidence to support a model where RelA uses the ribosome together with uncharged tRNAs to respond to amino acid starvation.

***Small alarmone synthetases***

Small alarmone synthetases (SASs) are enzymes containing only a (p)ppGpp synthetase domain. These enzymes are predominantly found in Firmicutes (Ronneau and Hallez, 2019). *B. subtilis* encodes two SASs, SAS1 (RelQ/YjbM) and SAS2 (RelP/YwaC), both containing a single synthetase domain that shares essential residues with the synthetase domain of RelA (Srivatsan

and Wang, 2008; Steinchen and Bange, 2016). While pppGpp is the major product of RelA, work has shown that the SASs in *B. subtilis* preferentially synthesize ppGpp from GDP (Steinchen et al., 2015).

A recent study has proposed the mechanism of (p)ppGpp synthesis by SAS1 is a two-step catalytic model whereby the ATP binds initially, followed by the binding of GDP or GTP (Steinchen et al., 2015). A crystal structure of SAS1 shows that is forms a tetramer, with two pppGpp molecules binding at an allosteric site between two dimers. Modifications of this allosteric site prevent pppGpp binding and consequently synthetase activity, showing that pppGpp binding activates SAS1 (Steinchen et al., 2015). This allosteric activation of SAS1 is specific for pppGpp rather than ppGpp. The current hypothesis is that RelA production of pppGpp during the stringent response in turn activates SAS1 in a positive feedback manner to promote more rapid accumulation of (p)ppGpp.

**(p)ppGpp physiology**

***(p)ppGpp regulation of GTP synthesis***

Among the first set of proteins identified as direct targets of (p)ppGpp were enzymes involved in nucleotide metabolism. The necessity of this regulation was emphasized in a study showing that a decrease in GTP levels is essential for *B. subtilis* to survive amino acid starvation (Kriel et al., 2012). GTP depletion in cells first occurs by production of (p)ppGpp, as GTP is a substrate. Several enzymes are then bound by (p)ppGpp to inhibit GTP biosynthesis. In *E. coli* the enzyme GuaB, which catalyzes the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP), was shown to directly bind (p)ppGpp (Gallant et al., 1971; Pao and Dyes, 1981). Work in *B. subtilis* found that GuaB retains its activity even at 300-500 µM

(p)ppGpp, suggesting that GuaB is unlikely to be a primary (p)ppGpp target in *B. subtilis* (Kriel et al., 2012).

Instead, a more recent study has shown that in *B. subtilis* (p)ppGpp targets the purine synthesis enzyme guanylate kinase (GMK), which converts GMP to GDP, but (p)ppGpp is unable to bind *E. coli* GMK (Liu et al., 2015). Regulation of GMK in *B. subtilis* is critical for adaptation to nutrient starvation. Additional work in *B. subtilis* has shown that (p)ppGpp also binds the purine salvage enzyme hypoxanthine-guanine phophoribosyltransferase (HPRT) that converts hypoxanthine or guanine bases to IMP or GMP nucleotides, respectively (Anderson et al., 2019). (p)ppGpp has also been shown to bind HPRT in *E. coli*, suggesting that regulation of GTP levels during stress is important across bacterial species (Hochstadt-Ozer and Cashel, 1972).

Though regulation of nucleotide synthesis can differ slightly across bacterial species, it is clear that (p)ppGpp regulation of nucleotide metabolism is important for survival during stress. In both *E. coli* and *B. subtilis*, reducing nucleotide synthesis reduces the cell's overall capacity for transcription, translation, and replication and ultimately can help conserve a cell's energy during stress adaptation.

***(p)ppGpp regulation of transcription***

The best characterized (p)ppGpp target is the RNA polymerase (RNAP) in *E. coli* where (p)ppGpp binds two distinct sites and, together with the transcription factor DksA, destabilizes the promoter open complex (Barker et al., 2001; Ross et al., 2013, 2016; Gourse et al., 2018). This allows for decreased transcription of ribosomal RNA (rRNA) operons, reducing ribosome synthesis during stress. These RNAP binding sites are not conserved outside of proteobacteria, with other bacteria instead requiring alternative ways to regulate costly transcription. In *B.*

*subtilis*, for instance, rRNA transcription is heavily influenced by GTP, which is the initiating nucleotide for all of its rRNA operons (Krásny and Gourse, 2004).

Not all gene transcription is downregulated by (p)ppGpp. In fact, (p)ppGpp has been shown to be responsible for activating genes, such as amino acid biosynthesis genes. For example, in *E. coli* (p)ppGpp directly activates transcription from amino acid biosynthesis operons through its interactions with RNAP and DksA (Paul et al., 2005; Gourse et al., 2018; Sanchez-Vazquez et al., 2019). Firmicutes, though, rely on indirect regulation of rRNA transcription through (p)ppGpp regulation of GTP levels. A transcription factor in Firmicutes, CodY, regulates over 200 genes, among which are branched chain amino acid biosynthesis genes (Levdikov et al., 2006; Brinsmade et al., 2010; Gaca et al., 2015). CodY is activated through direct binding to GTP, thus (p)ppGpp can indirectly alter the CodY regulon by reducing GTP levels (Handke et al., 2008; Geiger and Wolz, 2014).

Other amino acid biosynthesis genes can also be affected during starvation in *B. subtilis* in a CodY-independent manner. For instance, decreases in GTP levels is often associated with an increase in ATP levels, promoting expression of many $\sigma^A$-dependent promoters which utilize ATP as the initiating nucleotide (Krásný et al., 2008). Additionally, decreasing rRNA transcription allows for more RNA polymerases to be available to transcribe other genes, such as biosynthesis genes like *ilv* and *leu* (Tojo et al., 2008, 2010). Altogether, this shows that (p)ppGpp has a critical role in deactivating rRNA transcription and upregulating amino acid biosynthesis genes to adapt to nutrient limitation.

***(p)ppGpp regulation of translation***

Translation is the largest energy consumer during cellular growth. In addition to regulating transcription of ribosomal RNA, it has been shown that (p)ppGpp inhibits translation initiation, elongation, and ribosome assembly (Bennison et al., 2019). (p)ppGpp inhibits the translation initiation factor IF2, and the elongation factors Ts, Tu, and G (Miller et al., 1973; Hamel and Cashel, 1974; Rojas et al., 1984; Milon et al., 2006; Mitkevich et al., 2010; Kanjee et al., 2012). The inhibition of ribosome assembly stems from (p)ppGpp inhibition of GTPases, which interact with the 70S ribosomes or the individual subunits in a GTP-dependent manner (Britton, 2009; Corrigan et al., 2016). These GTPases function at all levels of ribosome maturation, thus they can act as checkpoints during the ribosome maturation process (Britton, 2009; Bennison et al., 2019).

***(p)ppGpp regulation of DNA replication***

***Regulation of DNA replication initiation***

Several decades ago it was observed that during stringent conditions in *E. coli* new rounds of replication were blocked (Levine et al., 1991; Schreiber et al., 1995; Ferullo and Lovett, 2008). For many years it was assumed that this decrease was due to a reduction in *dnaA* transcript levels (Schreiber et al., 1995). Recently though, a more detailed explanation has emerged. During normal cell growth, transcription occurs frequently at *oriC*, introducing negative supercoiling which promotes replication initiation (Figure 3ii) (Lark, 1972; Liu and Wang, 1987; Asai et al., 1990; Theisen et al., 1993; Magnan and Bates, 2015). However, by reducing transcription, (p)ppGpp is indirectly creating an unfavorable energy landscape at *oriC*, and increasing the energy required for DNA melting (Figure 3iii) (Kraemer et al., 2019).

***Regulation of DNA replication elongation***

Though replication initiation was found to be inhibited after amino acid starvation in Gram-negative bacteria such as *E. coli*, the same was not observed in Gram-positive bacteria such as *B. subtilis*. In 2007 a novel regulatory mechanism of DNA replication was identified that inhibited the progression of the replication fork at any time during replication elongation (Figure 3iv) (Wang et al., 2007). Treatment of *B. subtilis* cells with arginine hydroxamate (RHX) to mimic nutrient starvation and induce (p)ppGpp synthesis resulted in rapidly halting replication forks in a reversible and nondisruptive manner (Wang et al., 2007). Regardless of when RHX was added, cells would almost immediately inhibit fork progression (Wang et al., 2007).

A critical part of fork progression and halting it is preserving fork stability. Unstable replication forks collapse and can be characterized by the accumulation of abnormal replication intermediates, bound by the DNA damage repair protein recombinase A, or RecA. RecA binds to exposed single-stranded DNA or double-strand breaks and exchanges strands with a similar sequence in dsDNA (Cox, 2007; Bell and Kowalczykowski, 2016; Del Val et al., 2019). Fluorescently labelled RecA can be used to determine whether DNA damage is present when systems are disrupted (Renzette et al., 2005). During nutrient starvation no *B. subtilis* cells were observed to have RecA foci when compared cells treated with DNA damaging agents (Wang et al., 2007). It was later seen that cells unable to synthesize (p)ppGpp formed RecA foci upon RHX treatment (unpublished), clearly showing that (p)ppGpp can stop the replication fork but through an unknown mechanism that allows it to retain stability.

Importantly, it was found that (p)ppGpp was binding to DNA primase in order to non-disruptively halt the replication fork (Wang et al., 2007). Since primase is essential for lagging strand synthesis it is an ideal target for regulation as its inhibition can decrease both lagging and leading strand synthesis of the chromosomal DNA (Pagès and Fuchs, 2003). Regulation of primase could also result in allosteric inhibition of other proteins in the replication complex as primase is known to directly interact with several other proteins (Arai et al., 1981; Sun and Nigel Godson, 1996; Tougu and Marians, 1996a, 1996b; Georgescu et al., 2014; Chodavarapu et al., 2015). For instance, primase and the replicative helicase directly interact and regulate each other's activity so it is possible that inhibiting primase activity may result in inhibition of helicase activity since additional data suggested that (p)ppGpp was able to inhibit helicase-dependent primosome activity *in vitro* (Johnson et al., 2000; Van Eijk et al., 2016).

Additional work then found that (p)ppGpp inhibited DNA primase in *E. coli* similarly to in *B. subtilis*, though less potently *in vivo* than in *B. subtilis* (Figure 3iii) (Maciag et al., 2010; Rymer et al., 2012; Denapoli et al., 2013; Maciag-Dorszyńska et al., 2013). A structural study of the RNA polymerase domain of *S. aureus* DNA primase showed that (p)ppGpp inhibits primase by binding to the active site in a manner similar, but not identical, to the NTP substrates (Rymer et al., 2012). The structure shows that primase activity is impeded by (p)ppGpp blocking entry of an incoming NTP as well as interfering with the binding of an initiating 5'-NTP or the extensible 3'-end of the RNA-DNA heteroduplex (Rymer et al., 2012).

**DNA primase**

The DNA primase (DnaG) is a single polypeptide of around 60 kDa that is responsible for priming the DNA by laying down a short RNA chain from which DNA polymerase can then

extend (Rowen and Kornberg, 1978). DnaG was discovered alongside six other genes done in a study with *E. coli* strains having temperature sensitive DNA synthesis mutations (Carl, 1970). Since the discovery of DNA primase, primer synthesis has been shown to play a critical role in the regulation of replicative processes such as replisome assembly (Makowska-Grzyska and Kaguni, 2010), the control of fork progression (Salzberg et al., 1998; Lee et al., 2006; Chintakayala et al., 2009), the regulation of Okazaki fragment length (Wu et al., 1992) and replication restart (Heller and Marians, 2006). Thus, primer synthesis and DnaG itself seems to be a central node to regulate bacteria replication.

Primase is a member of a class of enzymes known as nucleic-acid polymerases. Polymerases can be further classified into 13 families, according to their sequence or structural similarity: 12 families of single-subunit polymerases, and a single family of multi-subunit RNA polymerases (Cramer et al., 2008; Johansson and Dixon, 2013). One of these single-subunit polymerase families is the DnaG-type primases, using the same substrates and performing the same reaction as the other single-subunit polymerases, but sharing no other similarities to the canonical polymerases (Johansson and Dixon, 2013; Guilliam et al., 2015). The differences between DnaG-type primases and the canonical polymerases begins with the global domain structure of DnaG. Polymerases typically have a classic fingers-palm-thumb domain organization, while DnaG is comprised of three other types of domains: an N-terminal Zinc Binding Domain (ZBD), a central active site containing RNA Polymerase Domain (RPD), and a C-terminal Helicase Interaction Domain (HID/CTD) (Figure 4) (Frick and Richardson, 2001). Though these domains would appear to be functionally related to other polymerases, the elements themselves are structurally, and perhaps functionally, unrelated. Specifically, the fingers, palm,

and thumb domains of canonical polymerases work together in substrate recognition and catalysis (Brautigam and Steitz, 1998), whereas the DnaG domains are simply loosely tethered to each other and appear to have distinct functions (Larson et al., 2010).

**DNA primase domains**

DNA recognition and specificity are conferred through the N-terminal ZBD, a domain with a single, globular fold that resembles the zinc-ribbon motifs found in other nucleic acid binding proteins (Pan and Wigley, 2000). The precise mechanism by which the ZBD carries out this recognition remains poorly characterized, however. The formation and extension of RNA primers occurs in the central RPD. There are no similarities to this domain and that catalytic palm domain of canonical polymerases. Instead it is more closely related to topoisomerases, OLD family nucleases, and RecR via the TOPRIM fold (Aravind et al., 1998; Keck et al., 2000; Podobnik et al., 2000). Several RPD structures have been solved that illustrate a high degree of structural conservation between bacterial DnaGs (Keck et al., 2000; Podobnik et al., 2000; Rymer et al., 2012).

Lastly, the C-terminal HID directs the interaction between primase and the replicative helicase by binding to the N-terminus of the helicase (Bailey et al., 2007; Chintakayala et al., 2008). Crystal structures from three bacterial species have shown that the HID is a helical domain structurally similar to the N-terminal domain of the replicative helicase (Oakley et al., 2005; Syson et al., 2005; Abdul Rehman et al., 2013). The HID is poorly conserved among bacterial species, which could reflect the high specificity of the primase-helicase interaction (Koepsell et al., 2006; Larson et al., 2010).

**DNA primase substrates**

In addition to the structural differences, DnaG-type primases also differ enzymologically from canonical polymerases. At least two unique characteristics set them apart: start-site sequence specificity and autoregulation of product length. In isolation, *E. coli* DnaG synthesizes 8-12 nucleotide long primers (Kitani et al., 1985), using only the middle base of the CTG or CAG triplet sequences as a start site (Swart and Griep, 1995; Khopde et al., 2002). In *B. subtilis*, isolated DnaG can synthesize primers as short as 5-7 nucleotides long, using the middle base of the CTA or CTT triplet sequences as a start site (Larson et al., 2010; Rannou et al., 2013). In all instances, the 3' base in the recognition sequence is omitted from the primer product. Primase catalyzes the synthesis of short RNA oligonucleotides through a series of steps: (i) DNA template binding, (ii) NTP binding, (iii) initiation, (iv) elongation to a functional primer, and (v) primer handoff to DNA polymerase. The RNA polymerase domain (RPD) then binds NTPs – first it binds the NTP needed for the 3' end of the initial dinucleotide (GTP in the recognition sequences above) before binding the nucleotide needed for the 5'-end of the primer (ATP in both cases above). Initiation begins upon formation of the dinucleotide and release of the inorganic pyrophosphate ($PP_i$) from the two bound NTPs. The growing nucleotide chain then moves to the site originally occupied by the ATP so that next nucleotide to be added on the 3'-end can be recruited.

The sequence specificity and length regulation has been found to be a highly conserved feature of the DnaG-type primases (Koepsell et al., 2006; Larson et al., 2010). Initial work looking at sequence specificity and length regulation originated from a variant of T7 gp4 that lacked the ZBD (Bernstein and Richardson, 1988). The truncated protein retained its priming

ability (albeit at a reduced level), but no longer was able to discriminate against primer sequence and length. An identical role for the *E. coli* ZBD was later established through a series of studies (Sun et al., 1994; Griep and Lokey, 1996; Powers and Griep, 1999). Recent work has shown the first structure of the ZBD directly interacting with the DNA template, showing that the ZBD stays bound to the initiation site and radially moves away from the replisome ring as the primer is elongated (Hou et al., 2018). This movement is limited by the covalent connection of the ZBD and RPD, limiting the primer length. As the ZBD moves away from the active site, binding of the DNA in the active site is destabilized, eventually disengaging the primer-template hybrid from the RPD.

**DNA primase interactions with the replicative helicase**

The interaction with the replicative helicase is critical, as the helicase separates the duplex DNA into individual strands and most polymerases require a single-stranded template. DnaG-type primases are no different, which has likely led to the evolution of this direct interaction with the replicative helicase (Marians, 1992; Wu et al., 1992; Frick and Richardson, 2001; Wang et al., 2007). Previous work has suggested that by interacting with the helicase, primase is limited to priming only at the replication fork, rather than at any available ssDNA with the appropriate sequence (Corn and Berger, 2006). Additional work has also suggested that this interaction couples primer synthesis to the process of replication in general (Zhu et al., 2009).

It is worth noting that DnaGs from mesophilic bacteria such as *E. coli* and *B. subtilis* form only transient interactions with DnaB (the replicative helicase), observable indirectly through activity assays (Johnson et al., 2000; Koepsell et al., 2006). One reason for this may be due to

the repeated cycles of primase-helicase association and disassociation, an important feature in replication systems with separate proteins (Corn and Berger, 2006; Lee et al., 2006). For instance, intermediate complexes form with other replisome components over the course of replication, which could necessitate dissociation of the primase from the helicase (Yuzhakov et al., 1999). For example, in bacteria it was originally found that a three-point-switch between primase, SSB, and the χ subunit of the clamp loader complex guides the primer to the replicative polymerase, since it has been demonstrated the Pol III cannot assemble onto primed DNA while the primase is still bound (Yuzhakov et al., 1999). To load onto a primed template the clamp loader unit makes contact with SSB, which also interacts with primase (Shereda et al., 2008). It is suspected that the χ subunit displaces the primase, allowing for Pol III to be loaded and extend the RNA primer. (Yuzhakov et al., 1999). However, this model has not been supported by recent work, which instead suggests the interaction between χ and SSB is more important in the coupling of leading and lagging strand synthesis (Georgescu et al., 2014). Beyond restricting priming events to occur only at the replication fork along with coupling primer synthesis to primer hand-off, this primase-helicase association greatly stimulates the rate of primer synthesis (Johnson et al., 2000; Koepsell et al., 2006) and reduces sequence specificity for the priming start site (Johnson et al., 2000).

**Interaction of the RPD with the ZBD**

The primase-helicase interaction has been suggested to also promote an association of the DnaG N-terminal ZBD with the central RPD of DnaG *in trans*. Briefly, an *in trans* interaction is where the ZBD of one primase monomer would associate with the RPD of a second primase monomer. This *in trans* interaction has been shown to lead to additional stimulation of the

primer synthesis rate, and further ensures that primer synthesis is initiated at the appropriate sequence (Corn et al., 2005). Further work demonstrated that this *trans* interaction is functionally important for a bacterial primase-helicase array, shown using chimeric bacterial enzymes. In this study, the ZBD from one species was swapped onto the RPD of a different species and vice versa. The chimeras only achieved optimal activity in the presence of helicase, using mixtures of the chimeric enzymes (Larson et al., 2010).

It is unlikely that bacterial primases engage in a *cis* (where the ZBD of one primase monomer interacts with the RPD it is tethered to) interaction. In both *E. coli* and *A. aeolicus* DnaG, the ZBD and RPD have a compact conformation that they adopt. It is possible for them to have an extended conformation in high salt, but it is unclear from a crystal structure of *Aa*DnaG ZBD-RPD how the ZBD could associate with the RPD (Corn et al., 2005). There is also DNA binding evidence that supports two DnaG protomers associating on a single template strand (Khopde et al., 2002). Lastly, the structural datum suggests that the distance between the ZBD and RPD increases during primer extension, making a *cis* interaction unlikely (Hou et al., 2018).

The *trans* interaction between the ZBD and the RPD is one of the biggest differences between the DnaG family of polymerases and the canonical polymerases. There are still outstanding questions about how this *trans* interaction assists with primer synthesis, substrate recognition, and catalysis. What is clear though is that the unique manner of this collaboration is unlike those of the fingers and thumb domains of other single-subunit polymerases.

There are approximately 50 to 100 copies of primase in *E. coli*, and it is one of the only proteins to regularly disassociate from the replication fork (Kornberg and Baker, 1992). This

disassociation makes it an ideal target for regulating DNA replication (Trakselis et al., 2003). On top of its ability to disassociate from the fork, primase is known to interact with several proteins, most notably the replicative helicase and single-stranded binding proteins (SSB) (Kornberg and Baker, 1992).

**Importance of (p)ppGpp in regulating DNA replication**

Regulation of replication by (p)ppGpp extends beyond periods of starvation. It has also been shown that basal levels of (p)ppGpp are critical to maintaining cellular homeostasis, independent of its role in the stringent response. For instance, when bacteria, such as the Gram-negative *E. coli* and Gram-positive *Enterococcus faecalis*, are unable to synthesize (p)ppGpp, they display hallmarks of metabolic dysregulation, even when grown in nutrient rich media (Traxler et al., 2008; Dalebroux and Swanson, 2012; Gaca et al., 2015). The source of the dysregulated metabolism in these (p)ppGpp$^0$ cells remains unclear but there are a couple factors that may contribute. First, without (p)ppGpp there may be an unbalanced metabolism and inefficient energy expenditure, such as an inability to properly regulate purine metabolism(Anderson et al., 2019) . Secondly, an increased basal rate of mutagenesis caused by an increase in replication-transcription conflicts and compromised DNA repair pathways (Tehranchi et al., 2010; Kamarthapu et al., 2016). Cells unable to prevent conflicts will have replication forks collide with transcriptional machinery. The conflict can result in fork collapse. Recruitment of RecA will then occur, leading to recombination that can result in one large chromosome instead of two distinct chromosomes.

Since replication and transcription occur on the same DNA template they have the inherent potential to interfere with each other, especially since the replication fork moves

about 10 times faster than the transcription machinery (Hirose et al., 1983; Gotta et al., 1991; Mirkin and Mirkin, 2005). These conflicts can lead to an increase in mutagenesis, particularly if the collision is head-on (Merrikh et al., 2012; Sankar et al., 2016). Ideally these conflicts would be prevented, such as when *E. coli* cells that inhibit transcriptional pausing via DksA (Tehranchi et al., 2010). Recently is was shown that DksA inhibits transcriptional pausing by increasing the fidelity of the RNA polymerase in *E. coli* both *in vitro* and *in vivo*, likely preventing the pauses that occur from nucleotide misincorporation that then lead to collisions with the replication (Zenkin et al., 2006; Dutta et al., 2011; Roghanian et al., 2015; James et al., 2017). (p)ppGpp was observed to potentiate this function of DksA, though the precise mechanism of this increase in fidelity remains unknown.

Some recent evidence has suggested that (p)ppGpp functions in preserving genomic integrity (McGlynn and Lloyd, 2000; Trautinger et al., 2005; Denapoli et al., 2013; Madison et al., 2014). (p)ppGpp$^0$ *E. coli* was shown to be sensitive to DNA damaging agents, while cells that produced excess (p)ppGpp under normal exponential growth conditions were more resistant to the damaging agents than the parent control (Kamarthapu et al., 2016). Also, since (p)ppGpp regulates transcription by directly binding RNAP in *E. coli*, it follows that (p)ppGpp assists transcription coupled DNA repair.

**Scope of dissertation**

My thesis work investigates the regulation of DNA primase by the stress alarmone (p)ppGpp. We reveal insights into the molecular mechanisms of (p)ppGpp regulation of replication by characterizing its diverse effects on DNA primase. First, I show that *B. subtilis* DNA primase is most strongly inhibited at high levels of (p)ppGpp and low levels of GTP. Next, I

show that (p)ppGpp reduces overall primer synthesis, likely reducing the ability of DNA primase to reassociate to the DNA after it falls off. My work has found that (p)ppGpp reduces the processivity of DNA primase, forcing primase off the DNA sooner than it normally would in a single round of priming. Lastly, we revealed an unexpected effect of (p)ppGpp regulation of DNA primase – at low levels of GTP (p)ppGpp increases the fidelity of primase, limiting the error-prone readthrough observed in its absence. Altogether this work advances our understanding of (p)ppGpp regulation of replication elongation and leads into potential future work on how to study the replication fork during stress.

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**Table 1. The DNA replication machinery of *Bacillus subtilis* and *Escherichia coli***

<table>
<tr><th>Role in DNA Replication</th><th>Bacillus subtilis</th><th>Escherichia coli</th><th></th></tr>
<tr><td>Initiator</td><td>DnaA</td><td>DnaA</td><td></td></tr>
<tr><td>Replicative Helicase</td><td>DnaC</td><td>DnaB</td><td></td></tr>
<tr><td>Helicase Loader</td><td>DnaI</td><td>DnaC</td><td></td></tr>
<tr><td>DNA Remodeling</td><td>DnaB, DnaD</td><td>-</td><td></td></tr>
<tr><td>DNA Primase</td><td>DnaG</td><td>DnaG</td><td></td></tr>
<tr><td>DNA polymerization</td><td>PolC, DnaE</td><td>DnaE</td><td rowspan="7">Together assembles the fully functional DNA Polymerase</td></tr>
<tr><td>3′-5′ exonuclease activity</td><td>PolC</td><td>DnaQ</td></tr>
<tr><td>θ proofreading subunit</td><td>HolE</td><td>HolE</td></tr>
<tr><td>DNA Polymerase Clamp</td><td>DnaN</td><td>DnaN</td></tr>
<tr><td>Clamp Loaders τ and γ</td><td>DnaX</td><td>DnaX</td></tr>
<tr><td>Clamp loaders δ and δ′</td><td>HolA & HolB</td><td>HolA & HolB</td></tr>
<tr><td>Clamp loaders Χ and Ψ</td><td></td><td>HolC & HolD</td></tr>
<tr><td>Single-stranded binding protein</td><td>SSB</td><td>SSB</td><td></td></tr>
</table>

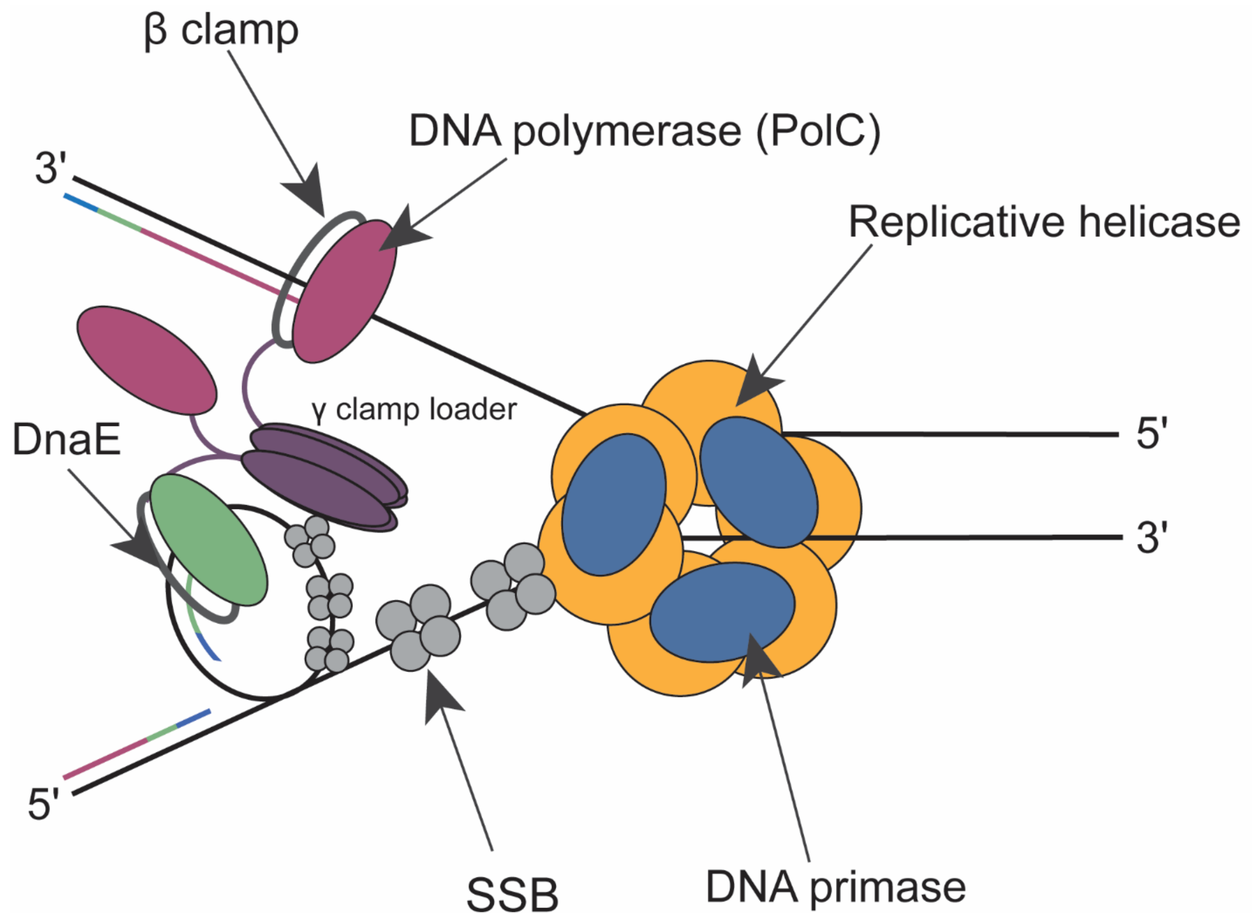


**Figure 1. Simplified overview schematic of the *Bacillus subtilis* replication fork**. The parental DNA (black line) is unwound by the replicative helicase (orange). The DNA primase DnaG (blue) physically interacts with the helicase and synthesizes short RNA primers (blue line) along the lagging strand (bottom strand). The primer is then extended by the DNA polymerase DnaE (green), before being handed off to the primary polymerase PolC (pink). Depicted on the lagging strand is DnaE extending the primer with an extra PolC tethered to the clamp loader. This PolC will be switched in once DnaE has finished its brief extension the the primer. The β clamp (grey) ensures ensures the processivity of the polymerase and coordinates leading- and lagging-strand synthesis through its interaction with the clamp-loader (purple) comprised of five subunits: ΧΨγδ´δ.

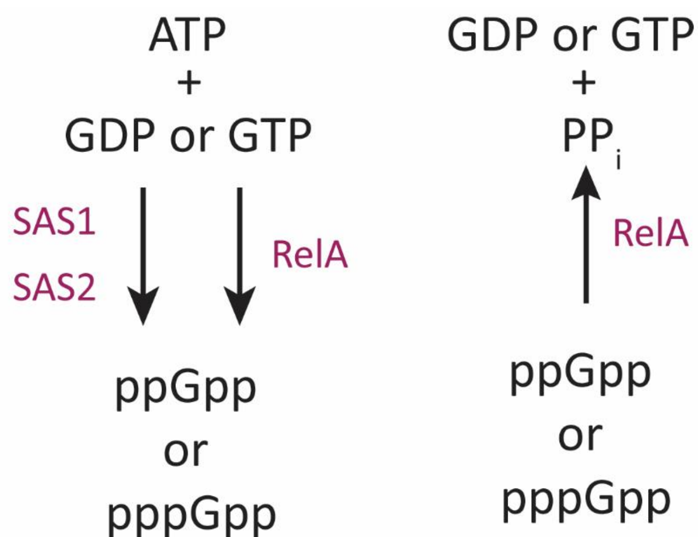


**Figure 2. (p)ppGpp synthesis in *Bacillus subtilis*.**
(p)ppGpp is synthesized by the addition of a pyrophosphate group from ATP to the 3´-hydroxyl group of either GDP or GTP to produce ppGpp or pppGpp, respectively. *Bacillus subtilis* synthesizes (p)ppGpp using the bifunctional synthetase and hyrdrolase RelA, and the monofunctional synthetases SAS1 (YjbM/RelQ) and SAS2 (YwaC/RelP). (p)ppGpp is hydrolyzed by RelA to produce GDP or GTP and $PP_i$.

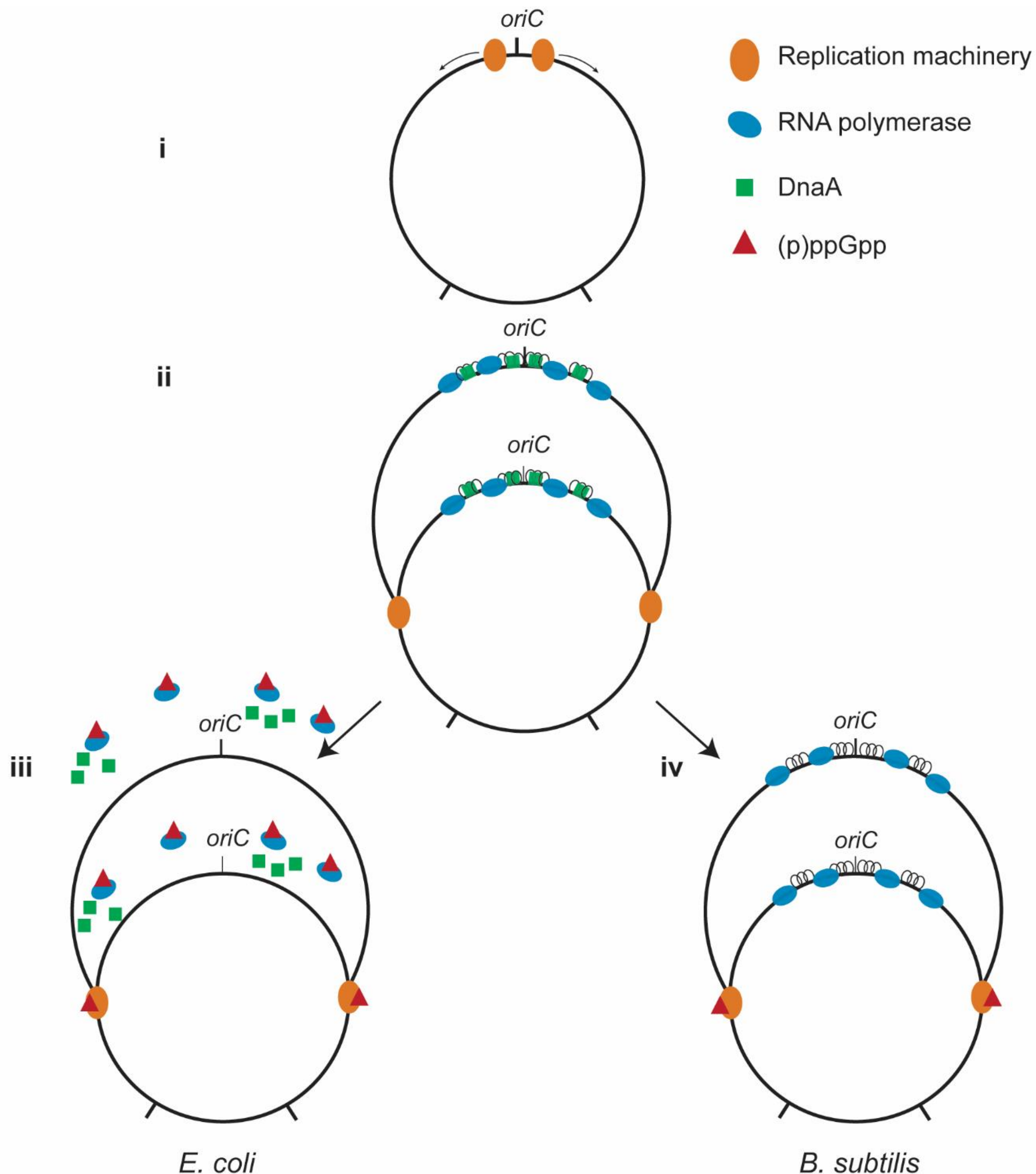


**Figure 3. (p)ppGpp regulation of replication in different bacterial species.**
(i) Replication of the bacterial chromosome initiates at the origin, oriC, and the replication forks (orange) proceed away from the origin bidirectionally. (ii) During replication, the RNA polymerase (blue) transcribes genes, introducing a negative supercoil in the DNA near the origin, allowing for the initiator protein DnaA (green) to easily bind. (iii) When (p)ppGpp (red) is induced in *E. coli*, (p)ppGpp binds the RNA polymerase, reducing the amount of negative supercoil at the origin so DnaA does not bind as readily. (p)ppGpp will also mildly bind the replication fork, inhibiting fork progression. (iv) In *B. subtilis* however, (p)ppGpp only binds the replication fork, halting fork progression anywhere during replication elongation.

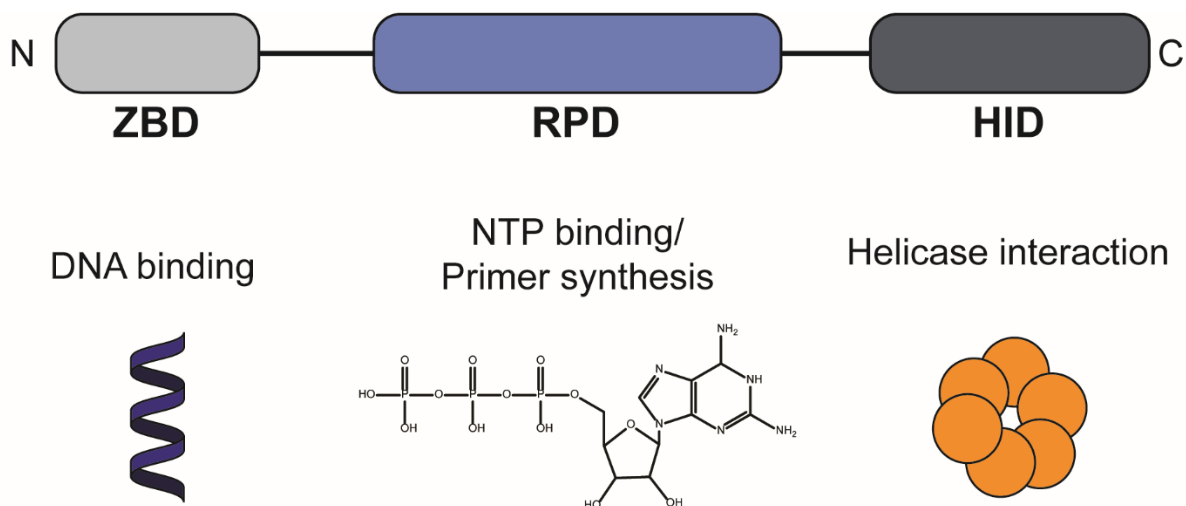


**Figure 4. The domains of the bacterial DNA primase DnaG.**
Schematic of the primary structure of DnaG. The N-terminal zinc binding domain important for DNA recognition. The active RNA polymerase domain involved in primer synthesis, which uses NTPs like GTP (shown). The C-terminal helicase interaction domain that physically interacts with the N-terminus of the replicative helicase monomers.

# Chapter 2: The alarmone (p)ppGpp has diverse effects on *Bacillus subtilis* DNA primase

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Contribution:

C.N.G. performed experiments, analyzed data, made all the figures, and wrote the chapter.

**Abstract**

Primase is an essential component of DNA replication machinery, responsible for synthesizing RNA primers that prime leading and lagging strand DNA synthesis. Bacterial primase activity can be regulated by the starvation-inducible nucleotide (p)ppGpp. This regulation contributes to a timely inhibition of DNA replication upon amino acid starvation in the bacterium *Bacillus subtilis*. Here we characterized the effect of (p)ppGpp on *B. subtilis* primase elongation. High (p)ppGpp concentration, together with low GTP concentration, synergistically inhibit primase activity, corroborating with structure of (p)ppGpp binding site on primase that overlaps with GTP and explaining the strong inhibition of replication elongation in *B. subtilis*. Using a single-nucleotide-resolution primase assay, we dissected the effect of ppGpp on the initiation, extension and processivity of *B. subtilis* primase. We found that ppGpp has a mild effect on initiation, but strongly inhibits primer extension and reduces primase processivity. Finally, we found that lowering GTP results in priming infidelity while ppGpp protects priming fidelity. These results highlight the importance of (p)ppGpp in protecting replisome integrity and genome stability in fluctuating nucleotide concentrations upon onset of environmental stress.

**Introduction**

DNA replication is a highly regulated cellular process to ensure faithful and complete duplication of genetic material. In most bacteria, DNA replication initiates from a single origin of replication on the circular chromosome, the bidirectional replication forks continue elongating until they reach the terminus of replication. DNA replication is performed by a multicomponent cellular machine composed of the replicative helicase, the leading and lagging strand DNA polymerases, the DNA primase that produces RNA primers for both leading and lagging strand DNA synthesis, and other accessory replication proteins. The DNA primase functions through a multi-step process: it first binds ssDNA and uses two ribonucleotides to form an RNA dinucleotide at a preferred start site, then extends the RNA primer in a 5′ to 3′ direction, and terminates priming by handing off the RNA primer to the DNA polymerase (Frick and Richardson, 2001). The bacterial DNA primase (DnaG) comprises three domains: an N-terminal Zinc-binding domain (ZBD) involved in binding DNA, a primer synthesizing RNA polymerase domain (RPD), and a C-terminal helicase interaction domain (HID or CTD) that interacts with the replicative helicase (Figure 1A).

A regulatory mechanism of primase activity by the starvation-inducible nucleotide (p)ppGpp has been discovered in bacteria (Wang et al., 2007b). (p)ppGpp is the collective name of nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) that signal nutrient and other stresses in bacteria (Cashel, 1996). Crystal structures of the active-site-containing RPD of *Staphylococcus aureus* primase bound to ppGpp or pppGpp show that (p)ppGpp binds to a partially overlapping site of the NTP substrates, suggesting that (p)ppGpp binds the DnaG active site to directly obstruct NTP binding (Rymer et al., 2012). *In*

*vitro* analyses have shown that (p)ppGpp inhibits the production of RNA by primase from multiple bacterial species (Wang et al., 2007a; Maciag et al., 2010; Rymer et al., 2012). This regulation may underlie a nutritional regulation of replication observed in the Gram-positive bacterium *Bacillus subtilis*, where replication elongation is strongly inhibited upon amino acid starvation in a (p)ppGpp dependent, non-disruptive manner. This regulation is proposed to protect genome integrity from being disrupted by nutrient stress (Wang et al., 2007a).

Despite strong evidence for an interaction between (p)ppGpp and primase, key questions remain. The inhibitory effect of (p)ppGpp is based on measurement of bulk RNA synthesis by primase (Wang et al., 2007a; Maciag et al., 2010; Rymer et al., 2012), and it remains unclear how (p)ppGpp affects primer initiation, extension and processivity. In addition, the similar *in vitro* effects of (p)ppGpp on primase activity from both *E. coli* and *B. subtilis* contrast with strong differences *in vivo*, where (p)ppGpp strongly inhibits replication elongation in *B. subtilis* but has only a very mild effect in *E. coli* (Maciag-Dorszyńska et al., 2013)(Wang et al., 2007b; Denapoli et al., 2013).

To provide a thorough understanding of *B. subtilis* primase activity and its regulation by (p)ppGpp, we adapted an *in vitro* priming assay to examine the initiation, extension, processivity, and fidelity of *B. subtilis* primase. We then examined the effect of (p)ppGpp on each of these aspects and revealed diverse and surprising effects of (p)ppGpp on priming initiation, extension, processivity, and fidelity. Because amino acid starvation in *B. subtilis* results in (p)ppGpp production and concomitant GTP depletion, the interplay between (p)ppGpp and GTP on priming is also examined, with strong implications in regulation of replication in nutrient-stressed *B. subtilis* cells.

## Materials and Methods

### Protein Purification

*Bacillus subtilis* DnaG full-length protein over-expression construct was generated by PCR amplification of genomic DNA from using oligos oJW2646 and oJW702, and insert into the pLIC plasmid pJW269. The DnaG ΔCTD(Δ440-603) construct was generated similarly, except that oligos oJW2646 and oJW2649 were used instead. Recombinant proteins were expressed in BL21 (DE3+) cells and purified by Ni –affinity chromatography over 1-mL HisTrap column. Lysis buffer consisted of 300 mM NaCl, 20 mM imidazole, 40 mM HEPES pH 7.8-8.0. Stepwise elution was carried out from 100% lysis buffer to 100% of an otherwise equivalent buffer containing 500 mM imidazole. Proteins were then dialyzed into a storage buffer that consisted of 40 mM HEPES pH 7.8, 300 mM NaCl, 10% Glycerol, 1 mM EDTA, and 1 mM dithiothreitol. Purity was assessed using polyacrylamide gel electrophoresis and Coomassie staining and protein preparations were confirmed to be ≥ 95% pure. Concentration was determined by absorption at 280 nm using the following coefficients: 46,760 $M^{-1}cm^{-1}$ (full-length), 32,320 $M^{-1}cm^{-1}$ (ΔCTD). Protein activity and binding to (p)ppGpp with and without the His-tag was assessed and no discernable difference was observed (data not shown).

### Synthesis, Purification, and Quantification of (p)ppGpp

(p)ppGpp was prepared *in vitro* using RelSeq1–385 and GppA, purified and quantified as described (Mechold et al., 2013). 6 mM GTP was mixed with 8 mM ATP, 300 µg RelSeq1NH385, 0.5 mM dithiothreitol, 25 mM bis-Tris propane pH 9.0, 15 mM MgCl2 and allowed to incubate at 37°C for 2-5 hours to generate pppGpp. ppGpp was then produced upon addition of GppA and allowed a 1 hour incubation. (p)ppGpp was purified with an anion exchange column (HiTrap

QFF 1 mL; GE Healthcare) using a binding buffer (0.1 mM LiCl, 0.5 mM EDTA, 25 mM Tris pH 7.5). Stepwise elution was carried out by increasing LiCl from 0.1 mM to 500 mM.

**Fluorescent Detection of Primer Synthesis**

Primer synthesis assays were based on a previously established primase activity assay from ProFoldin (Hudson, MA) (Koepsell et al., 2005). All reactions were carried out in 40 mM HEPES pH 7.5, 1% glycerol, 1 mM magnesium acetate, 1 mM manganese chloride, and 5 mM dithiothreitol. Reactions containing full-length BsDnaG included 100 nM enzyme. Reactions containing a truncated BsDnaG lacking the C-terminal domain included 200 nM enzyme. Reaction mixtures were incubated at 30°C for 5 minutes prior to addition of the NTP mixes. Reactions included 0.4 mM ATP, CTP, UTP, and the indicated concentrations of GTP. For measurement of product, reaction mixes at respective time points (5 minutes, 10 minutes, 20 minutes, and 30 minutes) were stopped by mixing with 5 µl of 0.5 M EDTA pH 8.0 to a final concentration of 100 mM. Stopped reactions were then stained with the ProFoldin primase activity assay dye and allowed to incubate in the dark for 5 minutes. Raw fluorescence of reactions was measured in a BioTek Synergy2 multi-mode microplate reader and was then background corrected with a no enzyme control to give fluorescence intensity.

For inhibitor titrations, ppGpp and pppGpp were prepared and stored in desiccated form until immediately prior to use. Dry pellets were resuspended in in Tris 50mM pH 7.5, 0.5mM EDTA buffer and the concentration was measured by absorbance at 258 nm using the extinction coefficient 13,700 $M^{-1}cm^{-1}$. Inhibitor dilutions were prepared in TE and mixed with an equal volume of an incomplete reaction mixture containing the reaction buffer, DnaG, and template, and allowed to incubate at 30°C for 5 minutes prior to addition of NTPs. Reactions

were incubated at 37°C for 30 minutes and time points were taken at 5, 10, 20, and 30 minutes where they were immediately stopped as the standard GTP titrations.

The data were normalized to the average maximum fluorescence intensity and fit by nonlinear regression:

$V_0 = V_{max}$*[GTP]/($K_{mpseudo}$ + [GTP])

In which $V_{max}$ is the maximal enzyme rate (AFU/min), $K_{mpseudo}$ is a pseudoapparent $K_m$ that corresponds to the concentration of GTP that yields one-half of $V_{max}$.

**Radiolabeled Primer Extension**

Reactions contained 40 mM HEPES, pH 7.5, 1% Glycerol, 1 mM magnesium acetate, 1 mM manganese chloride, 5 mM dithiothreitol, 6.4 µM primase, and 500 nM ssDNA template. Templates used are listed in Table 1. The reaction mixtures had 373 nM [γ-$^{32}$P]ATP (5 µCi/nmol, Perkin Elmer). Reactions were allowed to briefly incubate at room temperature with ppGpp at the indicated concentrations before addition of the unlabeled NTPs (GTP at the indicated concentrations and 1 mM UTP). After addition of the unlabeled nucleotides, the reactions were incubated 30 min at 30 °C. Reactions were stopped upon addition of 95% formamide, 5 mM EDTA, 0.09% xylene cyanol FF, heated for 5 min at 95 °C, and separated in a denaturing 20% polyacrylamide gels (acrylamide/bisacrylamide ratio, 19:1; 7 M urea) for 2 hours at 2000 V. A Typhoon Phosphorimager was used to detect luminescence following exposure of the gel to a phosphor screen. Data were quantified using Image-J software. Lengths of RNA primers were determined against RNA standards with the predicted primer sequence based on the template used.

RNA standards were prepared using an RNA oligonucleotide with the sequence complementary to the primase template. The 5′-end was labeled with [$\gamma$-$^{32}$P]ATP (Perkin Elmer) and separated from ATP using a nucleotide clean up kit (Qiagen). 120 nM purified, labeled RNA was then hydrolyzed for 5 minutes at 45°C in 300 nM NaOH. Hydrolysis reactions were neutralized with 2 volumes of 2 M Tris-HCl (pH 7-7.5). Stop buffer (95% formamide, 5 mM EDTA, 0.09% xylene cyanol FF) was added and the hydrolyzed fragments were heated for 2 minutes at 95°C and quickly cooled on ice (Supplemental Figure 1).

**Differential Radial Capillary Action of Ligand Assay (DRaCALA)**

DRaCALA was performed with pure protein and radioactive ligand as described (Roelofs et al., 2011). [5′$\alpha$-$^{32}$P] (p)ppGpp was synthesized according to modified protocols of (p)ppGpp synthesis (Mechold et al., 2002). The reaction contained 25 mM bis-Tris propane (pH 9.0), 15 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP, 2 µM $Rel_{Seq}$ (1-385), and 37.5 µCi [$\alpha$-$^{32}$P] GTP (Perkin Elmer). The reaction was incubated at 37°C for 1 hour. ppGpp was then produced by addition of the enzyme GppA for an 1 hour. The reaction was diluted in 0.5 mL of Buffer A (0.1 mM LiCl, 0.5 mM EDTA, 25 mM Tris-HCl pH 7.5) prior to adding to a 1 mL HiTrap QFF strong anion exchange column (GE Healthcare) equilibrated with 10 column volumes (CV) of 83% Buffer A + 17 % Buffer B (Buffer B: 1 M LiCL, 0.5 mM EDTA, 25 mM Tris-HCl pH 7.5). [$\alpha$-$^{32}$P]-(p)ppGpp was eluted with a mixture of 50% Buffer A + 50% Buffer B. Fractions of 1 mL were collected from the elution.

DRaCALA reactions using purified DnaG or DnaG domains contained 40 mM HEPES pH 7.5-7.8, 1 mM MnCl2, 10 mM MgAc, 150 mM NaCl, 5% Glycerol, 5 mM DTT, 10 µM DnaG (diluted in 40 mM HEPES pH 7.5-7.8, 150 mM NaCl, 5% Glycerol, 5 mM DTT) and $^{32}$P-(p)ppGpp

(1:100 final dilution of first elution fraction of $^{32}$P-(p)ppGpp purification). Reactions were incubated for 10 minutes at room temperature. Two microliters from each reaction were spotted in duplicate on Protran BA85 nitrocellulose (GE Healthcare) via pipette or a replicator pinning tool (VP 408FP6S2; V and P Scientific, Inc). Spots were allowed to dry and radioactivity was detected with phosphorimaging (Typhoon FLA9000). Fraction bound of $^{32}$P-(p)ppGpp was calculated as described (Roelofs et al., 2011).

**Results**

**pppGpp, together with decreased GTP, strongly inhibits *B. subtilis* DNA primase**

To determine the effects of (p)ppGpp on *Bacillus subtilis* primase, we cloned and purified *B. subtilis* primase DnaG recombinantly in *E. coli*. We first applied the differential radial capillary action of ligand assay (DRaCALA) (Orr and Lee, 2017) to quantify the interaction between DnaG and $^{32}$P-labeled pppGpp. DRaCALA relies on the different migration properties of protein and ligand on nitrocellulose. Protein diffuses slowly but ligand diffuses rapidly when a solution is spotted on nitrocellulose. However, ligand interacting with protein co-migrates with the protein (Fig. 1B). The interaction can be quantified as the fraction of total ligand bound to the protein. DRaCALA showed that pppGpp bound to recombinantly expressed, purified DnaG. Unlabeled pppGpp effectively competed for binding to DnaG, indicating that the observed DRaCALA result was not due to protein aggregation or precipitation of $^{32}$P-pppGpp. GTP was effective in competing with $^{32}$P-pppGpp for binding DnaG. Although DnaG has three domains, the RNA polymerase domain (RPD) was sufficient for strong binding (Fig. 1B). These results are similar to those obtained for *E. coli* DnaG (Rymer et al., 2012), and indicate that pppGpp binds to *B. subtilis* RPD active site in competition with the substrate GTP.

Next, we used an *in vitro* fluorescence-based assay to measure the effect of (p)ppGpp on the enzymatic activity of *B. subtilis* primase. Primase was mixed with NTPs and a single-stranded DNA template (Table 1, oJW2384) containing the preferred initiating sequence for primase (Koepsell et al., 2006; Rannou et al., 2013). The product was measured by fluorescence of a dye that intercalates between the bases of RNA-DNA heteroduplexes (Figure 1C). Using this assay, we verified that (p)ppGpp has a modest inhibitory effect on *B. subtilis* primase, in agreement with prior reports (Wang et al., 2007a; Maciag et al., 2010; Rymer et al., 2012) (Figure 1D-E). We also assessed the ability of (p)ppGpp to compete with GTP by varying the concentrations of GTP and the inhibitor. Our results show that (p)ppGpp inhibits *B. subtilis* in a mixed inhibitory manner similar to its effects on *E. coli* primase (Rymer et al., 2012). ppGpp caused an increase in the apparent pseudo-Michaelis-Menten constant ($K_{Mpseudo}$) and both ppGpp and pppGpp decreased the maximum initial rate of the reaction ($V_{max}$) (Figures 1D-E, Table 2).

Since both the RPD and the N-terminal domain of DnaG are required for primase activity, whereas the C-terminal domain of DnaG plays a regulatory role, we examined the specific effects of (p)ppGpp on a DnaG variant lacking the CTD (DnaGΔCTD) (Griep, 1995; Tougu and Marians, 1996; Johnson et al., 2000; Corn and Berger, 2006; Koepsell et al., 2006; Lee et al., 2006; Kuchta and Stengel, 2010; Rymer et al., 2012; Van Eijk et al., 2016; Catazaro et al., 2017). The DnaGΔCTD variant was active in priming, and its activity was inhibited by (p)ppGpp similar to full-length DnaG (Figure 1F). Taken together, our data suggest that (p)ppGpp binding of the RPD inhibits *B. subtilis* DnaG.

In *B. subtilis*, (p)ppGpp inhibits multiple enzymes along the GTP biosynthesis pathway, resulting in depletion of GTP levels. Therefore, cellular nucleotide concentrations in *B. subtilis* during amino-acid starvation include both high (p)ppGpp and low GTP (Kriel et al., 2012; Liu et al., 2015). Because high levels of (p)ppGpp and lower levels of GTP strongly inhibits DNA primase activity (Figure 1G), this could explain the nearly complete arrest of replication elongation during amino acid starvation in *B. subtilis* (Wang et al., 2007a).

**Initiation and extension of RNA primers by *B. subtilis* primase**

The experiments above and prior published work evaluating the effects of (p)ppGpp on primase relied on bulk measurement of all RNA-DNA heteroduplexes produced. However, there are various facets of primase action, including initiation of primer synthesis, primer elongation, and primer release. To understand how (p)ppGpp regulates primase activity, we performed primase assays in the presence of [γ-$^{32}$P]ATP to specifically label the 5' end of each primer synthesized. The RNA primers were then denatured and resolved via gel electrophoresis using urea-PAGE (Figure 2A). We were able to separate primers as small as dinucleotides (formed at the primer initiation step) to the maximum templated size (29 nucleotides). We also observed a small amount of primers longer than 29 nucleotides, which we found to be due to impurity of the template (Supplemental Figure 2). Therefore, we use PAGE-purified oligonucleotides as the ssDNA templates for the remaining experiments. Importantly, detectable primer formation depended on the presence of DNA primase and increased proportionally as primase concentration increased (Figure 2B).

**ppGpp inhibits primer extension**

Using the single nucleotide resolution priming assay, we characterized primer extension by visualizing production of primers over time, using oJW3319 as a template (Table 1). Dinucleotide formed rapidly, followed by a 6-nucleotide band within 5-10 minutes, with a band corresponding to the full-length primer of 21 nucleotides increasing in intensity throughout the duration of the reaction (Figure 3A). Thus, we can monitor several aspects of primase activity with urea PAGE, including primer initiation (the formation of the dinucleotide) and primer extension (the formation of primers > 2 nucleotides) (Thirlway and Soultanas, 2006; Rannou et al., 2013).

We next examined the effect of 1mM ppGpp on both the initiation and extension of primers. In the first 2 minutes, ppGpp did not have a strong effect on total primer synthesis (Figure 3B), and the total amount of dinucleotide synthesis was not significantly affected by ppGpp (Figure 3C). However, ppGpp strongly reduced the subsequent extension step compared to the reaction without ppGpp (Figure 3D). This suggests that the main effect of ppGpp is on primer extension.

In these assay conditions, the ssDNA template was in 12.5-fold molar excess to DNA primase, thus DNA primase may finish priming a ssDNA template and then associate with a new one. This means we are measuring multiple rounds of priming. To examine the effect of ppGpp on the extension of primers without the complication of primer initiation, we performed a reaction in which DnaG was in 12.8-fold molar excess to template (Figure 4A). We found that lower levels of substrate nucleotide GTP (0.1 mM) reduced primer extension compared to a higher concentration (1 mM GTP) (Figure 4A). Importantly, when we added increasing amounts of ppGpp and measured priming activity 30 minutes after the reaction started, we observed a

clear and strong reduction in the lengths of primers (Figure 4A). Correspondingly, the amount of full-length product (21 nucleotides) and intermediate primers (3-20 nucleotides) also decreased as ppGpp concentration increased (Figure 4B-C, respectively). The total number of primers synthesized remained relatively constant until high concentrations (0.8 and 1 mM) of ppGpp were reached, at which point a very modest reduction was observed (Figure 4D). The dinucleotide formed via primer initiation also remained constant for all but the highest two concentrations of ppGpp, at which a modest reduction was observed (Figure 4E). Altogether, our data suggest that ppGpp strongly and primarily inhibits primer extension and very modestly reduces primer initiation at high concentrations.

**ppGpp decreases the processivity of DNA primase**

We next sought to determine if this inhibition of primer extension was due to simply slowing down the primase, or due to decreased primase processivity. In order to do so, we applied an assay often used in characterizing RNA polymerase processivity – the heparin competition assay (Pfeffer et al., 1977; Leirmo and Gourse, 1991; Reddy et al., 1992; Reha-krantzs and Nonay, 1994). Heparin is commonly used to block protein rebinding to DNA or RNA through its helical structure and polyanionic nature (Chammas et al., 2017). Therefore, we added heparin alongside the NTPs to a pre-incubated reaction mix of primase, ppGpp, and the ssDNA template, where the amount of ssDNA was 12.5 times greater than primase. Primase pre-bound to the template would initiate the *de novo* synthesis of the primer and extend processively. Once primase fell off, it would be prohibited from rebinding to the partially extended RNA to continue extension due to the presence of heparin. Compared to the same assay without heparin (Figure 3B-C), in which the primers gradually extend to the full length,

the reactions with heparin yielded primers which were predominantly 8 or 10 nucleotides long (Figure 5A-C). This suggests that primase synthesizes 8-10 nucleotide primers per priming event and requires reassociation to extend primers to the full length of the DNA template.

When ppGpp was added to the reaction the total number of primers was comparable to that without ppGpp (Figure 5D). However, primase was unable to synthesize higher order primers (> 7 nucleotides) in the presence ppGpp (Figure 5B, C, and E). These data, together with the previous time course data support the conclusion that ppGpp is inhibiting primer extension by decreasing primase processivity, resulting in primase falling off the DNA sooner. Alternatively, ppGpp may slow down the primase extension rate so primase tends to fall off early before finishing priming.

**ppGpp protects priming fidelity from lowering concentrations of GTP**

Finally, we examined the effect of ppGpp on priming fidelity using a read-through assay on a template with a single site requiring incorporation of an CTP after a 7-nucleotide extension (Supplemental Figure 3). When we withheld CTP from reactions with DnaG in 12.8-fold molar excess to ssDNA, the majority of priming products were 7 nucleotides. This is the expected length when primase is halted at the starved site. However, there were readthrough primers longer than the expected 7 nucleotides (Figure 6A, 6C), which requires either misincorporation of NTPs at the starved site, or primer-template slippage. When we switched to an alternative template that still contained starvation pause site (Figure 6B, Table 1, oJW3668), we observed similar readthrough primers up to 23 nucleotides, the length expected for a completely template-directed primer (Figure 6B). This is not unexpected, because primase has an active site with an open conformation, suggesting a high error rate. Unexpectedly, at lower

concentrations of GTP (0.1mM), the readthrough product greatly increased compared to 1 mM GTP in the reaction (Figure 6A-D), suggesting that nucleotide imbalance may greatly promote priming errors.

We found a strong effect of ppGpp on promoting fidelity. As ppGpp levels increased, the read-through products decreased (Figure 6C). In the low GTP case, the correctly sized 7 nucleotide primer increased as ppGpp increased (Figure 6D). The total number of primers synthesized did not change with increasing ppGpp, suggesting that ppGpp is regulating fidelity without stopping priming initiation (Figure 6E).

The DnaGΔCTD variant performed similarly to full-length primase, with extensive priming readthrough at 0.1 mM GTP but not at 1 mM GTP (Supplemental Figure 4A-C). Adding ppGpp decreased the readthrough primers observed, akin to the decrease observed with the full-length protein (Supplemental Figure 4C). Altogether, these suggest that ppGpp protects priming fidelity most likely through its competition with the substrate nucleotides.

**Discussion**

Bacterial primase is an essential replication enzyme that is evolutionarily divergent from eukaryotic primase. In Gram positive bacteria, a starvation-signaling molecule (p)ppGpp directly inhibits primase activity. In this work, we pinpoint the effect of ppGpp on extension, processivity and fidelity of *B. subtilis* primase. We found that ppGpp strongly inhibits primer extension and reduces primase processivity. Interestingly, increasing (p)ppGpp lowers GTP levels in *B. subtilis*, and we show that while lowering GTP and increasing (p)ppGpp each reduce primase activity, (p)ppGpp also prevents lowered GTP level from promoting priming errors.

These results reveal the importance of ppGpp in regulating DNA replication in the context of unbalanced nucleotide concentrations upon nutrient stress.

**Differential regulation of replication in different bacterial species**

Our study explains the difference of (p)ppGpp-dependent control of replication elongation between *E. coli* and *B. subtilis*. *In vitro* work has shown that (p)ppGpp inhibits DNA primase similarly across species (Wang et al., 2007a; Maciag et al., 2010; Rymer et al., 2012). However, *in vivo*, ppGpp-dependent inhibition of replication elongation upon starvation is far stronger in *B. subtilis* than in *E. coli*. In *E. coli*, amino acid starvation regulates replication mainly through decreased initiation from the origin of replication (*oriC*) (Schreiber et al., 1995; Ferullo and Lovett, 2008). (p)ppGpp inhibits replication initiation in *E. coli* indirectly by regulating transcription of genes proximal to the origin of replication, thus controlling its supercoiling status and facilitating strand opening (Kraemer et al., 2019). Although we previously observed a dose-dependent inhibition of replication elongation by (p)ppGpp upon amino acid starvation in *E. coli* (up to ~10% in wild type cells, and ~30% in a mutant (Δ*gppA*) with elevated pppGpp) (Denapoli et al., 2013), inhibition of *B. subtilis* replication elongation under similar starvation conditions is far stronger (nearly complete) (Denapoli et al., 2013; Maciag-Dorszyńska et al., 2013). This disparity between *in vivo* and *in vitro* results can be explained by our finding that in *B. subtilis*, inhibition of DNA primase is strongest when (p)ppGpp levels are high and GTP levels are low (Figure 1). In *B. subtilis*, (p)ppGpp has a stronger inhibitory effect on GTP production than in *E. coli*, resulting in GTP depletion by more than 10-fold (Kriel et al., 2012; Liu et al., 2015; Anderson et al., 2019). This would result in far lower primase activity, and thus replication elongation, in *B. subtilis* cells. In *E. coli*, amino acid starvation induces (p)ppGpp but

does not reduce GTP levels to the same extent as in *B. subtilis*, explaining the mild dose-dependent replication elongation inhibition observed in starved *E. coli* (Denapoli et al., 2013).

There are additional differences in the replication machineries between these organisms. For example, in *E. coli*, the RNA primer generated by primase is handed to the high fidelity DNA polymerase for replication. However, in *B. subtilis*, the RNA primer will be first handed to a DNA polymerase DnaE to extend a short DNA primer, then handed to the high fidelity DNA polymerase PolC. Future work is needed to examine the effect of (p)ppGpp in ternary complexes with these additional replication proteins and compare their effects between organisms.

**Primase regulation and non-disruptive inhibition of replication elongation in bacteria**

Throughout replication, forks may encounter obstacles that trigger replisome disassembly, requiring replication restart to rescue these abandoned forks for cell survival (Michel et al., 2018). Failure to reactivate stalled or collapsed forks is a source of genome instability or lethality. Regulation of replication that results in non-disruptive fork arrest, however, can prevent these deleterious events from taking place. Since DNA primase is known to interact with and stimulate the activity of the replicative helicase, inhibition of primase activity by (p)ppGpp may in turn inhibit helicase movement, thus pausing replication fork progression (Van Eijk et al., 2016).

Although our work was performed with primase in the absence of other proteins, it nonetheless offers important insights into primase action that will be helpful to elucidate how primase may function in concerted action with DNA helicase and polymerase. Previous work with *B. subtilis* primase found that primers 5-15 nucleotides in length were synthesized at

physiologically relevant primase concentrations (Rannou et al., 2013). This agrees with our observation that *B. subtilis* primase synthesizes primers 6-10 nucleotides long before dissociating from the DNA. (p)ppGpp decreases priming processivity, thus during the stringent response, primers may have altered length, which may relate to how it halts progression of the replication fork.

**Primase fidelity and genome stability in bacteria**

We identified an unexpected effect of (p)ppGpp on reducing priming readthrough at a starved NTP site, implicating (p)ppGpp in promoting primase fidelity. Because (p)ppGpp slows down extension by primase, (p)ppGpp may promote primase fidelity by increasing kinetic proofreading. The theoretical framework for kinetic proofreading was first established for ribosomes and DNA polymerase (Hopfield, 1974). In transcription, (p)ppGpp has been shown to indirectly promote fidelity of RNA polymerase through affecting the transcription factor DksA (Roghanian et al., 2015). DnaG priming is expected to be error prone due to the open conformation of its active site. Because RNA primers are eventually degraded and replaced by DNA, lack of priming fidelity has not been regarded problematic. However, loss of fidelity my affect how a primer is handed off to DNA polymerase. This is especially apparent when GTP levels are depleted during nutrient starvation, resulting in strongly elevated priming error. Therefore, regulation of priming by (p)ppGpp may protect fidelity of priming, allowing its appropriate handoff to the DNA polymerase, thus ensuring processive genome duplication and genome integrity.

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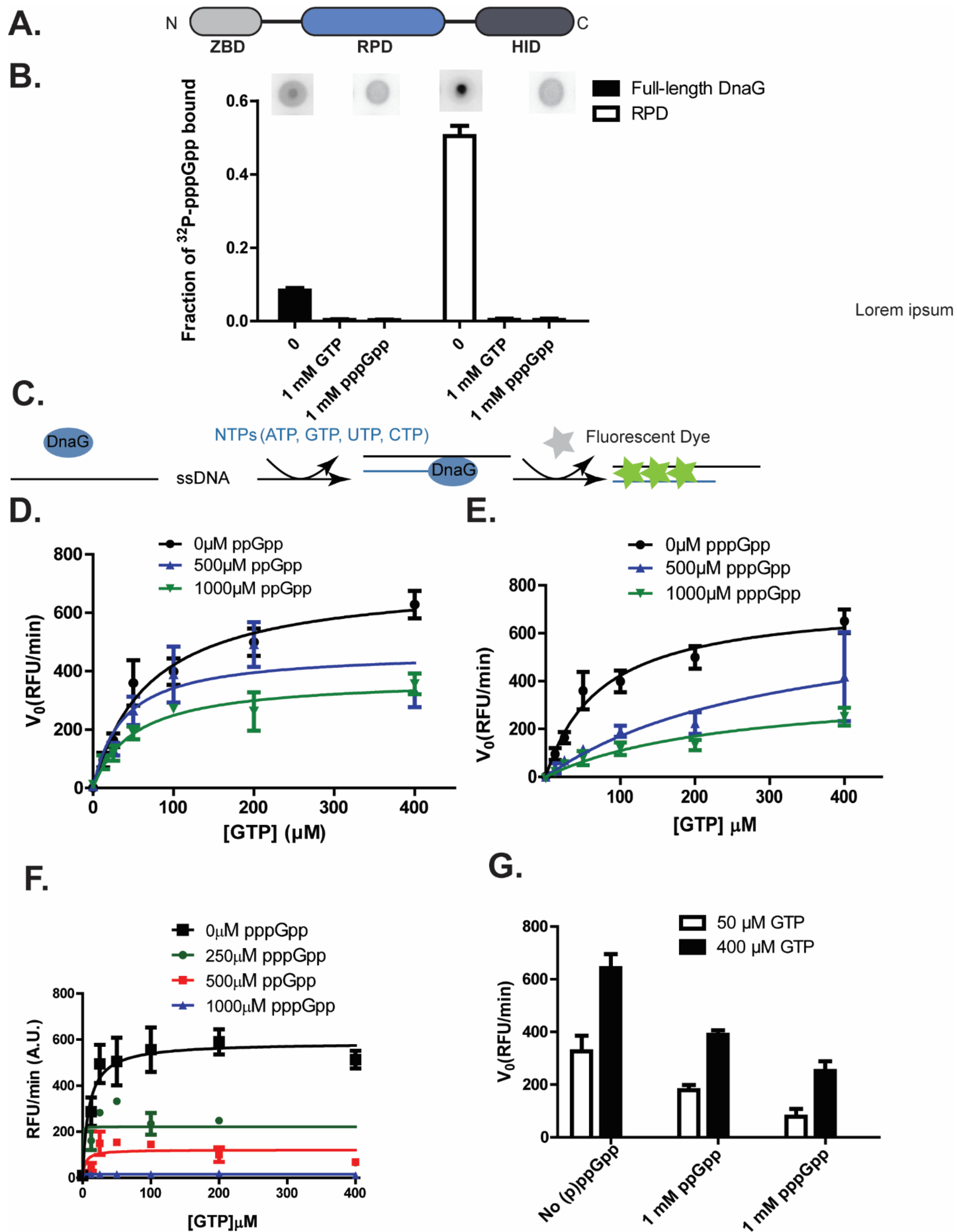

A.
N
ZBD
RPD
HID
C
B.
Fraction of $^{32}$P-pppGpp bound
Full-length DnaG
RPD
0
1 mM GTP
1 mM ppGpp
0
1 mM GTP
1 mM pppGpp
Lorem ipsum
C.
DnaG
ssDNA
NTPs (ATP, GTP, UTP, CTP)
DnaG
Fluorescent Dye
D.
0μM ppGpp
500μM ppGpp
1000μM ppGpp
V0(RFU/min)
[GTP] (μM)
E.
0μM pppGpp
500μM pppGpp
1000μM pppGpp
V0(RFU/min)
[GTP] μM
F.
0μM pppGpp
250μM pppGpp
500μM ppGpp
1000μM pppGpp
RFU/min (A.U.)
[GTP]μM
G.
50 μM GTP
400 μM GTP
V0(RFU/min)
No (p)ppGpp
1 mM ppGpp
1 mM pppGpp

**Figure 1. Low levels of GTP coupled with high levels of (p)ppGpp strongly inhibit B. subtilis DNA primase.**

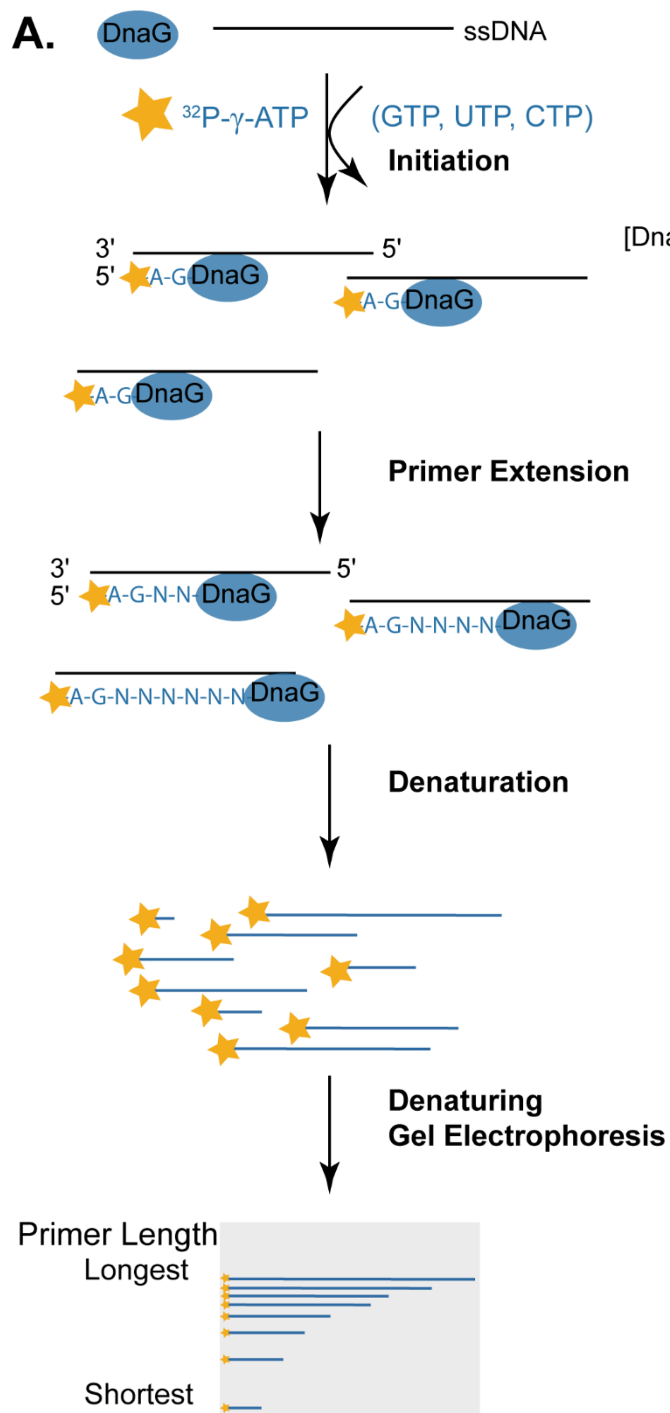
(A) The bacterial primase DnaG is composed of three domains: the N terminal zinc binding domain (ZBD), the RNA polymerase domain (RPD) and the C terminal helicase interacting domain (CTD).

(B) DRaCALA images of interactions between 10 µM B. subtilis DNA primase and 32P-pppGpp, as well as competition with 10 µM ssDNA template, 1 mM GTP, or 0.5 mM unlabeled pppGpp.

(C) Schematics of the fluorescent based primase assay. DNA primase (DnaG) is mixed in with a ssDNA template and allowed to briefly incubate with or without (p)ppGpp before the addition of NTPs. Aliquots of the reaction are taken at various time points, and DNA-RNA heteroduplexes are measured with a dye that interchelates the double-stranded heteroduplex. The level of fluorescence is dependent on the amount of primers synthesized.

(D-F) Inhibition of full-length DnaG primer synthesis by ppGpp and pppGpp (C and D respectively) and (E) DnaGΔCTD variant inhibition by ppGpp or pppGpp. GTP-dependent de novo primer synthesis activity was measured against increasing concentrations of ppGpp or pppGpp. Points represent averages of ≥ 2 replicates. Error bars represent ± SEM for > 2 replicates and the range for 2 replicates. Curves were fit as described in the Materials and Methods.

(G) Fluorescent based primer synthesis assays show that DNA primase is most strongly inhibited at low levels of GTP (50 µM) and high levels of (p)ppGpp (1000 µM), when compared to the not as strong inhibition seen at high levels of GTP (400 µM).


A.
DnaG
ssDNA
32P-γ-ATP
(GTP, UTP, CTP)
Initiation
3'
5'
A-G
DnaG
Primer Extension
A-G-N-N-
A-G-N-N-N-N
A-G-N-N-N-N-N-N
Denaturation
Denaturing
Gel Electrophoresis
Primer Length
Longest
Shortest

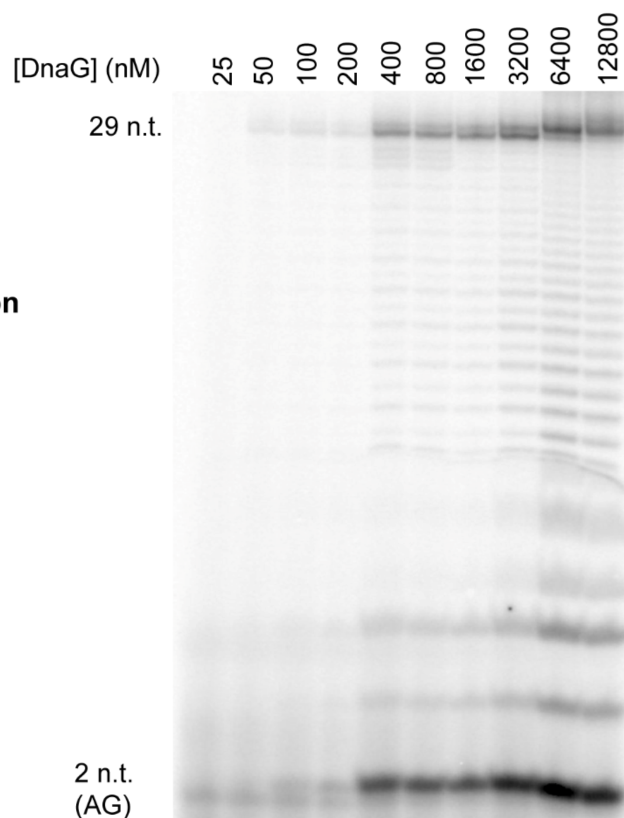

B.
[DnaG] (nM)
25
50
100
200
400
800
1600
3200
6400
12800
29 n.t.
2 n.t.
(AG)

**Figure 2. Primase activity can be monitored with urea-PAGE denaturing gel**. (A) Schematic of radioactive primase activity assay for primer detection of varying primer lengths. Each detected primer is labelled only at the 5' end of the RNA. Products were denatured and resolved with 7M Urea 20% PAGE. (B) Titration of DNA primase with 500 nM ssDNA, 0.4 mM NTPs (CTP, UTP, GTP) and 373 nM 32P-γ-ATP. The expected product size was 29 nucleotides but many intermediate primers were observed. These intermediates were in part due to the impurities from the synthesis of the template oligonucleotide that can be removed via PAGE purification.

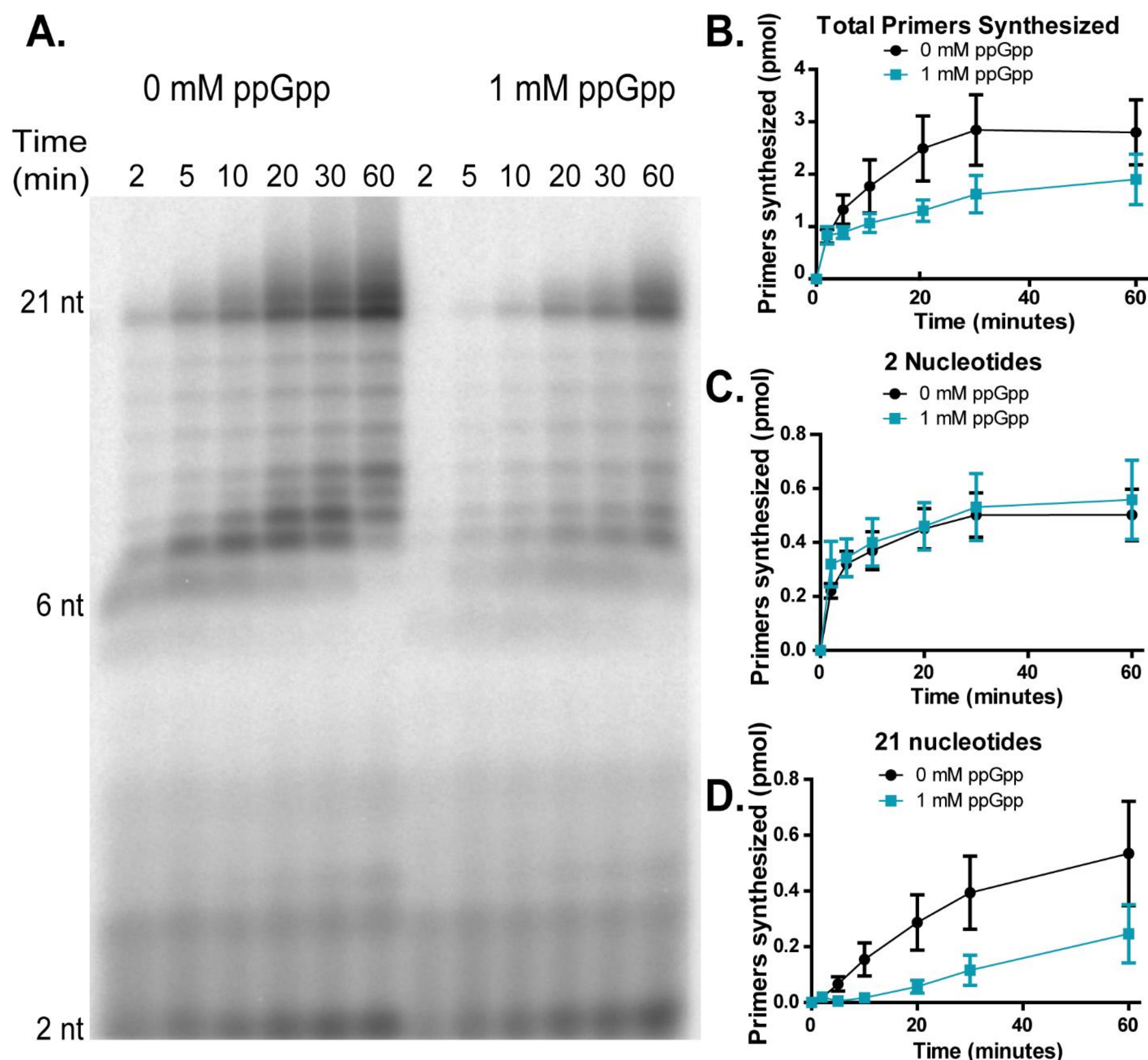



**Figure 3. ppGpp inhibits primer formation.** (A) Representative gel of primer synthesis over the course of an hour with 400 nM DnaG and 5 μM ssDNA. On the left is the synthesis without ppGpp. Reactions on the right contain 1 mM ppGpp. (B) Quantification of the full-length primers synthesized over the course of an hour without ppGpp (black line) and with ppGpp (blue line). (C) Quantification of the total number of primers synthesized over the course of an hour. In the absence of ppGpp (black line) the reaction was able to plateau by 30 minutes. Points represent averages of duplicate reactions, and the range is indicated by error bars. (D) Quantification of the initiating dinucleotide formed over the course of an hour without ppGpp (black line) and with ppGpp (blue line). Points represent n=3, and the SEM is indicated by error bars.

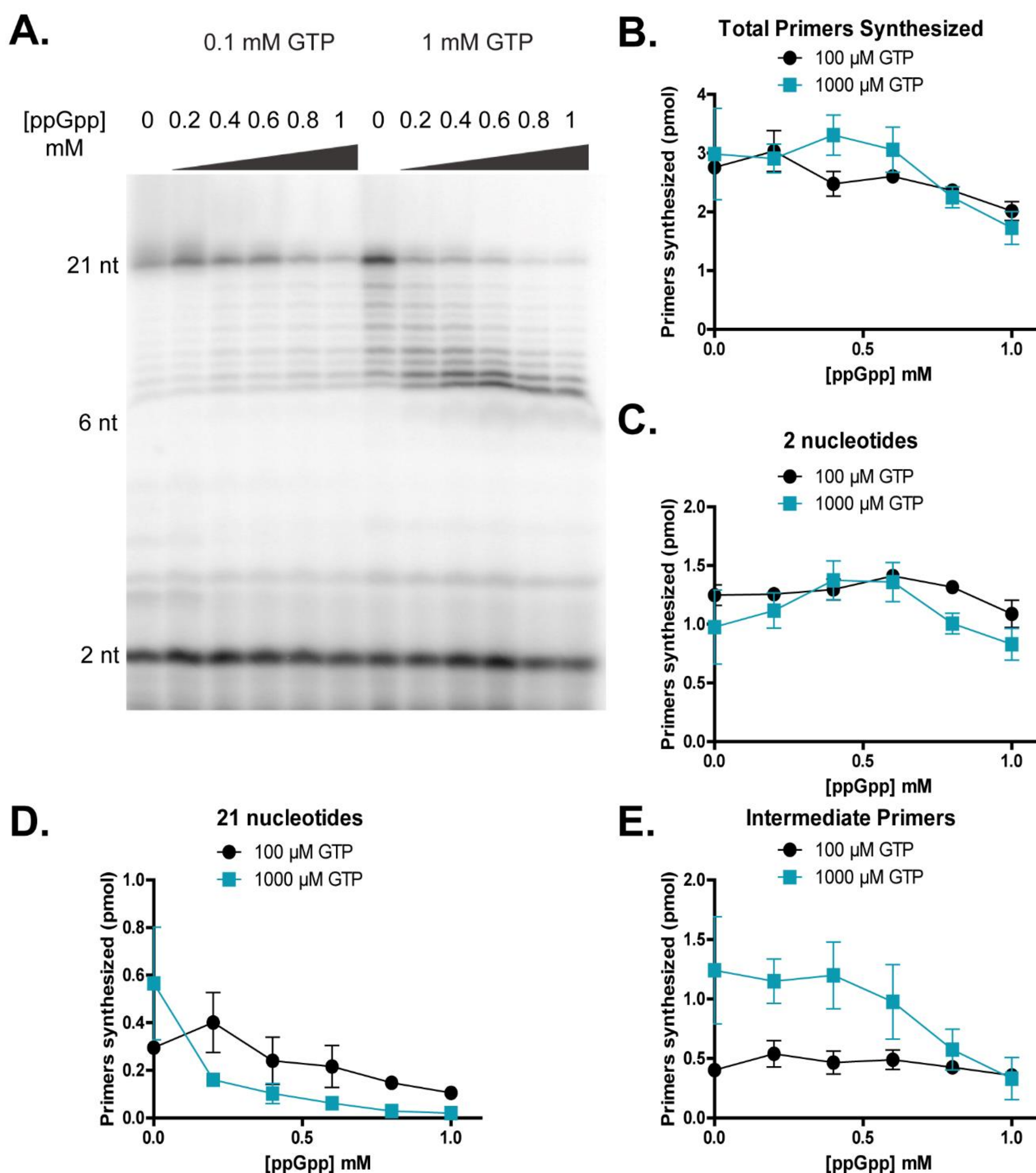



**Figure 4. (p)ppGpp inhibits elongation of RNA primers synthesized by *B. subtilis* DNA primase.** (A) Representative gel showing 5' labeled RNA primers produced with 6.4 μM DnaG, 500 nM oJW3319, and 1 mM each UTP and CTP, and indicated amount of GTP. RNA primer lengths were determined by comparison to a 21 nucleotide RNA standard with an identical sequence. (B-F) Quantification of primers synthesized over increasing amounts of ppGpp for the total number of primers (B), the initial dinucleotide (C), the full-length primer (D), and the intermediate primers between 2 and 21 nucleotides (E), and the total number of primers synthesized (E). Points represent averages of n=3, and the SEM is indicated by error bars.

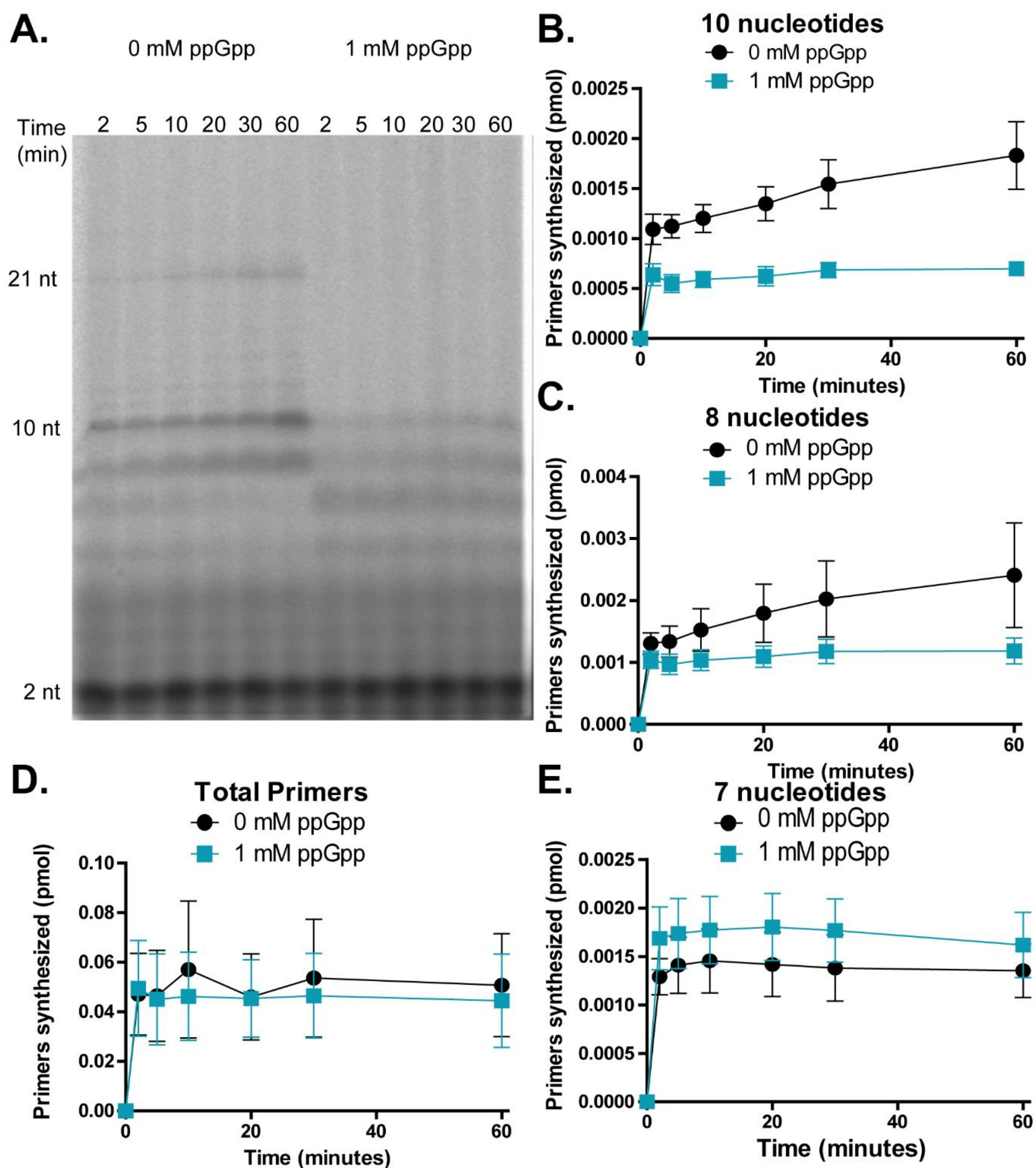



**Figure 5. ppGpp reduces the processivity of DNA primase.** (A) Representative gel of a primase activity time course in the presence of heparin with 400 nM DnaG and 5 μM ssDNA. An initial 'burst' phase is observed in the first two minutes shows that primase mostly forms 8 and 10 nucleotide (B and C) long primers before disassociation. Primase primarily synthesized 8 or 10 nucleotide long primers over the course of one hour, with the majority forming in the first 2 minutes. Addition of ppGpp reduced synthesis of 8 and 10 nucleotide long primers. A slight elevation in the amount of 7 nucleotide long primer was observed in the presence of ppGpp but was not signficant (D) while the total amount of primers initiated (excluding abortive dinucleotides) was mostly unchanged (E). Points represent averages of n=3, and the SEM is indicated by error bars.

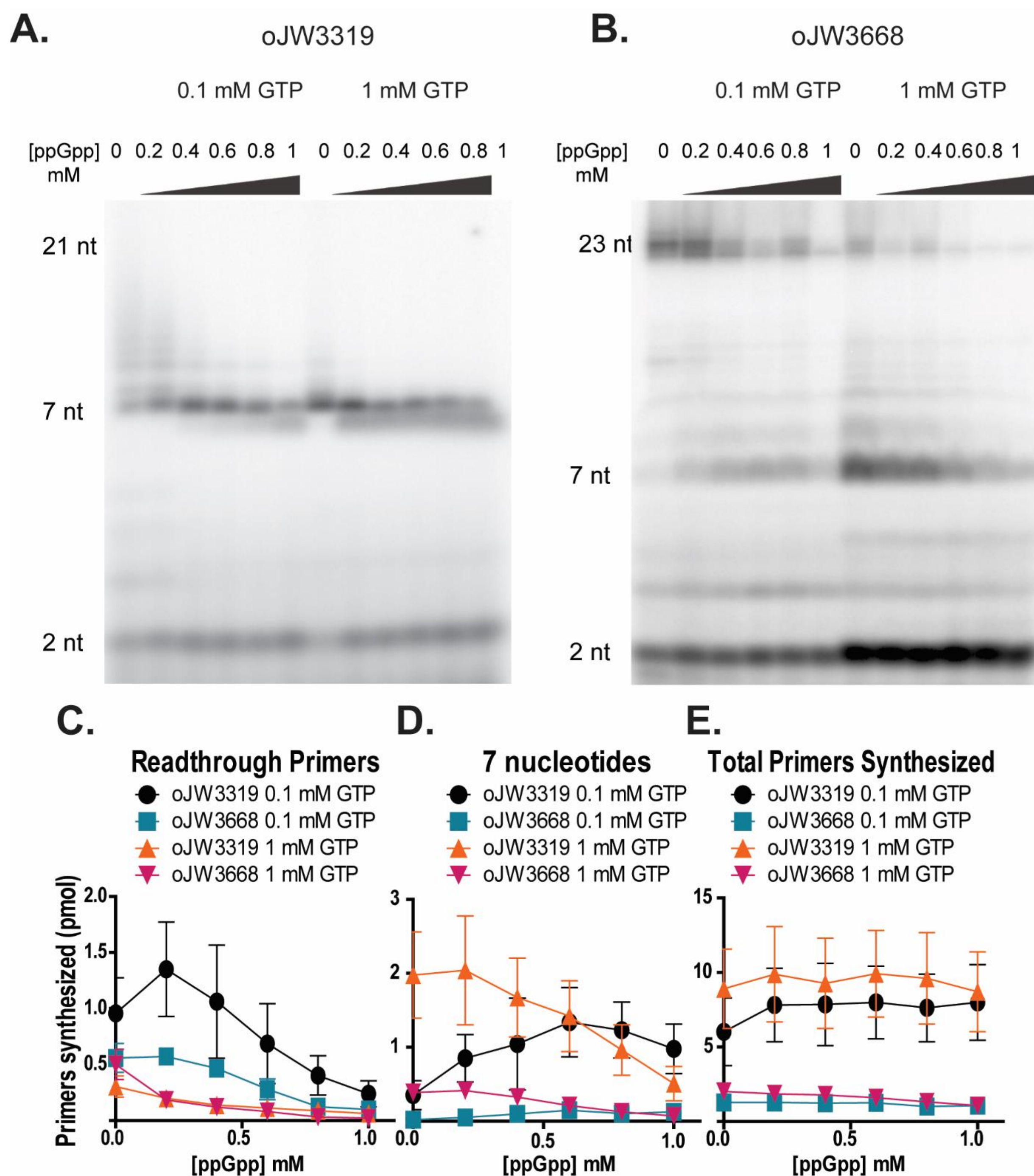
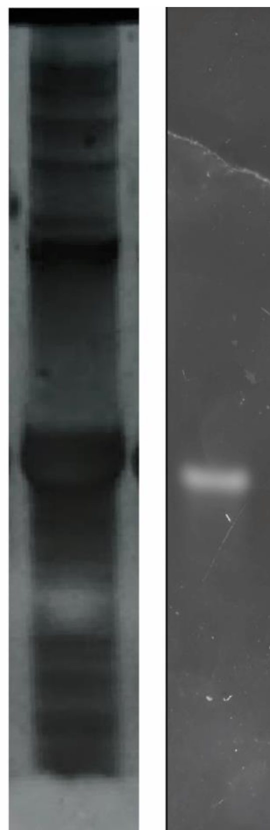
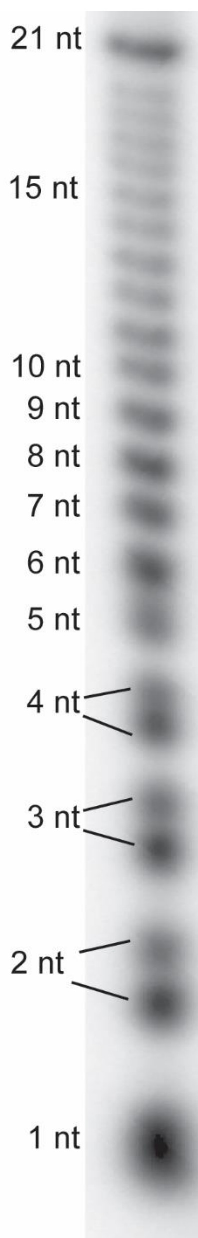



**Figure 6. ppGpp reduces priming readthrough at low levels of GTP.** (A, B) Representative gels of primase reactions with 6.4 μM DnaG and 500 nM oJW3319 or oJW3668, respectively, showing that primase reads through a pause site at low levels of GTP in the absence of ppGpp (expected product 7 nucleotides). (C-F) All line charts have the same y-axis units. Quantification of primers synthesized over increasing amounts of ppGpp for the readthrough primers (C), the expected 7 nucleotide primer (D), the total number of primers (E). Points represent averages of n=3, and the SEM is indicated by error bars.

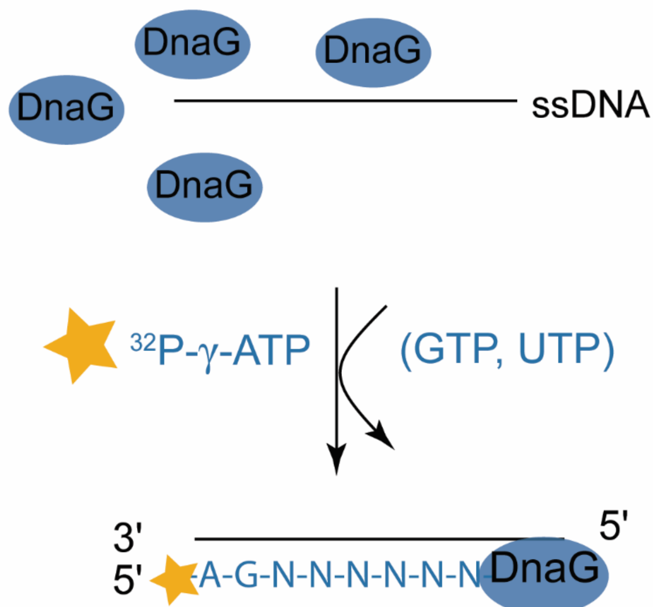



**Supplemental Figure 1. Oligonucleotide used in initial fluorescent based primase assay.** (Left) Run on 7M Urea 20% PAGE, the template used is not pure, but instead a heterogenous population. (Right) PAGE purified template shows the template is now pure and a purely homogenous population.

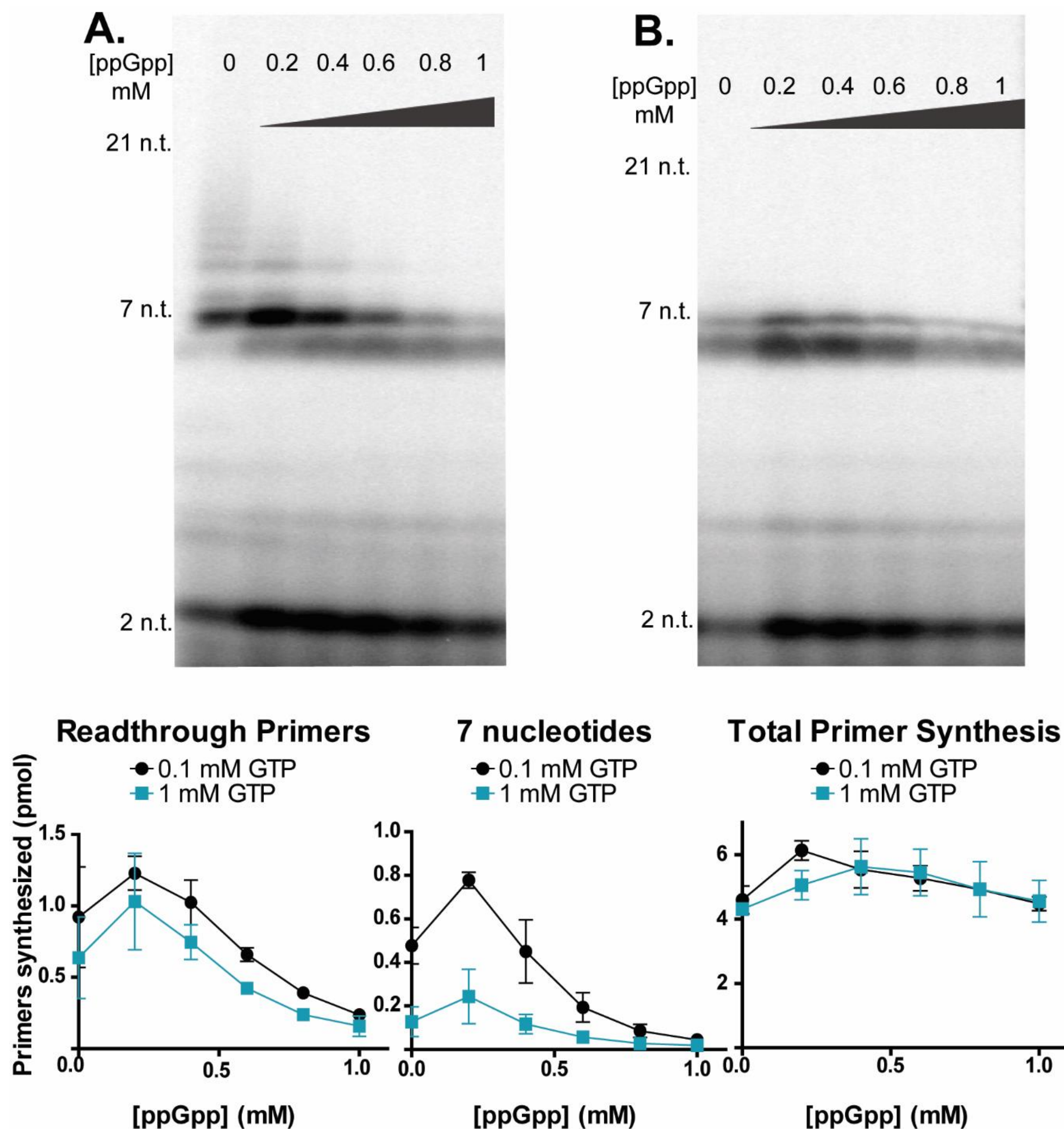



**Supplementary Figure 2. RNA standard ran alongside primase reactions.** Standard was generated by labeling the 5' end of an RNA with a sequence identical to that of the primase reaction product. The labeled RNA was subjected to NaOH hydrolysis to generate the RNAs of different lengths. The hydrolysis was able to occur on either the 2' or 3'-OH which is why on the very short RNAs (2-4) there are two different species.

```
 3′ -             A GAG AGATCCCCC GAG AAG AGA GAG AGA - 5′
Expected primer: 5′ -  pppAGGGGG - 3′
```




**Supplemental Figure 3. Schematic of the readthrough experiment done.** (Top) Sequence of ssDNA template going into the reaction. In bold and italics is the preferred primase start site. In bold is the pause site since CTP is withheld from the reaction. (Bottom) DNA primase is shown to saturate the ssDNA, 12.8:1. NTPs are added except for the CTP, which by withholding should produce a pause site that gives rise to the expected primer.




**Supplemental Figure 4. ppGpp binding to the RPD of DnaG is sufficient to inhibit priming readthrough at 0.1 mM GTP.** (A) Representative gel of primase activity of the DnaGΔCTD variant (6.4 µM, 500 nM oJW3319) showing that priming readthrough is still observed at 0.1 mM GTP and decreases upon addition of ppGpp. (B) Representative gel of primase activity of the DnaGΔCTD variant (6.4 µM, 500 nM oJW3319) at 1 mM GTP shows that no priming readthrough is observed. (C-E) Quantification of the total primers synthesized (C), the readthrough primers synthesized (D) and the amount of expected 7 nucleotide primer synthesized. Points represent averages of n=3, and the SEM is indicated by error bars.

**Table 1. List of oligonucleotides used as templates in primase assays. CTA start site is bolded as is the pause site G.**

| Name | Sequence | |
|---|---|---|
| oJW2646 | TACTTCCAATCCAATGCAATGGGAAATCGGATACCAGATGA | Forward primer to amplify *Bs dnaG* |
| oJW702 | TTATCCACTTCCAATGTTATTA)TTATTTTAAAGATCGGTTCAATG | Reverse primer to amplify full-length *dnaG* |
| oJW2649 | TTATCCACTTCCAATGTTATTACTGCTCAGTTAAAGACTCCT | Reverse primer to amplify the *dnaGΔCTD* |
| oJW2384 | CAGACACACACACACACACACACACACA**CTA**CACACA | Template used in primase assay in Figures 1 & 2 |
| oJW3319 | AGAGAGAGAGAAGA**G**CCCC**CTA**GAGAGA | Template used in primase assay in Figures 3-6 |
| oJW3668 | CACACACCAACAACCA**G**CACA**CTA**GAGAA/3InvdT/ | Template used in primase assay in Figure 6 |

**Table 2. Kinetic parameters related to Figure 1.**

Figure 1B

| [ppGpp] (µM) | $V_{max}$ (AFU) ± SEM | $K_{Mpseudo}$ (µM) ± SEM |
|---|---|---|
| 0 | 668.7 ± 54.9 | 35.5 ± 10.8 |
| 500 | 467.8 ± 63.7 | 36.8 ± 18.8 |
| 1000 | 376.2 ± 43.0 | 51.4 ± 20.5 |

Figure 1C

| [pppGpp] (µM) | $V_{max}$ (AFU) ± SEM | $K_{Mpseudo}$ (µM) ± SEM |
|---|---|---|
| 0 | 667.5 ± 140.4 | 40.6 ± 29.1 |
| 500 | 554.0 ± 38.4 | 37.8 ± 9.1 |
| 1000 | 455.5 ± 88.8 | 22.7 ± 18.1 |

Supplemental Figure 1

| [nucleotide] (µM) | $V_{max}$ (AFU) ± SEM | $K_{Mpseudo}$ (µM) ± SEM |
|---|---|---|
| 0 | 587.0 ± 46.8 | 8.87 ± 4.23 |
| 250 pppGpp | 221.9 ± 36.5 | 0.14 ± 4.42 |
| 500 ppGpp | 455.5 ± 88.8 | 22.7 ± 18.1 |
| 1000 pppGpp | 15.4 ± 4.8 | not determined |

## Chapter 3: Summary and Future Directions

C. N. G. wrote the entire chapter.

In my thesis work, I have investigated the mechanism by which the bacterial alarmone (p)ppGpp regulates the replication protein DNA primase. My work has shown that *B. subtilis* primase is more strongly inhibited at lower levels of GTP and high levels of (p)ppGpp. This regulation at low levels of GTP is physiologically relevant due to the reduction of GTP during the stringent response in *B. subtilis*. Through an optimized single nucleotide resolution assay, I determined that (p)ppGpp limits the extension of RNA primers and slows reassociation to the DNA. Further, I showed that this inhibition of extension occurs at high micromolar concentrations of (p)ppGpp, and that when (p)ppGpp reaches low millimolar concentrations, primase initiation is inhibited. Additionally, through primase experiments with heparin, I have shown that under normal conditions, *B. subtilis* DNA primase lays down 10 nucleotides before dissociating from the DNA. I showed (p)ppGpp regulation of DNA primase reduces primase processivity, since primase with (p)ppGpp in the reaction was unable to lay down 10 nucleotides before dissociation. Finally, I showed (p)ppGpp increases the fidelity of DNA primase at low levels of GTP. In the absence of (p)ppGpp, DNA primase reads through a pause that is induced by withholding a single nucleotide. As (p)ppGpp was added into the reaction, the amount of observed readthrough decreased. My work has shown that all these diverse effects of primase regulation are through (p)ppGpp interacting with the active site of its RNA polymerase domain. Although we used *in vitro* assays to characterize primase activity in isolation, we have gained important insights into the mechanism of primase regulation by (p)ppGpp.

**GTP levels differ between bacterial species upon induction of stress**

My thesis work has provided a potential explanation for the differing level of replication regulation by (p)ppGpp in different bacterial species. *In vitro* work has shown (p)ppGpp inhibition of DNA primase to be similar across species (Wang et al., 2007; Maciag et al., 2010; Rymer et al., 2012). *In vivo* work has shown a different story however, indicating a much stronger inhibition in *B. subtilis* than in *E. coli* (Denapoli et al., 2013; Maciag-Dorszyńska et al., 2013). The modest inhibition of replication elongation in *E. coli* can be exacerbated by deleting *gppA*, a phosphatase that converts pppGpp to ppGpp (Denapoli et al., 2013). In my own work I have shown that total primase activity inhibition is strongest at low levels of GTP and high levels of pppGpp, though high levels of ppGpp also inhibit total primase activity. Due to recent work showing that ppGpp and pppGpp can have different roles in a cell, it is tempting to speculate that pppGpp is the primary inhibitor of replication elongation in bacteria (Steinchen et al., 2015). We can even speculate that perhaps inhibition of elongation is stronger in *B. subtilis* than in *E. coli* due to its preferential production of pppGpp, whereas *E. coli* preferentially synthesizes ppGpp (Hara and Sy, 1983).

The disparity between the *in vivo* and *in vitro* results can be explained by our work showing that (p)ppGpp inhibition of *B. subtilis* DNA primase is strongest at high levels of (p)ppGpp and low levels of GTP. This was not observed with *E. coli* primase *in vitro* (Rymer et al., 2012). GTP levels are tightly regulated by (p)ppGpp in *B. subtilis*, even at basal levels, through inhibition of GTP biosynthesis enzymes such as HPRT, GMK, and GuaB (Kriel et al., 2012; Liu et al., 2015; Anderson et al., 2019). GTP levels in *E. coli* are not as stringently regulated during a stress response as they are in *B. subtilis*. For example, *B. subtilis* GMK, a

kinase responsible for converting GMP to GDP, is inhibited by (p)ppGpp whereas *E. coli* GMK is not (Liu et al., 2015). GTP regulation in *B. subtilis* is critical for cell survival. It would be interesting in a future study to see if lowering GTP levels, likely with a drug, and increasing pppGpp levels in *E. coli* cells (through a *gppA* deletion) would result in inhibition of replication elongation akin to that seen in *B. subtilis*. Ultimately this would be testing to see if lowered GTP levels coupled with high pppGpp strongly inhibits replication elongation in bacteria besides *B. subtilis*.

**Primase processivity and primase dissociation from the replication fork**

An unanticipated revelation from my work is the effect of (p)pGpp on primase processivity, a topic that is absent in the current literature. It has been known that primase is a non-processive and error-prone enzyme, but there had been no published reports looking at how long the synthesized RNA primers were *in vitro* in the presence of heparin. This absence of work looking at primase processivity is likely because RNA primers are removed, thus their only significance was the necessity of these RNA primers from which DNA polymerase could then begin its DNA duplication.

Additionally, our data suggest (p)ppGpp is affecting reassocation to the DNA during primer synthesis assays. In our time course assay with saturating amounts of ssDNA template, the total primers being synthesized is greater in the absence of ppGpp. We see that in these assays that full-length primers spanning the length of the template are synthesized over time. A reduction of shorter primers is observed while the amount of longer primers increases.

Taken together with the data that show that DnaG falls off the DNA after synthesizing 8-10 nucleotide primers, our results suggest that DnaG needs to reassociate with the DNA to

make full-length template-dependent primers. The reduction in the total number of primers synthesized and the amount of template-dependent primer synthesized when (p)ppGpp is added into the reaction suggests that (p)ppGpp is preventing DnaG from reassociating to the DNA. This is especially noteworthy because DnaG is one of the few proteins at the replication fork that is known to leave the fork and then revisit throughout replication. DNA primase is needed continuously throughout replication to prime the lagging strand every 1-2 kb (Kornberg and Baker, 1992). If primase is unable to be recruited to the replication fork then the lagging strand will not be synthesized, since DNA polymerase is unable to duplicate DNA *de novo*.

An interesting way to test the effect of (p)ppGpp on primase reassociation to the replication fork would be to examine Okazaki fragment synthesis in a separation of function primase mutant that is unable to bind (p)ppGpp but still retains enough activity to support viable growth. Structural work as well as my own data have confirmed that (p)ppGpp binds the active site of its RPD. Given the highly conserved nature of the active site residues, it could be a challenge to generate a viable but (p)ppGpp-insensitive mutant. Should such a variant be obtained Okazaki fragment synthesis should be examined, as well as replication fork progression (through gene expression microarrays or other similar experiments) and stability (through RecA-GFP recruitment).

It would also be interesting to test primase binding to DNA *in vitro*. In my work, I tested if saturating levels of DNA could disrupt (p)ppGpp binding and observed no effect. However, it would be interesting if, using radiolabeled ssDNA template, a future study could assess if (p)ppGpp disrupted DNA binding. Our *in vitro* primer synthesis data suggest that it would, but a lack of difference in ssDNA binding could also offer insights. In the event of no difference in

DNA binding, then would could hypothesize that (p)ppGpp is highly specific to inhibiting reassociation of primase to DNA, and perhaps then it is preventing binding to an RNA:DNA heteroduplex. Follow-up experiments could be conducted to test the heteroduplex as a ligand to determine if its binding is disrupted, similar to work previously done with the RPD of *E. coli* DnaG (Rymer et al., 2012).

**Readthrough, fidelity, and mutagenesis**

My work showed unexpectedly that (p)ppGpp regulation of DNA primase reduces priming readthrough, implicating an increase of priming fidelity. The fidelity of primase has never been examined, given that RNA primers are ultimately removed. Recently it has been shown that (p)ppGpp, together with DksA, has been shown to increase the fidelity of *E. coli* RNA polymerase (Roghanian et al., 2015). With RNA polymerase the increase in fidelity causes a reduction in the number of pausing events. Misincorporation events ultimately can slow down transcription, induce backtracking and thus cause a temporary pause of transcription. If left unchecked, these pauses can result in replication-transcription collisions. The increased fidelity of RNA polymerase leads to a reduction in transcriptional pausing, and by extension, a reduction in the number collisions. The only other factors known to regulate transcription fidelity are the Gre factors (Zenkin and Yuzenkova, 2015). GreA and GreB are cleavage factors that act on backtracked RNA polymerase elongation complexes, resolving these complexes more than preventing them (Zenkin and Yuzenkova, 2015).

Increased fidelity has never been reported for DNA primase. What is interesting about the increase in priming fidelity I observed is that the amount of pausing increases rather than decreases. Since primase is known to be the most error-prone enzyme in replication, with a

misincorporation estimated to occur every 20 nucleotides (Griep, 1995), it is possible that fidelity is the by-product of this regulation and not the sought after result. I suspect that by making primase more selective about its substrates, (p)ppGpp is slowing primase, which in turn could slow and stop the progression of the replication fork due to primase's interactions with other replication proteins (see below for a more detailed discussion).

If replication goes unchecked during periods of nutrient starvation, fidelity in replication may be lost due to a potential dNTP imbalance, an effect known to increase mutagenesis (Nordman and Wright, 2008; Kumar et al., 2011; Schaaper and Mathews, 2013). Given that NTPs are the precursor to dNTPs, it would follow that by creating an imbalance in cells, fidelity could be disrupted. There has been previous work in *E. coli* that has shown that imbalanced dNTP pools in a cell increase mutagenesis (Schaaper and Mathews, 2013).

Since *B. subtilis* GTP levels decrease during the stringent response, this would perhaps create an imbalanced dNTP pool (Kriel et al., 2012). Without proper regulation of replication, cells might experience an increase in mutagenesis. By halting the replication fork in a non-disruptive manner then, cells are no longer as susceptible to mutagenesis as a result of an imbalanced dNTP pool. It would be interesting in future work to study the mutagenesis of *B. subtilis* cells with a primase mutant no longer regulated by (p)ppGpp.

**Primase interacts with several proteins at the replication fork**

Though my work has only looked at primase in isolation, it offers important insights into how the replication fork might be regulated during stress. Since DnaG has been shown to interact with other replication proteins such as SSB and the replicative helicase, it is likely that inhibiting primase may inhibit these proteins (Wu et al., 1992; Shereda et al., 2008). For

example, early work has shown that the interaction between primase and the replicative helicase impacts the synthesis of Okazaki fragments (Tougu and Marians, 1996). Since then, it has also been shown that primase and helicase affect each other's activity in a species specific manner (Lu et al., 1996; Johnson et al., 2000; Koepsell et al., 2006; Van Eijk et al., 2016). It has also been shown that helicase modulates the size of primers synthesized by DnaG (Johnson et al., 2000; Thirlway and Soultanas, 2006).

It is tempting to speculate that regulation of DnaG activity could by extension regulate the activity of the helicase. Previous work has demonstrated a physical interaction between the DNA polymerase and the primosome via a τ-DnaB contact (Kim et al., 1996). Another study, done in the bacteriophage T7, has also shown that the interaction between DNA polymerase and the replicative DNA helicase is required for fork processivity (Hamdan et al., 2007). Given this work, it is not far reaching to hypothesize that regulating primase activity could halt the replicative helicase and the DNA polymerase.

How (p)ppGpp can regulate the replication fork in a non-disruptive manner though, remains to be established. Recent work has found that DNA breakage is not a frequent occurrence in cells, and that it appears to happen in only approximately 18% of cells in each generation (Sinha et al., 2018). They propose that the replicative helicase remains on the DNA at intact but inactivated replication forks, and the replication restart proteins reactivate the fork. I suspect that regulation of DNA primase, through its interactions with other replication proteins, halts the replication fork but allows for the fork to remain intact. This would allow for the DNA to remain undamaged, and for replication to then resume via a restart pathway when the stress is alleviated.

Future studies could perhaps look at RecA recruitment in cells that have a disrupted primase-helicase or helicase-polymerase interaction to determine if (p)ppGpp is affecting the replication fork through their interactions with primase. Additionally, it would be interesting to study if the replication fork remains intact and on the DNA upon exposure to nutrient starvation. I think this would be especially interesting if it could be done in cells with fluorescently labelled replication fork proteins.

In addition to its interactions with the replicative helicase and with SSB, primase has also been shown to interact with the error-prone DNA polymerase DnaE, found in low G-C Firmicutes (Velten et al., 2003; Smits et al., 2010; Rannou et al., 2013). While DnaE resembles the PolIII-α in *E. coli*, it is not involved in the bulk replication of DNA, and it briefly extends the RNA primers (Sanders et al., 2010). This resembles the replication system in eukaryotes which requires two polymerases (δ and ϵ) for replication (Dervyn et al., 2001; Le Chatelier et al., 2004).

Recent work has suggested that DnaE might play a more prominent role in lagging strand synthesis, akin to the DNA polymerase δ found in eukaryotes (Johnson et al., 2015; Paschalis et al., 2017). The 2017 study showed that DnaE is not as error-prone *in vivo* as it is *in vitro*, due to its interaction with DnaN: DnaE misincorporations are proofread by the 3' > 5' exonuclease activity, and misincorporations are also removed by the mismatch repair system (Paschalis et al., 2017). It is possible then if DnaE plays a more prominent role in replication than previously proposed, that regulation of primase could by extension regulate the activity of DnaE. This idea needs further testing though, particularly to fully determine the role of DnaE in replication of low G-C Firmicutes.

**Final perspectives**

The field of DNA primase regulation has remained stagnant throughout my graduate school tenure. When I joined the lab, the last work published on (p)ppGpp and DNA primase was in 2013 and we still did not know why the inhibition of replication elongation is stronger in *B. subtilis* than in *E. coli*, nor did we fully understand the importance of this regulation. We also did not know of a potential ligand binding site on the C-terminal helicase interaction domain of DNA primase. My work has advanced this field, pushing it forward by characterizing the mechanism of DNA primase regulation by (p)ppGpp. My work has begun characterization of the processivity of primase, and how (p)ppGpp clearly diminishes this processivity. Additionally, I unexpectedly uncovered that (p)ppGpp increases the fidelity of DNA primase. I expect that this forces primase to slow down, and by extension the rest of the replication fork will slow before finally halting. For many years the question I have received has been – "Why bother regulating DNA primase if the RNA primers are just going to be removed anyway?" The best way to answer this is to look beyond primase in isolation and consider that primase associates with many proteins at the replication fork such as the replicative helicase, the error-prone polymerase DnaE, and SSB. On top of that, primase is one of the few proteins that regularly dissociates and reassociates to the replication fork, making it a prime target for binding to ligands such as (p)ppGpp. Though my work has begun to shed light on the importance of regulating DNA primase, more work remains to be done in future studies. My work only considered primase in isolation, and I think it is critical that regulation of primase in the context of the replication fork is the next step to understanding how this regulation is non-disruptive to cells.

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## Appendix I: Characterizing (p)ppGpp binding to DNA primase domains.

Contribution:

C. N. G. performed most of the experiments and wrote the appendix.

Andrew Voter from the Keck lab performed the fluorescence polarization experiment.

**Summary**

(p)ppGpp has recently been shown to have two distinct binding sites on the RNA polymerase of *E. coli* (Ross et al., 2013, 2016). Additionally, recent work found a site on the CTD of *S. aureus* DnaG that can bind ligands when those ligands were provided in millimolar concentrations (Catazaro et al., 2017). Previously had shown a crystal structure of the RPD of *S. aureus* bound to (p)ppGpp, sharing part of the NTP binding site (Rymer et al., 2012). Since bacterial DNA primase has three distinct domains (Frick and Richardson, 2001), I sought to determine if (p)ppGpp was able to bind any of the other domains. I constructed the domain variants of primase (ZBD, RPD, HID; Figure 1A) and tested binding of (p)ppGpp to these variants with DRaCALA. I observed that (p)ppGpp does not bind the ZBD, but it binds the RPD and HID as well as the full-length primase (Figures 1B, 1C). Assessing the binding affinity of all these variants determined that the RPD and HID bind to (p)ppGpp more strongly than the full-length ($K_d$ 500 nM, 2 µM, 8 µM, respectively; Figure 1D). This may be due to the full-length protein being larger and having flexible linkers connecting the domains. I suspect the domains are moving around, limiting access to the binding sites on primase. The final thing I tested was with Andrew Voter from the Keck lab, to determine if (p)ppGpp bound to the same site as the C-terminal tail of SSB (Shereda et al., 2008) (SSBct). Using fluorescence polarization with a fluorescently labelled *B. subtilis* SSBct, we tested if unlabeled pppGpp could compete with the SSBct. Preliminary results show that pppGpp was not an effective competitor, compared with unlabeled SSBct (Figure 1E), indicating that (p)ppGpp is not competing with SSB binding on DNA primase. An outstanding question that remains with this work is where does (p)ppGpp bind to the HID? Is it interfering with the interaction between primase and helicase? Further work is

necessary to address these questions, and to confirm our results that (p)ppGpp binds to the HID.

**Material and Methods**

**Primase Protein Purification**

BsDnaG full-length, ZBD, RPD, and CTD constructs were generated and the coding DNA sequence was verified for all constructs (FunctionalBio). All proteins were expressed in BL21 DE3 cells and purified by Ni –affinity chromatography over 1-mL HisTrap column. Lysis buffer consisted of 300 mM NaCl, 20 mM imidazole, 40 mM HEPES pH 7.8-8.0. Stepwise elution was carried out from 100% lysis buffer to 100% of an otherwise equivalent buffer containing 500 mM imidazole over X column volumes. Proteins were then exchanged into a storage buffer that consisted of 40 mM HEPES pH 7.8, 300 mM NaCl, 10% Glycerol, 1 mM EDTA, 1 mM DTT. Purity was assessed using polyacrylamide gel electrophoresis and Coomassie staining and protein preparations were confirmed to be >= 95% pure Concentration was determined by absorption at 280 nm using the following coefficients: 46,760 M-1cm-1 (full-length), 5960.00 M-1cm-1 (ZBD), 5500.00 M-1cm-1 (RPD), and 12950.00 M-1cm-1 (HID).

**Synthesis, Purification, and Quantification of (p)ppGpp**

(p)ppGpp was synthesized in vitro using RelSeq1–385 and GppA, purified, and quantified as described (Mechold et al., 2013). 6 mM GTP was mixed with 8 mM ATP, 300 µg RelSeq1NH385, 0.5 mM dithiothreitol, 25 mM bis-Tris propane pH 9.0, 15 mM MgCl2 and allowed to incubate at 37°C for 2-5 hours to generate pppGpp. ppGpp was then synthesized upon addition of GppA and allowed a brief 1 hour incubation. (p)ppGpp was purified with an anion exchange column (HiTrap QFF 1 mL; GE Healthcare) using a binding buffer (0.1 mM LiCl,

0.5 mM EDTA, 25 mM Tris pH 7.5). Stepwise elution was carried out from 100% binding buffer to 50% of an otherwise equivalent buffer containing 1 M LiCl.

**Differential Radial Capillary Action of Ligand Assay (DRaCALA)**

DRaCALA was performed with pure protein and radioactive ligand as described (Roelofs et al., 2011). [5′ α-32P] (p)ppGpp was synthesized according to modified protocols of non-radioactive and radioactive (p)ppGpp synthesis (Mechold et al., 2002). The reaction contained 25 mM bis-Tris propane (pH 9.0), 15 mM MgCl2, 0.5 mM DTT, 2 mM ATP, 2 µM RelSeq (1-385), and 37.5 µCi [α-32P] GTP (Perkin Elmer). The reaction was incubated at 37°C for 1 hour. The reaction was diluted in 0.5 mL of Buffer A (0.1 mM LiCl, 0.5 mM EDTA, 25 mM Tris-HCl pH 7.5) prior to adding to a 1 mL HiTrap QFF strong anion exchange column (GE Healthcare) quilibrated with 10 column volumes (CV) of 83% Buffer A + 17 % Buffer B (Buffer B: 1 M LiCL, 0.5 mM EDTA, 25 mM Tris-HCl pH 7.5). 32P-(p)ppGpp was eluted with a mixture of 50% Buffer A + 50% Buffer B. Fractions of 1 mL were collected from the elution.

DRaCALA reactions using purified DnaG or DnaG domains contained 40 mM HEPES pH 7.5-7.8, 1 mM MnCl2, 10 mM MgAc, 150 mM NaCl, 5% Glycerol, 5 mM DTT, 10 µM DnaG (diluted in 40 mM HEPES pH 7.5-7.8, 150 mM NaCl, 5% Glycerol, 5 mM DTT) and 32P-(p)ppGpp (1:100 final dilution of first elution fraction of 32P-(p)ppGpp purification). Reactions were incubated for 10 minutes at room temperature. Two microliters from each reaction were spotted in duplicate on Protran BA85 nitrocellulose (GE Healthcare) via pipette or a replicator pinning tool (VP 408FP6S2; V and P Scientific, Inc). Spots were allowed to dry, and radioactivity was detected with phosphorimaging (Typhoon FLA9000). Fraction bound of $^{32}$P-(p)ppGpp was calculated as described (Roelofs et al., 2011). Data were analyzed in GraphPad Prism v5.02 and

binding curves were fitted to the equation Y = (Bmax × Xh) / (Kdh + Xh), where h is the Hill coefficient.

**Fluorescence polarization**

Expression and purification of the fluorescein-labeled, and unlabeled *Bacillus subtilis* SSB C-terminus tail (SSBct) was performed in the Keck lab using methods adapted from previous work SSBct (Voter et al., 2016; Dubiel et al., 2019). The FP measurement was carried out in a black 96-well plate (ThermoFisher). Fluorescence polarization was done using an N-terminally fluorescein-labeled *B. subtilis* SSBct, unlabeled *B. subtilis* SSBct, unlabeled pppGpp, and the purified C-terminal HID of *B. subtilis* DnaG. The DnaG-CTD was preincubated with the labeled SSBct in 40 mM HEPES pH 7.5-7.8, 1 mM MnCl2, 10 mM MgAc, 5% Glycerol, 5 mM DTT, for at least 20 minutes, covered with foil. Unlabelled SSBct or pppGpp was added to the reaction in increasing amounts and FP values were measured on a Biotek Synergy 2 plate reader.

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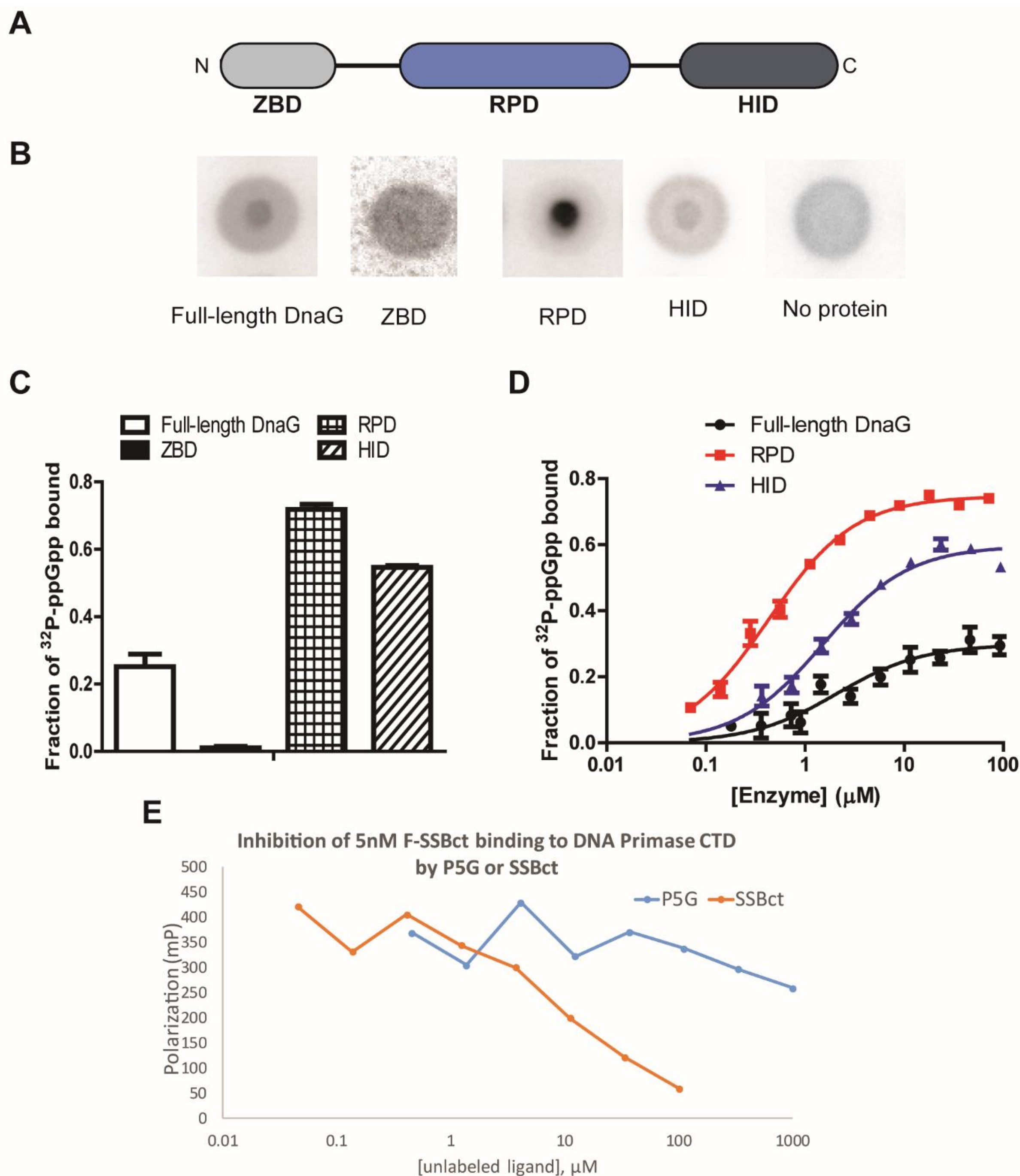


**Figure 1. (p)ppGpp binding to DNA primase domains.**
A) Domain architecture of bacterial DNA primase. The N-terminal zinc-binding domain (ZBD), the active RNA Polymerase domain (RPD), and the C-terminal helicase interaction domain (HID). B) DRaCALA of individual primase domains to $^{32}$P-pppGpp. C) Quantification of fraction of $^{2}$P-pppGpp bound to the respective primase prep. D) Binding affinity of DNA primase and its two domains that bind pppGpp show that the RPD ($K_d$ ~500 nM) and HID ($K_d$ ~2 μM) bind to pppGpp more strongly than the full-length DnaG ($K_d$ ~8 μM). E) Fluorescent polarization to assess if pppGpp (blue line) inhibits binding of the C-terminal tail of SSB.