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Multiple mechanisms for overcoming lethal over-initiation of DNA replication

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Abstract

DNA replication is highly regulated and primarily controlled at the step of initiation. In bacteria, the replication initiator DnaA and the origin of replication *oriC* are the primary targets of regulation. Perturbations that increase or decrease replication initiation can cause a decrease in cell fitness. We found that multiple mechanisms, including an increase in replication elongation and a decrease in replication initiation, can compensate for lethal over-initiation. We found that in *Bacillus subtilis*, under conditions of rapid growth, loss of *yabA*, a negative regulator of replication initiation, caused a synthetic lethal phenotype when combined with the *dnaA1* mutation that also causes replication over-initiation. We isolated several classes of suppressors that restored viability to *dnaA1 ΔyabA* double mutants. Some suppressors (*relA*, *nrdR*) stimulated replication elongation. Others (*dnaC*, *cshA*) caused a decrease in replication initiation. One class of suppressors decreased replication initiation in the *dnaA1 ΔyabA* mutant by causing a decrease in the amount of the replicative helicase, DnaC. We found that decreased levels of helicase in otherwise wild-type cells were sufficient to decrease replication initiation during rapid growth, indicating that the replicative helicase is limiting for replication initiation. Our results highlight the multiple mechanisms cells use to regulate DNA replication.

KEYWORDS
Bacillus subtilis, DNA helicase, DNA replication, replication elongation, replication initiation

1 | INTRODUCTION

DNA replication is a highly regulated, essential process across all domains of life. Most organisms control DNA replication based on environmental cues and nutrient conditions to ensure each daughter cell has a complete copy of the genome. Multiple mechanisms are used to coordinate DNA replication with other cellular processes, including metabolism and cell division (reviewed in Kellogg & Levin, 2022). Failure to properly regulate DNA replication can result in a variety of consequences, including cell division defects, anucleate daughter cells, DNA damage, and in higher organisms, disease (Magdalou et al., 2014; O'Donnell et al., 2013).

Bacteria typically have a single circular chromosome and replication initiates bi-directionally from a single origin of chromosomal replication, *oriC*. Under nutrient-rich growth conditions, certain bacteria, including *Escherichia coli* and *Bacillus subtilis*, can undergo multifork replication; that is, they initiate a new round of DNA replication before the previous round has finished. Multifork replication results in each daughter cell receiving a chromosome with active replication forks and multiple origins, enabling cells to divide more

quickly, while still ensuring each daughter cell receives a completed chromosome (reviewed in: Jameson & Wilkinson, 2017; Katayama et al., 2017; Skarstad & Katayama, 2013). In wild-type cells, there is nutritional control of replication initiation. During growth in nutrient-poor conditions, there is typically less replication initiation compared with that under growth in nutrient-rich conditions. Over-initiation can lead to replication fork collapse and the DNA damage response, in addition to problems with cell division and chromosome segregation (Bach & Skarstad, 2004; Katayama, 2001; O'Donnell et al., 2013; Simmons et al., 2004). The flexibility in regulating the rate of replication initiation gives some bacteria the advantageous ability to reliably adjust growth and division based on environmental and internal cues.

In bacteria, DNA replication initiation is primarily regulated by controlling the levels and-or activity of the replication initiator, DnaA. DnaA is AAA+ ATPase that binds both ADP and ATP (reviewed in: Davey et al., 2002; Ozaki & Katayama, 2009). The ATP-bound form is active for replication initiation and binds cooperatively to sites located in *oriC*. This cooperative binding causes the formation of a nucleoprotein helical filament that melts the AT-rich DNA unwinding element (DUE) (reviewed in: Jameson & Wilkinson, 2017; Katayama et al., 2017; Leonard & Grimwade, 2005; Ozaki & Katayama, 2009). In *B. subtilis*, DnaA recruits additional proteins required for chromosome organization and helicase loading (DnaD and DnaB), followed by loading of the replicative helicase (DnaC) by the helicase loader (DnaI). After additional unwinding by the helicase, the remaining replication machinery is recruited to the origin and DNA replication proceeds bi-directionally (reviewed in: Jameson & Wilkinson, 2017; Kaguni, 2006; Leonard & Grimwade, 2011; Mott & Berger, 2007).

The levels and activity of DnaA directly affect replication initiation. For example, increased expression of *dnaA* causes over-initiation in both *E. coli* and *B. subtilis* (Atlung et al., 1987; Ogura et al., 2001; Skarstad et al., 1989) and various *dnaA* mutations have been characterized that either enhance or inhibit DNA replication initiation (Guo et al., 1999; Moriya et al., 1990; Murray & Errington, 2008; Scholefield & Murray, 2013). One such mutation in *B. subtilis*, *dnaA1* {a serine to phenylalanine change at amino acid 401F (S401F)} causes a temperature-sensitive growth phenotype. Replication initiation is inhibited at non-permissive temperatures where the DnaA1 mutant protein is unstable (Moriya et al., 1990).

DnaA is regulated by mechanisms that prevent its cooperative binding at the origin. In *E. coli*, this is primarily accomplished by regulating the availability of ATP-bound DnaA, either through sequestration or titration (*seqA/datA*) or regulated inactivation of DnaA (RIDA) by Hda (reviewed in: Kaguni, 2006; Katayama et al., 2017; Leonard & Grimwade, 2011). However, in gram-positive bacteria like *B. subtilis*, DnaA is regulated by direct interactions with several proteins that affect cooperative binding, including YabA (Bonilla & Grossman, 2012; Jameson & Wilkinson, 2017; Merrikh & Grossman, 2011; Scholefield et al., 2012; Scholefield & Murray, 2013; Wagner et al., 2009). YabA binds directly to DnaA and prevents the necessary cooperative binding at DnaA boxes in *oriC*, thereby inhibiting the formation of the nucleoprotein filament and melting of the origin (Merrikh &

Grossman, 2011; Scholefield & Murray, 2013). YabA also stimulates the dissociation of DnaA from *oriC* (Schenk et al., 2017). A null mutation in *yabA* leads to increased and asynchronous replication initiation (Goranov et al., 2009; Hayashi et al., 2005; Noirot-Gros et al., 2002).

We found that the *dnaA1* mutation causes over-initiation at permissive temperatures, and that combining *dnaA1* with a null mutation in *yabA* resulted in a more extreme over-initiation phenotype that was lethal when cells were grown in rich medium. We leveraged this conditional synthetic lethal phenotype to isolate suppressors that restored viability to the cells that had extreme over-initiation of replication, with the aim of elucidating novel mechanisms of regulation of DNA replication. By selecting for survival under conditions of rapid growth (LB medium), we identified mutations affecting five different genes that suppressed the lethal phenotype of the *dnaA1 ΔyabA* double mutant. We found that null mutations in *dnaC* and *cshA* suppressed lethality by decreasing replication initiation, whereas a null mutation in *nrdR* and a mutation affecting the (p)ppGpp synthetase domain of *relA* suppressed lethality by stimulating replication elongation, likely to keep pace with increased initiation. We found that mutations that decrease levels of the replicative helicase, DnaC, were sufficient to limit DNA replication initiation under conditions of high replication initiation, either in the over-initiating *dnaA1 ΔyabA* background or during rapid growth of otherwise wild type cells. In addition to elucidating novel genes that can regulate replication initiation, the results of this screen highlight the multiple ways cells can regulate DNA replication, by employing changes in other cellular processes to compensate for replication over-initiation.

2 | RESULTS

2.1 | Phenotypes of *dnaA1 ΔyabA* mutants

dnaA1(S401F) of *B. subtilis* causes a temperature sensitive phenotype due to loss of replication initiation at non-permissive temperatures (Moriya et al., 1990). DnaA is also a transcription factor and at permissive temperature, the DnaA1 mutant protein has increased activity as a transcription factor (Burkholder et al., 2001). We found that the *dnaA1* mutation also caused increased replication initiation at permissive growth temperatures. We measured replication initiation in the *dnaA1* mutant by marker frequency analysis of the origin (*ori*) and terminus (*ter*) regions of chromosomes for cells grown in defined minimal medium with glucose as a carbon source. Increased *ori/ter* typically indicates increased initiation (or decreased elongation, see below). The *dnaA1* mutant had an approximately 30% increase in *ori/ter* (Table 1), consistent with an increase in initiation of DNA replication. The increase in replication initiation is likely due to an increase in the activity of the DnaA1 mutant protein at the permissive temperature, similar to its increased activity as a transcription factor (Burkholder et al., 2001).

YabA is a negative regulator of replication and functions to inhibit cooperative binding of DnaA to DNA (Merrikh & Grossman, 2011;

Strain	Relevant genotype	Relative *ori/ter*[a]	Doubling time[b]
AG1866	Wild type	1.00 ± 0.04	52.0 ± 1.3
KPL2	*dnaA1*	1.28 ± 0.12	60.8 ± 1.1
MEA64	Δ*yabA*	1.38 ± 0.05	58.0 ± 1.7
CAL2320	*dnaA1* Δ*yabA*	1.84 ± 0.26	101.6 ± 8.1
MEA537	*dnaA1* Δ*yabA* Δ*nrdR*	0.84 ± 0.09	56.1 ± 6.0
MEA102	*dnaA1* Δ*yabA* *relA102*(Δ264–295)[c]	1.04 ± 0.31	80.3 ± 2.9
MEA370	*dnaA1* Δ*yabA* P*dnaC*(T7)	1.11 ± 0.02	53.9 ± 2.5
MEA303	*dnaA1* Δ*yabA* Δ*cshA*	1.55 ± 0.21	83.1 ± 1.6
MEA323	*dnaA1* Δ*yabA* Δ*ccrZ*	1.36 ± 0.28	54.1 ± 2.5

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

[b]Strains were grown at 37°C in minimal glucose medium. OD_{600} was measured throughout exponential growth phase and plotted versus time. Doubling times were determined from the linear portion of the plot log(OD_{600}) versus time. A minimum of 3 biological replicates were included for average and standard error of the mean.

[c]MEA102 is the originally isolated suppressor mutation in *relA*. All other strains are reconstructed in a clean background that was not mutagenized.

Noirot-Gros et al., 2002). As such, null mutations in *yabA* cause an increase in replication initiation (Goranov et al., 2009; Hayashi et al., 2005). It is possible that the increase in replication initiation caused by the *dnaA1* mutation could be due to an inability of YabA to properly inhibit the mutant DnaA. If true, then a *dnaA1* Δ*yabA* double mutant would have the same phenotype as a Δ*yabA* single mutant.

Based on the known biochemical functions of DnaA and YabA in replication initiation, we conclude that the *dnaA1* Δ*yabA* double mutant has an increase in replication initiation. It is likely that there is also a decrease in replication elongation, caused indirectly by the over-initiation (see below). If true, then the increase in *ori/ter* is due to a combination of increased replication initiation and decreased replication elongation.

In addition, *ori/ter* is influenced by the availability of nutrients (among other things) and there is typically a correlation between *ori/ter* and growth rate, although growth rate per se does not determine *ori/ter* (Murray & Koh, 2014; Sauer et al., 2016). In the nutrient rich conditions, growth rate and *ori/ter* are higher than in nutrient poor conditions (Murray & Koh, 2014; Nouri et al., 2018). The *dnaA1* Δ*yabA* single and double mutants had a slower growth rate (mass doubling) than wild-type cells, yet still had an increase in *ori/ter* (Table 1).

2.1.1 | Growth and replication phenotypes of *dnaA1* Δ*yabA* double mutants

We found that a *dnaA1* Δ*yabA* double mutant had a severe growth defect and an increase in *ori/ter* compared with either single mutant. We int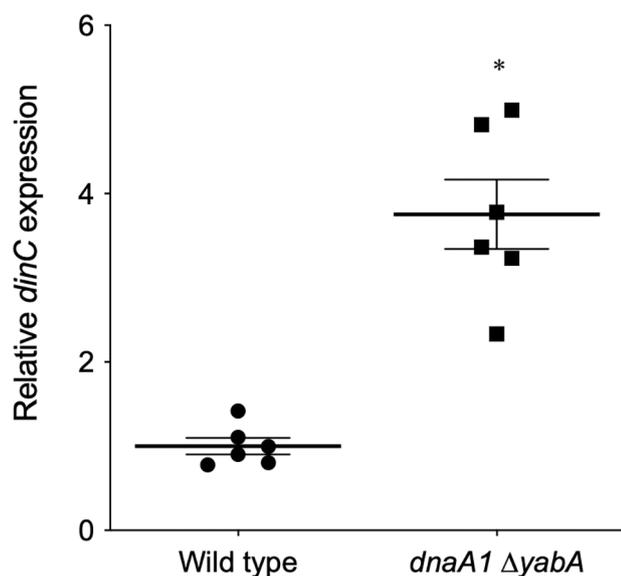roduced a *yabA* null mutation (Δ*yabA*::*spc*, simply referred to as Δ*yabA*) into a *dnaA1* mutant. Immediately, it was apparent that the double mutant had some sort of growth defect. It made small colonies on agar plates with minimal medium. The *dnaA1* Δ*yabA* mutant had a doubling time of 102 min compared with 52 min for the isogenic wild type strain in defined minimal liquid medium, and the *ori/ter* ratio was greater than that of either single mutant and ~80–85% greater than that of wild-type cells (Table 1). These results indicate that the effects of *dnaA1* and Δ*yabA* were roughly additive and we conclude that the primary defect of the *dnaA1* mutant is not loss of interaction between DnaA1 and YabA.

2.1.2 | SOS response in the *dnaA1* Δ*yabA* mutant

Too much replication initiation can lead to collapse of replication forks (Charbon et al., 2014; Simmons et al., 2004) and induction of the *recA*-dependent SOS response (Kreuzer, 2013; Simmons et al., 2008). We found that the SOS response was induced in the *dnaA1* Δ*yabA* double mutant growing in defined minimal glucose medium. We used RT–qPCR to measure mRNA levels of the DNA damage-inducible gene *dinC* (Cheo et al., 1991; Gillespie & Yasbin, 1987; Goranov et al., 2006; Love et al., 1985) relative to that of so-called housekeeping genes *rpoD* (*sigA*) and *gyrA*. During growth in minimal medium, levels of *dinC* mRNA were increased approximately 4-fold in the *dnaA1* Δ*yabA* double mutant, relative to that in the wild-type cells (Figure 1). These results indicate that there is induction of the SOS response in the *dnaA1* Δ*yabA* double mutant. We infer that this is likely because of the replication fork collapse caused by too much replication initiation. This could explain the increase in doubling time of the population and the small colony size of the *dnaA1* Δ*yabA* double mutant.

2.1.3 | dnaA1 ΔyabA synthetic lethal phenotype in rich medium

In addition to the compromised growth in minimal medium, we found that the dnaA1 ΔyabA double mutant did not grow in LB liquid medium nor did it form colonies on LB agar plates. Thus, the dnaA1 ΔyabA double mutant had a conditional lethal phenotype that was dependent on the growth medium. Based on the phenotypes in minimal medium, the known effects of over-replication, and the increase in the frequency of replication initiation in rich compared with the minimal medium, we suspect that the lethal phenotype in LB medium is due to over-initiation of replication. This conditional phenotype (able to grow in minimal but not rich medium) provided an opportunity to select for suppressor mutations that would enable survival on rich medium. We anticipated that at least some of these suppressors would affect replication initiation or replication elongation.

2.2 | Isolation of suppressors of the synthetic lethal phenotype of the dnaA1 ΔyabA double mutant

We isolated 45 independent suppressors of a dnaA1 ΔyabA double mutant that restored the ability to grow on LB agar plates. Briefly, 45 independent cultures of the dnaA1 ΔyabA double mutant (strain CAL2320) were grown in defined minimal medium and then an aliquot from each was plated on LB agar and grown at 37°C. Suppressor mutants (revertants) that were able to grow arose at a frequency of approximately 1 per 10^5 cells. To ensure independent suppressor mutations, a single colony was chosen from each plate for further analyses. We were most interested in pseudo-revertants that were not in dnaA. Since we used a deletion–insertion of yabA, it was not possible to get suppressor mutations in yabA. To eliminate suppressors with mutations in dnaA, we amplified by PCR and sequenced dnaA from each of the suppressor strains. Five of the suppressors had at least one additional mutation in dnaA and these were not analyzed further.

We chose 30 independent suppressors for whole genome sequencing to determine the gene altered in each suppressor. Most strains had multiple mutations but comparing them enabled us to focus on a handful of extragenic suppressors. There were several classes of suppressors that each had a mutation in the same gene, and we focused on these mutations for this work (Table 2). There were other strains that had mutations in multiple genes that did not appear in other isolates, or mutations in and around dnaA-oriC-dnaN,

FIGURE 1 SOS response is elevated in dnaA1 ΔyabA compared to wild type. Strains were grown at 37°C in minimal glucose medium to mid-exponential phase and collected to isolate RNA. cDNA was synthesized using reverse transcriptase and RT-qPCR was used to measure expression of dinC, a gene activated during the DNA damage response. dinC expression was normalized to housekeeping genes, sigA and gyrA. Error bars represent standard error of the mean of 6 biological replicates (p-value < .001). Wild type (AG1866); dnaA1 ΔyabA (CAL2320).

TABLE 2 Mutations that suppress the conditional synthetic lethal phenotype of a dnaA1 ΔyabA double mutant

Gene	Occurrences	Nature of mutation
nrdR	1	Δ329 bp; deletion of 329 bp starting at nucleotide 151 of the open reading frame
	1	change of arg to leu at amino acid 12 (R12L)
relA	1	Nonsense mutation at amino acid S600
	1	Δ264–295 amino acids in the synthetase domain of RelA (of 734 total amino acids in RelA)
dnaC	10	ΔT; loss of a T in the 8-T tract in the promoter region of dnaC (58 bp upstream of the start codon)
cshA	1	T27I; in the first RecA-like domain
	2	H312Y; in the second RecA-like domain
	1	Δ169 bp in C-terminal domain starting at nt position 1264 of the open reading frame
ytmP (ccrZ)	1	R61P
	1	A17V

Strain	Genotype (reference)
AG174	*trpC2 pheA1* (Perego et al., 1988; Smith et al., 2014)
AG1866	*trpC2 pheA1* Tn917ΩHU163 (*mls*)[a]
CAL2055	*trpC2 pheA1 ΔyabA::spc*
CAL2320	*trpC2 pheA1 ΔyabA::spc dnaA1*-Tn917ΩHU163 (*mls*)
KPL2	*trpC2 pheA1 dnaA1*-Tn917ΩHU163 (*mls*) (Burkholder et al., 2001)
MEA73[b]	*trpC2 pheA1 ΔyabA::spc dnaA1*-Tn917ΩHU163 (*mls*) PdnaC(T7)
MEA64	*trpC2 pheA1 ΔyabA::spc* Tn917ΩHU163 (*mls*)
MEA102[b]	*trpC2 pheA1 ΔyabA::spc dnaA1*-Tn917ΩHU163 (*mls*) *relA102*(Δ264–295)
MEA187	*trpC2 pheA1 ΔnrdR::kan*
MEA250	*trpC2 pheA1 ΔcshA::kan*
MEA303	*trpC2 pheA1 ΔyabA::spc dnaA1*-Tn917ΩHU163 (*mls*) *ΔcshA::kan*
MEA304	*trpC2 pheA1 ΔytmP::kan (ΔccrZ::kan)*
MEA323	*trpC2 pheA1 ΔyabA::spc dnaA1*-Tn917ΩHU163 (*mls*) *ΔytmP::kan (ΔccrZ::kan)*
MEA359	*trpC2 pheA1 ΔpurA::kan*
MEA360	*trpC2 pheA1 ycgO*::Pspank-*dnaC-cat*
MEA361	*trpC2 pheA1 ycgO*::Pspank-*dnaC-cat ΔdnaC::kan*
MEA362	*trpC2 pheA1* PdnaC(T7)
MEA370	*trpC2 pheA1 ΔyabA::spc dnaA1*-Tn917ΩHU163 (*mls*) PdnaC(T7)
MEA415	*trpC2 pheA1 polC*::Pspank-*polC-spc*
MEA537	*trpC2 pheA1 ΔyabA::spc dnaA1*-Tn917ΩHU163 (*mls*) *ΔnrdR::kan*

[a]The Tn917ΩHU163 is in *dck* and linked to *dnaA*.
[b]These were the original strains isolated from the suppressor screen. All the other strains had not been mutagenized.

or a rearrangement and duplication. We chose not to pursue these mutants in the current work.

Of the 20 mutants we decided to analyze, we identified 10 independent mutants that had the same mutation in the promoter region of *dnaC* (encoding the replicative helicase). Two or more independent mutations were also found in each of *nrdR*, *relA*, *cshA*, and *ytmP* {now called *ccrZ* (Gallay et al., 2021), see below} (Table 2). These results indicated that mutations in these genes were likely responsible for enabling the *dnaA1 ΔyabA* double mutant to grow on LB agar plates.

The frequency of suppressors (~10^{-5}) was likely indicative of null mutations in several genes and perhaps a high frequency of replication slippage in the homopolymaeric tract upstream of *dnaC*. In addition, there might be a higher mutation frequency due to the SOS response in the *dnaA1 ΔyabA* double mutant.

To confirm that mutations in the genes identified (Table 2) were responsible for suppression of the *dnaA1 ΔyabA* double mutant, we constructed defined mutations in each gene and introduced them into the *dnaA1 ΔyabA* mutant (Table 3). As the *dnaA1 ΔyabA* mutant is substantially less competent and can accumulate suppressor mutations, these defined alleles were first moved into a *dnaA1* single mutant. The *yabA* deletion was moved in last, and transformants (*ΔyabA::spc*) were selected for resistance to spectinomycin on agar plates with defined minimal medium to minimize selection of more suppressor mutations. These reconstructed mutants were then tested for growth in rich medium (LB) to confirm the mutations were sufficient for suppression. Each of the different classes of

suppressors had a decreased *ori/ter* ratio in minimal medium compared to the *dnaA1 ΔyabA* double mutant parent (Table 1), consistent with the notion that the conditional lethal phenotype of the parent was due to over-initiation of replication.

2.3 | Characterization of suppressor mutants

We characterized suppressors with mutations in *relA*, *nrdR*, *dnaC*, *cshA*, and *ytmP* (*ccrZ*), with extensive analysis of the mutations affecting *dnaC* (encoding the replicative DNA helicase; Table 2). In all the cases, we measured the relative copy number of the *oriC* region to that of *terC* (*ori* to *ter* ratios or *ori/ter*) in the suppressor strains compared with that of the *dnaA1 ΔyabA* double mutant. In all cases, there was a decrease in *ori/ter* in the suppressor mutants relative to that of the *dnaA1 ΔyabA* parent (Table 1; described later). Although *ori/ter* is frequently used as a readout of DNA replication initiation, it is important to note that changes in *ori/ter* could be due to changes in replication initiation or changes in replication elongation. A mutant with a decrease in *ori/ter* relative to that of the *dnaA1 ΔyabA* parent could have a decrease in replication initiation (lower *ori*) or an increase in replication elongation (higher *ter*), perhaps due to less fork arrest or more rapid elongation, or a combination of decreased initiation and increased elongation.

To determine if the suppressor mutations caused a decrease in replication initiation or an increase in replication elongation, we

measured the rate of total DNA synthesis by pulse labeling with ^{3}H-thymidine (see below). Briefly, if the decrease in *ori/ter* was due to a decrease in replication initiation, then there would be a decrease in DNA synthesis. If the decrease in *ori/ter* was due to an increase in replication elongation, then there would be an increase in DNA synthesis. If the decrease in *ori/ter* was due to a combination of decreased initiation and increased elongation, then whichever effect was larger would determine if DNA synthesis decreased or increased. Later, we describe the results that indicate that some suppressor mutations primarily affect replication initiation and others affect replication elongation.

## 2.4 | Mutations in *relA* and *nrdR* stimulate replication elongation

2.4.1 | *relA*

We isolated several mutations in *relA* (Table 2). The *relA* gene product interacts with the ribosome and is responsible for most of the synthesis and hydrolysis of (p)ppGpp in *B. subtilis* (Kriel et al., 2014; Nanamiya et al., 2008; Srivatsan & Wang, 2008; Wendrich & Marahiel, 1997). *B. subtilis* RelA has three domains, one for synthesis and one for hydrolysis of (p)ppGpp, and a third domain required for interaction with the ribosome (Wendrich & Marahiel, 1997). The suppressor mutations affected either the (p)ppGpp synthetase or ribosome binding domain, and none were in the hydrolase domain (Table 2). One of these mutants (MEA102) had an in-frame deletion that removes the conserved synthetase active site residue Asp264

(Kriel et al., 2014), indicating that this mutation causes a decrease in (p)ppGpp synthesis. Decreased levels of (p)ppGpp likely stimulate replication elongation in *B. subtilis* through reduced inhibition of the primase, DnaG (Wang et al., 2007). The presumed decrease in (p)ppGpp in the *relA* suppressor mutants would therefore indicate an increase in the processivity of the replication fork, which could cause the observed decrease in *ori/ter*.

We characterized one of the originally isolated suppressor mutants (MEA102) that contains a mutation in *relA* (*relA102*) that should inactivate the synthetase domain (Table 1; MEA102). We used the original mutant because we were unable to reconstruct an appropriate synthetase null in the *dnaA1 ΔyabA* background, largely due to lack of competence of the *relA* mutant and the *dnaA1 ΔyabA* mutant. The *relA102 dnaA1 ΔyabA* suppressor mutant had a decrease in *ori/ter* in cells growing in defined minimal medium compared to the parent *dnaA1 ΔyabA* double mutant (Table 1). This could indicate a decrease in replication initiation or an increase in replication elongation (or both).

In order to distinguish between effects on replication initiation versus elongation, we measured the rate of DNA synthesis by pulse-labeling cells with ^{3}H-thymidine and measuring incorporation into DNA (Methods). We found that there was an approximately 50% increase in the rate of DNA synthesis in the suppressor mutant (*relA102 dnaA1 ΔyabA*) compared with that in the parent (*dnaA1 ΔyabA*) (Figure 2a) in cells that were in mid-exponential growth in defined minimal medium. The increased rate of DNA synthesis in the suppressor mutant (Figure 2a), combined with the decrease in *ori/ter* (Table 1) indicated that the *relA* mutation primarily caused an increase in replication elongation (and not a decrease in replication
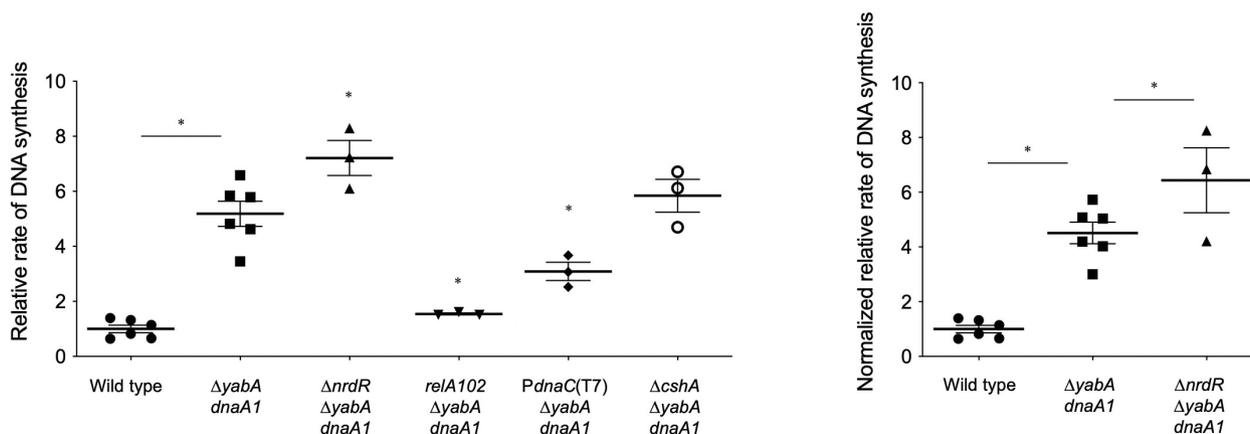
FIGURE 2 Relative rate of DNA synthesis. Strains were grown at 37°C in minimal glucose medium to mid-exponential phase. Relative rate of DNA synthesis was measured (incorporation of ^{3}H-thymidine into DNA relative to wild type). (a) Wild type (AG1866); *dnaA1 ΔyabA* (CAL2320); *dnaA1 ΔyabA relA102* (MEA102); *dnaA1 ΔyabA PdnaC(T7)* (MEA370); *dnaA1 ΔyabA ΔcshA* (MEA303); *dnaA1 ΔyabA ΔccrZ* (MEA323). MEA102 is the originally isolated suppressor mutation in *relA*. All other strains contain mutations reconstructed in a clean mutant background. Error bars represent standard error of the mean for at least 3 biological replicates. Significant differences compared to the parent strain (CAL2320) are indicated with an asterisk ($p < .05$). (b) Wild type (AG1866); *dnaA1 ΔyabA* (CAL2320); *dnaA1 ΔyabA ΔnrdR* (MEA537). ^{3}H-thymidine incorporation was normalized to the mean (of at least 3 biological replicates) cellular levels of dTMP in each strain as determined by mass spectrometry (Table 4; Methods). The average relative ^{3}H-thymidine incorporation of the *dnaA1 ΔyabA ΔnrdR* (MEA537) strain was 2.2. The individual data points were multiplied by 3.73 (Table 4) to correct for the increased dTMP pool size of that mutant. Error bars represent standard error of the mean of the normalized ^{3}H-thymidine incorporation for at least 3 biological replicates. Significant differences compared to the parent strain (CAL2320) are indicated ($p < .05$).

initiation). This effect is most likely because of an increase in the activity of DNA primase due to decreased inhibition by (p)ppGpp.

2.4.2 | nrdR

Two independent suppressor mutations were isolated in *nrdR* (negative regulator of ribonucleotide reductase genes), one of which was almost a complete deletion of the gene (Table 2), indicating that loss of *nrdR* likely suppressed the *dnaA1 ΔyabA* double mutant. To confirm that *nrdR* was responsible for suppression, we constructed a deletion of *nrdR* and made the triple mutant *ΔnrdR dnaA1 ΔyabA*. This mutant was indeed able to grow on LB agar, confirming that *ΔnrdR* is sufficient for suppression of the lethality of the *dnaA1 ΔyabA* mutant.

We measured the *ori/ter* ratio in the *ΔnrdR dnaA1 ΔyabA* strain and observed a significant decrease compared with that of the *dnaA1 ΔyabA* double mutant (Table 1). Again, the decrease in *ori/ter* could be due to either a decrease in replication initiation or an increase in replication elongation (or both). Because of the known function of NrdR, we anticipated that the suppressor had an increase in replication elongation.

NrdR is a repressor of genes encoding ribonucleotide reductases, and a deletion of *nrdR* causes an increase in the overall levels of nucleotides in the cell (Grinberg et al., 2006; Torrents et al., 2007). Presumably, this effect could help stimulate replication elongation in the *dnaA1 ΔyabA* mutant, which over-initiates and has an activated SOS response, likely due to fork collapse (see above), and therefore might require higher amounts of nucleotides for processive elongation.

Whereas incorporation of ^{3}H-thymidine in pulse-labeling experiments can be used to measure the rate of DNA replication, the amount of label incorporated is also influenced by the concentration of nucleotide (pool size) inside the cells. In most comparisons, there is no expectation that the in vivo concentrations of nucleotides change. However, since loss of *nrdR* is known to affect nucleotide biosynthesis and concentrations (Grinberg et al., 2006; Torrents et al., 2007), we expected that the concentration of nucleotides in the *ΔnrdR dnaA1 ΔyabA* mutant to be higher than that in the *dnaA1 ΔyabA* parent. Therefore, we measured pool sizes of dTMP in the relevant strains to correct for possible changes in the specific activity of the label inside cells.

As expected, we observed a significant increase in the concentration of dTMP in the *nrdR* suppressor compared to the wild type and the *dnaA1 ΔyabA* parent. We used mass spectrometry to measure levels of dTMP in an *nrdR* null mutant compared to wild type or the parent *dnaA1 ΔyabA* strains (Table 4). Cells were grown in defined minimal medium at 37°C and samples were taken for analysis (Methods). There was an increase in the concentration of dTMP of approximately 3–4-fold in the *ΔnrdR* mutants. Using this information, we could now compare rates of DNA synthesis between cells, correcting for the different intracellular concentrations of thymidine nucleotides.

TABLE 4 *ΔnrdR* has increased pools of dTMP

Relevant genotype (strain number)	Relative dTMP[a]
Wild type (AG1866)	1.00 ± 0.03
dnaA1 ΔyabA (CAL2320)	0.87 ± 0.17
ΔnrdR dnaA1 ΔyabA (MEA537)	3.73 ± 0.96

[a]Samples grown at 37°C in minimal glucose medium to mid-exponential phase were collected for metabolomic analysis using 40% methanol, 40% acetonitrile, 20% water with 0.1 M formic acid, and 500 nM each of 17 isotopically labeled amino acids for extraction buffer (see Methods). Values represent the mean and standard error of the mean of at least three biological replicates normalized to wild type.

We found that there was an increase in the rate of DNA synthesis in the *ΔnrdR dnaA1 ΔyabA* triple mutant compared with the (*nrdR*+) *dnaA1 ΔyabA* parent. Cells were grown to exponential phase in defined minimal medium at 37°C and pulse labeled with ^{3}H-thymidine (Methods, and as above). Taking into account the differences in the intracellular concentration of dTMP (Table 4), there was an approximately 40% increase in the rate of DNA replication in the *ΔnrdR dnaA1 ΔyabA* triple mutant compared to the *nrdR*+ double mutant (*dnaA1 ΔyabA*) parent (Figure 2b). Together with the decrease in *ori/ter*, these data indicate that the *ΔnrdR* mutation suppresses *dnaA1 ΔyabA* primarily by stimulating replication elongation and not by decreasing replication initiation.

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

Ten of the suppressors had a single base pair deletion in the promoter region of *dnaC*, the gene that encodes the replicative DNA helicase. The mutation *PdnaC(T7)* changes a run of eight thymines to seven, resulting in a decrease in the spacing between the −10 and −35 regions of the promoter driving expression of *dnaC*. This decrease in spacing likely causes a decrease in promoter activity.

To confirm that this mutation was responsible for suppression of the *dnaA1 ΔyabA* mutant phenotype, we constructed a single base pair deletion {*PdnaC(T7)*} and made the triple mutant *PdnaC(T7) dnaA1 ΔyabA*. This reconstructed mutant {*PdnaC(T7) dnaA1 ΔyabA*} grew in rich (LB) medium, confirming that the single base pair deletion in the promoter region of *dnaC* was sufficient to suppress the conditional lethal phenotype of the *dnaA1 ΔyabA* double mutant.

We hypothesized that the *PdnaC(T7)* mutation likely caused a change in expression of *dnaC*, and consequently a change in the amount of the replicative helicase (DnaC). We measured the amount of helicase protein in cells grown in minimal medium with glucose, using Western blots and probing with antibodies to DNA helicase. Both the wild type and *dnaA1 ΔyabA* had roughly the same amount of the replicative helicase, whereas the *dnaA1 ΔyabA PdnaC(T7)* suppressor had ~50% of wild-type levels (Figure 3a). A similar decrease in helicase protein was observed for the single mutant, i.e., *PdnaC(T7)* in otherwise wild-type (*dnaA+ yabA+*) cells (Figure 3b).

These results indicate the *PdnaC*(T7) mutation caused a decrease in the amount of the replicative helicase (DnaC).

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

Because the replicative helicase is needed for replication initiation (and elongation), we hypothesized that the decreased levels of helicase in the *PdnaC*(T7) mutants might reduce replication initiation and thereby be responsible for suppressing the over-initiation observed in the *dnaA1 ΔyabA* double mutant. We measured *ori/ter* ratios in the *PdnaC*(T7) suppressor and found that the *PdnaC*(T7) mutation caused a significant decrease in *ori/ter* close to the levels of wild type (minimal glucose; Table 1). To determine whether the decrease in *ori/ter* was

due to decreased initiation or increased elongation, the relative rate of DNA synthesis was measured. Similar to *ori/ter* results, the *PdnaC*(T7) mutation caused a decrease in total DNA synthesis compared to the *dnaA1 ΔyabA* parent (minimal glucose; Figure 2a). Taken together, these results indicate that the *PdnaC*(T7) mutation causes a decrease in DNA replication initiation in a *dnaA1 ΔyabA* background, presumably due to the decrease in the amount of the replicative helicase.

We found that the *PdnaC*(T7) mutation also caused a decrease in *ori/ter* in otherwise wild type cells under conditions of rapid growth in rich medium (LB). During rapid growth in LB medium, the *ori/ter* of the *PdnaC*(T7) mutant had a ~20% decrease compared to the isogenic wild type strain (Figure 4a). During slower growth in minimal glucose medium, the *PdnaC*(T7) mutant had no detectable change in *ori/ter* (Figure 5), despite the fact that it contained decreased amounts of the replicative helicase (Figure 3b). These results indicate that the decrease in the amount of the replicative helicase only has an effect on
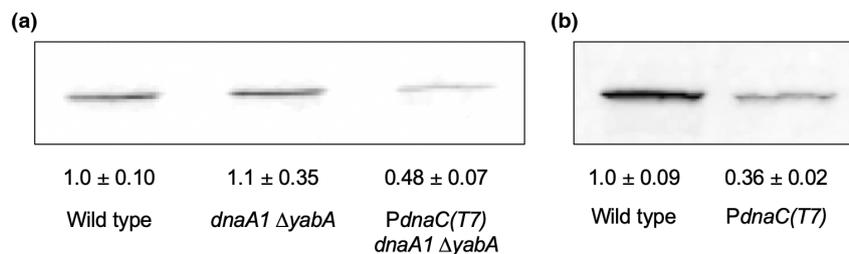
FIGURE 3 *PdnaC*(T7) causes decreased levels of DnaC protein. Strains were grown at 37°C in minimal glucose medium to mid-exponential phase. Levels of DnaC were determined by quantitative Western blot, normalized to optical density (OD_{600}, essentially normalizing to the amount of protein loaded per OD unit of cells; Methods). One representative blot is shown and values represent the average and standard error of the mean of at least 3 biological replicates. (a) Wild type (AG1866); *dnaA1 ΔyabA* (CAL2320); *dnaA1 ΔyabA PdnaC*(T7) (MEA370). (b) Wild type (AG174); *PdnaC*(T7) (MEA362).
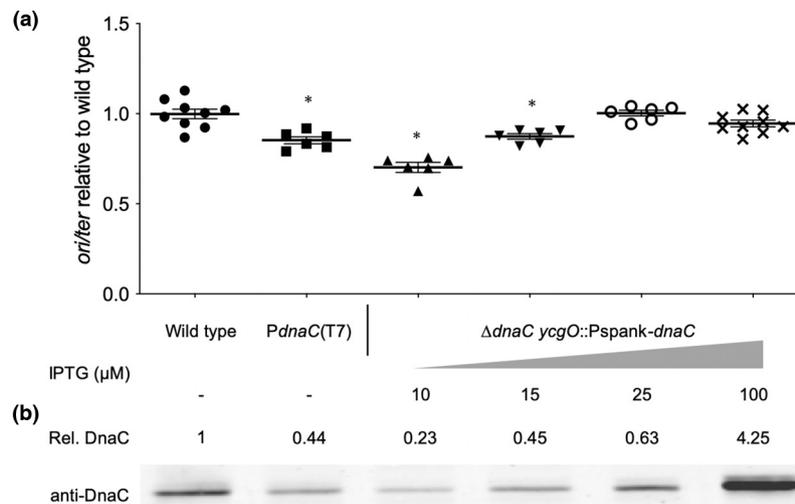
FIGURE 4 Decreased levels of DnaC are sufficient to decrease *ori/ter*. Strains were grown at 37°C in LB to mid-exponential phase. Expression from Pspank-*dnaC* was induced by addition of varied levels of IPTG (10 μM-100 μM). Wild type (AG174); *PdnaC*(T7) (MEA362); *ΔdnaC ycgO*::Pspank-*dnaC* (MEA361). (a) Samples were taken for genomic DNA isolation and used for qPCR to measure *ori/ter* as in Table 1. Error bars represent standard error of the mean for at least 6 biological replicates. Significant differences compared to the wild type (AG174) are indicated ($p < .05$). (b) At the same time, samples were taken to measure DnaC protein with quantitative Western blots as in Figure 3. This is a representative experiment but was repeated with 3 biological replicates.
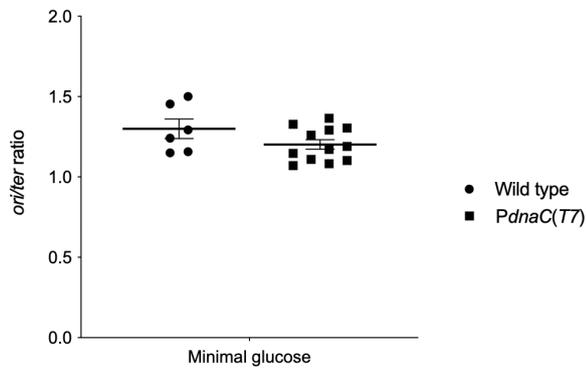
FIGURE 5 *PdnaC*(*T7*) does not affect *ori/ter* in slow growth (minimal glucose) conditions. Strains were grown at 37°C in minimal glucose medium to mid-exponential phase and cells were collected to isolate genomic DNA. qPCR was used to measure *ori/ter* as in Table 1. Error bars represent standard error of the mean for at least 6 biological replicates. Wild type (AG174); *PdnaC*(*T7*) (MEA362).

initiation when the cells are experiencing high rates of DNA replication initiation, such as fast growth (LB) or in the *dnaA1 ΔyabA* mutant.

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

2.7 | Decreasing levels of PolC does not decrease DNA replication initiation

In order to show that the decrease in DNA replication initiation was specific to decreased levels of the helicase, and not a general trend among other proteins involved in DNA replication initiation, we varied expression levels of DNA polymerase, *polC*, and measured *ori/ter*. We constructed an IPTG-inducible version of *polC* and measured *ori/ter* under a range of induction levels. Unlike the titration of *dnaC*, we did not observe a decrease in *ori/ter* as the levels of *polC* decreased (Figure 6). In fact, at the lowest level of induction we observed an increase in *ori/ter*. This agrees with a previous report that observed a decrease in elongation upon depleting levels of *polC* but no effect on initiation (Dervyn et al., 2001). At all the other levels of induction of *polC ori/ter* was indistinguishable from that of wild type (Figure 6).

Although the suppressor screen reported here was not saturated, there were no mutations isolated in other genes involved
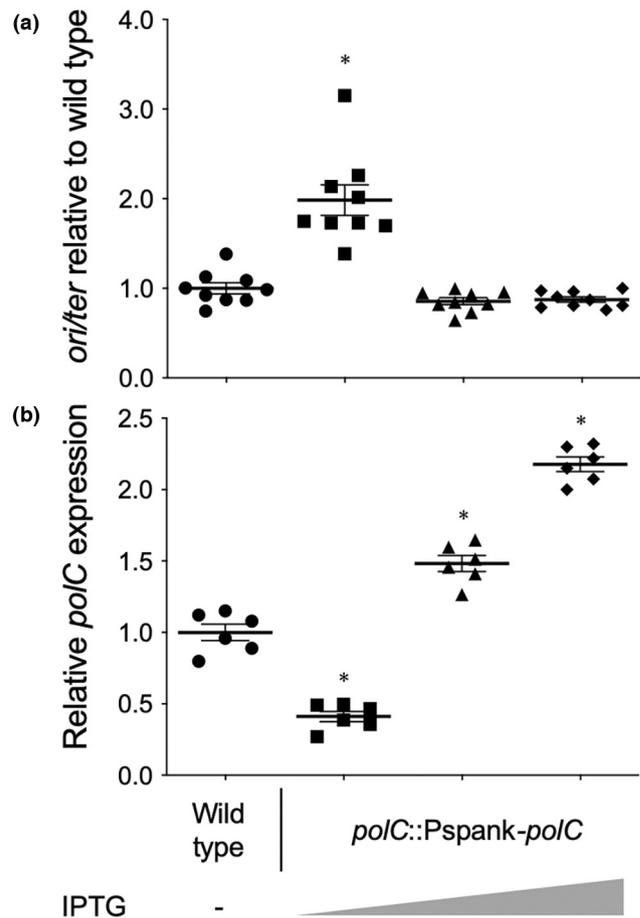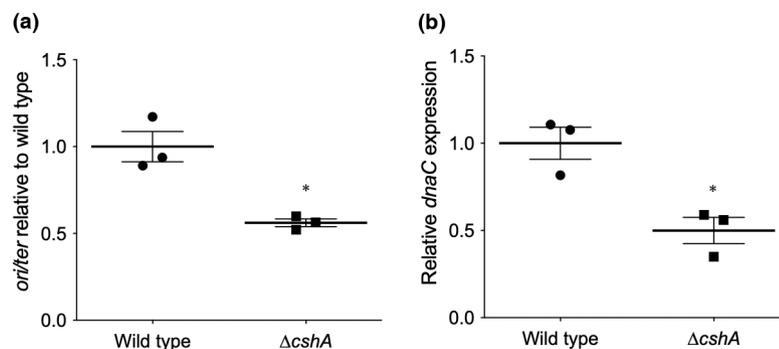

FIGURE 6 Decreased levels of *polC* did not decrease *ori/ter*. Strains were grown at 37°C in minimal glucose medium to mid-exponential phase and cells were collected to isolate RNA and genomic DNA. Expression from Pspank-*polC* was induced by addition of varied levels of IPTG. Error bars reflect standard error of the mean of at least 6 biological replicates. Wild type (AG174); *polC*::Pspank-*polC* (MEA415). Significant differences compared to the wild type (AG174) are indicated ($p < .05$). (a) gDNA was used for qPCR to measure *ori/ter* as in Table 1. Error bars represent standard error of the mean of 9 biological replicates. (b) RT-qPCR was used to measure expression of *polC* as in Figure 1. *polC* expression was normalized to housekeeping genes, *sigA* and *gyrA*. Error bars represent standard error of the mean of 6 biological replicates.

in DNA replication (apart from *dnaA*). These data indicate that the decrease in replication initiation may be specific to the decrease in levels of the helicase, and not a general phenomenon that applies to other proteins involved in DNA replication initiation.

2.8 | Suppressors in *cshA* and *ytmP* (*ccrZ*)

2.8.1 | *cshA*

Several mutations were isolated in *cshA*, a cold-shock RNA helicase associated with the RNA degradosome (Lehnik-Habrink et al., 2010), and a deletion of *cshA* was sufficient to suppress the growth

FIGURE 7 *dnaC* expression and *ori/ter* are decreased in Δ*cshA*. Strains were grown at 37°C in LB to mid-exponential phase and cells were collected to isolate RNA and genomic DNA. Error bars reflect standard error of the mean of 3 biological replicates. Wild type (AG174); Δ*cshA* (MEA250). Significant differences compared to the wild type (AG174) are indicated ($p < .05$). (a) gDNA was used for qPCR to measure *ori/ter* as in Table 1. (b) RT-qPCR was used to measure expression of *dnaC* as in Figure 1. *dnaC* expression was normalized to housekeeping genes, *sigA* and *gyrA*.

phenotype in the *dnaA1 ΔyabA* background. Similar to the other suppressors isolated, Δ*cshA dnaA1 ΔyabA* had a decrease in both *ori/ter* (Table 1) and the rate of DNA synthesis (Figure 2a) compared with *dnaA1 ΔyabA*, indicating that there was likely a decrease in DNA replication initiation. Nucleotide pools in the *cshA* mutants were not elevated compared to those in wild-type cells (92% ± 15%) or the *dnaA1 ΔyabA* mutant (58% ± 5%). Based on these results, we conclude that suppression by the *cshA* mutations was largely due to a decrease in replication initiation.

Given the role of *cshA* in mRNA turnover and ribosome biogenesis (Ingle et al., 2021; Lehnik-Habrink et al., 2010, 2013), we hypothesized that there may be a change in stability of certain mRNAs or the amount of one or more proteins associated with DNA replication. Given that we determined a decrease in expression of *dnaC*, encoding the replicative helicase, was sufficient to decrease DNA replication initiation (above), we measured levels of *dnaC* mRNA in a Δ*cshA* mutant using RT–qPCR. We found that there was an approximately 50% decrease in the amount of *dnaC* mRNA in Δ*cshA* (Figure 7b). This may contribute to the decrease in *ori/ter* but is likely not the sole factor because there is a greater decrease in *ori/ter* in Δ*cshA* than in the P*dnaC*(T7) mutant in otherwise wild-type cells grown in rich medium (Figures 4a and 7a). In addition, Δ*cshA* has a much slower growth rate compared to wild type, with Δ*cshA* having a doubling time of 51 min compared with 21 min for wild type in LB medium. This could contribute to the decrease in *ori/ter* but may also decrease the amount of DnaC needed for DNA replication initiation. It seems likely that suppression of the *dnaA1 ΔyabA* mutant is likely due to pleiotropic effects caused by loss of *cshA*.

2.8.2 | *ytmP* (*ccrZ*)

We isolated several mutations in a previously uncharacterized gene, *ytmP*, now called *ccrZ* (Gallay et al., 2021). The *ccrZ* gene product has regions of similarity to choline and ethanolamine kinases

(Zimmermann et al., 2018). We constructed a null mutation in *ccrZ* and this mutation was sufficient to suppress the conditional lethal phenotype of the *dnaA1 ΔyabA* mutant. The Δ*ccrZ* mutation caused a decrease in *ori/ter* relative to that of the *dnaA1 ΔyabA* parent (Table 1), indicating either a decrease in replication initiation and/or an increase in replication elongation. We found that DNA synthesis in the suppressor Δ*ccrZ dnaA1 ΔyabA* triple mutant was virtually the same as that in the parent *dnaA1 ΔyabA* double mutant (Figure 2a). Based on these results, we conclude that loss of *ccrZ* may suppress the *dnaA1 ΔyabA* mutant through multiple mechanisms. It is also possible that changes replication initiation frequency could cause indirect effects on elongation. A small decrease in initiation could prevent replication fork collapse, thereby allowing for more processive replication elongation, a decrease in *ori/ter*, and relatively little change in DNA synthesis.

A homolog of *B subtilis ccrZ* was identified as an essential gene in *Streptococcus pneumoniae*. In *S. pneumoniae*, *ccrZ* was found to couple DNA replication with cell division. The effects of *ccrZ* in *S. pneumoniae* and *B. subtilis* are described in other work (Gallay et al., 2021).

3 | DISCUSSION

3.1 | Suppressors of over-initiation of DNA replication

Work presented here highlights the multiple ways cells can compensate for over-initiation of DNA replication. Starting with a mutant that had an increase in replication initiation and a conditional lethal phenotype, we isolated suppressors that were capable of growing under otherwise non-permissive conditions (rich medium). Suppressors were found that cause a decrease in replication initiation and/or an increase in replication elongation. One suppressor was in the promoter region of the gene for the replicative DNA helicase and this was characterized in detail.

3.2 | Decreased levels of helicase as a novel mechanism of suppression for over-initiation of DNA replication

The majority of the suppressors we identified were in the promoter region of the replicative helicase, *dnaC*, and caused a decrease in the amount of helicase in cells. We found that in conditions in which cells have a relatively high frequency of replication initiation, either in the over-initiating double mutant (*dnaA1 ΔyabA*) or fast growth conditions (LB medium), decreasing the amount of helicase was sufficient to reduce the frequency of replication initiation. In contrast, we found that overproduction of helicase had no detectable effect on replication initiation.

In contrast, in *E. coli*, perturbing the ratio of helicase to helicase loader proteins inhibits replication initiation and elongation (Allen & Kornberg, 1991; Bruning et al., 2016; Skarstad & Wold, 1995). This is believed to be the result of an imbalance between the helicase and the loader proteins, which are normally at an approximately 1:1 ratio. To load the helicase ring onto the DNA, *E. coli* employs a "ring breaker" mechanism where the hexameric helicase loader breaks an already formed hexameric helicase ring and loads it around the DNA (Kornberg & Baker, 1992; reviewed in Davey & O'Donnell, 2003). An imbalance between the two proteins causes inhibitory associations between the helicase and the loader: excess loader causes continual reassociation between the helicase and loader, preventing replisome progression (Allen & Kornberg, 1991; Skarstad & Wold, 1995), whereas excess helicase reduces the probability of the helicase-loader complex formation (Bruning et al., 2016).

B. subtilis and some other gram-positive bacteria appear to use a "ring maker" mechanism where a monomeric helicase loader loads individual monomers around the DNA to create the hexameric ring required for DNA replication (reviewed in Davey & O'Donnell, 2003; Velten et al., 2003). Any inhibitory interactions would only affect monomer–monomer interactions, and would not inhibit an already assembled hexameric helicase and should have no effect on replication elongation. As shown aforementioned, overproduction of the replicative helicase (DnaC) had no effect on *ori/ter*, indicating that the amounts of helicase in *B. subtilis* have different effects than in *E. coli*.

Our results indicate that levels of the replicative helicase in *B. subtilis* are limiting under conditions with a high frequency of replication initiation. Estimates of the amount of the replicative helicase in rapidly growing cells of *B. subtilis* range from about 100–330 molecules (monomers) per cell (Goelzer et al., 2015). There are six helicase monomers per single replication fork (12 per bi-directional fork), and under conditions of rapid growth and multi-fork replication, cells can contain up to eight copies of *oriC* and perhaps 12–14 replication forks (LeBowitz & McMacken, 1986; Mangiameli et al., 2017; Reyes-Lamothe et al., 2010; Sauls et al., 2019).

We only observed an effect of altering the amount of the helicase under conditions with a high frequency of replication initiation, including growth in rich medium (LB) or in the *dnaA1 ΔyabA*

mutant. In the mutant background, where replication initiation is asynchronous and dysregulated, decreasing the amount of the helicase acts as a means to limit replication initiation. During slower growth, where lower concentrations of helicase protein are required, we observed no change in DNA replication initiation, indicating that helicase levels are not be limiting under these conditions. This makes sense given that higher levels of helicase are required when more DNA replication is taking place. It is also possible that under slow growth (minimal media) conditions, where wild type cells have an *ori/ter* of only about 1.2, there is not much room for a decrease in the amount of helicase to decrease replication initiation. Further, an increase in levels of helicase in an otherwise wild type background has no detectable effect on levels of initiation (Figure 4), likely due to the regulation of replication initiation by other factors, including YabA.

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

We did not isolate suppressor mutations in other genes known to be involved in replication initiation (apart from *dnaA*). Most proteins involved in DNA replication are essential and therefore any loss of function mutations would not be isolated in this screen. Our screen was able to isolate mutations that affect protein levels or activity, which are much less likely to occur. The *PdnaC* mutation we isolated, which occurred in the promoter region of *dnaC*, is one example of this type of mutation. The mutation that caused the decreased levels of helicase was a single base pair deletion in a run of thymines. However, we did not isolate any similar mutations in other essential DNA replication genes. This is not surprising, as these types of mutations occur very rarely and our screen was not performed to saturation. It is possible that the specific mutation in *PdnaC* occurs at higher frequency than promoter mutations in other DNA replication genes, due to the tendency of slippage by the replication machinery at poly-N tracts (reviewed in Strauss, 1999). There is also a high frequency of promoter mutations due to replication-transcription conflicts (Sankar et al., 2016) and *dnaC* is located on the lagging strand, perhaps increasing the likelihood of mutation. It is unique that *PdnaC* is so easy to mutate. Other genes involved with replication initiation, such as *dnaB–dnaI* and *dnaD*, are also on the lagging strand but have shorter poly-nucleotide tracts in their promoters (≤5) and not in the −10 to −35 region.

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

3.4 | Mutations that increase replication elongation can suppress growth defects caused by over-initiation

The conditional lethal phenotype of the *dnaA1 ΔyabA* mutant is most likely caused by replication over-initiation, leading to replication fork collapse. Increased replication initiation can lead to replication fork collapse (Simmons et al., 2004) which is evident due to the increased SOS response in our *dnaA1 ΔyabA* mutant (Figure 1). We isolated several mutations that suppress the over-initiation mutant by causing a decrease in replication initiation. We also isolated suppressor mutations that caused an increase in replication elongation. The faster replication elongation in our mutants could help overcome the over-initiation defect by either speeding up replication forks to prevent collisions, or stimulating repair of collapsed forks.

We determined that mutations in *relA*, likely resulting in a decrease in (p)ppGpp, appear to stimulate replication elongation. (p)ppGpp is known to inhibit elongation, specifically by inhibiting primase (DnaG) (Maciag et al., 2010; Wang et al., 2007). DNA primase synthesizes an RNA primer required for lagging strand DNA synthesis. Decreased inhibition of primase would help increase replication elongation. This could be by stimulating the rate of elongation in the mutant background thereby preventing replication fork collapse, by enhancing replication restart from already collapsed forks, or a combination of these two mechanisms.

Similarly, we determined that a null mutation in *nrdR* also suppressed the over-initiation mutant by stimulating replication elongation. Changes in expression of *nrd* genes, which encode nucleotide reductase needed for deoxyribonucleotide biosynthesis (reviewed in Nordlund & Reichard, 2006), in relation to perturbations in DNA replication have been well documented (Augustin et al., 1994; Goranov et al., 2005; Huang et al., 1998), establishing a link between DNA replication and nucleotide biosynthesis. In *E. coli*, DnaA itself directly activates the *nrdAB* operon (Augustin et al., 1994), indicating a positive correlation between increased DNA replication initiation and dNTPs. In *B. subtilis*, expression of the *nrdEF* operon (regulated by *nrdR*) increases upon replication fork arrest (Goranov et al., 2005). Increased dNTPs have been proposed to promote the RecA-dependent repair of stalled replication forks in *E. coli* (Robu et al., 2001).

Increased levels of dNTPs have been shown to speed up DNA replication in *E. coli*, indicating that the levels of dNTPs in the cell are not saturated (Zhu et al., 2017). Imbalances in the dNTPs pools and competition with the much more abundant rNTP pools also slow the replisome (Yao et al., 2013) and may be exacerbated in the double mutant. The increased levels of dNTPs in the *nrdR* mutant might suppress the over-initiation lethality by simply speeding up the rate of elongation, thereby preventing replication forks collapse. It is also possible that the excessive rounds of replication in the *dnaA1 ΔyabA* double mutant deplete the pools the of dNTPs, causing replication fork collapse, and the *nrdR* mutant alleviates this problem by increasing the dNTP pool sizes. Either of these mechanisms support

the model in which enhanced replication elongation helps overcome replication fork collapse caused by the severe over-initiation of *dnaA1 ΔyabA*.

As mentioned earlier, changes in one step of DNA replication may affect another. For example, a small decrease in initiation could prevent replication fork collapse, indirectly allowing for increased elongation. This could be a possible mechanism for the *ccrZ* suppressors, where there is a decrease in *ori/ter* but no obvious change in DNA synthesis. This could also contribute to the mechanism of suppression by other mutants, however based on what is known about some genes identified, we suspect there are at least some direct effects to stimulate elongation or the repair of collapsed replication forks.

3.5 | Multiple mechanisms for altering DNA replication and compensating for abnormal control

The diverse mutants isolated during this suppressor screen highlight the varied means that can be used to control detrimental DNA replication in a cell, a problem experienced across all domains of life. In some cases, organisms have found ways to adapt to increased DNA replication. For example, repeated rounds of DNA replication without segregation result in polytene chromosomes in *Drosophila* (Stormo & Fox, 2017). Typically however, uncontrolled DNA replication can lead to DNA damage, causing genome instability, chromosome partitioning defects, and cell death in bacteria (Bach & Skarstad, 2004; Katayama, 2001; O'Donnell et al., 2013; Simmons et al., 2004).

As with the suppressors discussed here, cells can overcome over-initiation by either reducing initiation events or finding a way to tolerate the increased origin firing to prevent fork collapse and DNA damage. Similar groupings of suppressors have been described in *E. coli* (reviewed in Charbon et al., 2018). Mutations that work by limiting initiation can act by affecting *oriC* binding or topology or limiting the activity or availability of DnaA. Those that allow the cell to tolerate over-initiation include mutations that decrease reactive oxygen species, alter DNA repair functions, and increase pools of dNTPs. This study emphasizes how cells can act at different steps in DNA replication to overcome a replication defect and highlights the many yet uncharacterized mechanisms they will use to maintain chromosome integrity.

4 | MATERIALS AND METHODS

4.1 | Media and growth conditions

Cells were grown with shaking at 37°C in Luria–Bertani (LB) medium (Miller, 1972) or S7 defined minimal medium with MOPS (3-[N-morpholino] propanesulfonic acid) buffer at a concentration of 50 mM rather than 100 mM supplemented with 1% glucose, 0.1% glutamate, trace metals, 40 μg/ml phenylalanine, and 40 μg/ml tryptophan (Jaacks et al., 1989). Standard concentrations

of antibiotics were used when appropriate (Harwood & Cutting, 1990). To induce expression of *dnaC* or *polC* from the LacI-repressible, IPTG-inducible Pspank, varied concentrations of IPTG were used as specified.

Cultures were typically grown to mid-exponential phase and diluted back to OD_{600} 0.05. OD_{600} was measured throughout exponential growth phase and plotted versus time. Doubling times were determined from the linear portion of the plot $log(OD_{600})$ versus time.

4.2 | Strains and alleles

The *E. coli* strain AG1111 (MC1061 F′ *lacI*^{q} *lacZ*M15 Tn*10*) (Glaser et al., 1993) was used for plasmid construction. *B. subtilis* strains were derived from JH642 (*pheA1 trpC2*) (Perego et al., 1988; Smith et al., 2014), are listed in Table 3, and were constructed by natural transformation using genomic DNA.

AG1866 was constructed by transforming AG174 with KPL2 genomic DNA and selecting for MLS (macrolide–lincosamide–streptogramin B) resistance and screening for temperature resistance at 50°C, resulting in a wild-type strain isogenic to KPL2 (Tn*917* Ω HU163 (*mls*)). The Tn*917* Ω HU163 allele is inserted in *dck* and linked to *dnaA*.

ΔyabA::*spc* (CAL2055) was constructed by replacing the *yabA* open reading frame in AG174 with a spectinomycin resistance cassette using long-flanking homology PCR. MEA64 is the result of backcrossing DNA from CAL2055 into AG1866 to generate a *ΔyabA*::*spc* (*dnaA*$^+$) strain isogenic to KPL2 and AG1866.

Deletions in *nrdR* (MEA187), *cshA* (MEA250), *purA* (MEA359), and *ytmP* (*ccrZ*) (MEA587) were constructed by replacing the open reading frames with a kanamycin resistance cassette (*kan*) using linear Gibson isothermal assembly (Gibson et al., 2009) of fragments containing ~1 kb of flanking homology for the indicated gene.

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

CAL2320 {*ΔyabA*::*spc dnaA1*-Tn*917*ΩHU163 (*mls*)} was constructed by transforming DNA from CAL2055 (*ΔyabA*::*spc*) into KPL2 (*dnaA1*-Tn*917*ΩHU163) and selecting on minimal medium plates with spectinomycin and confirming the presence of *dnaA1* by screening for temperature sensitivity at 50°C. All the derivatives of CAL2320 (except MEA370) were constructed by introducing the desired alleles into KPL2 (selecting on rich medium) and then introducing *ΔyabA*::*spc* (CAL2055) last, selecting on minimal medium plates to reduce selection for suppressors. MEA370 {*ΔyabA*::*spc dnaA1*-Tn*917*ΩHU163 (*mls*) P*dnaC*(T7)} was constructed in two steps. Genomic DNA containing *dnaA1*-Tn*917*ΩHU163 (*mls*) was used to transform MEA362 {P*dnaC*(T7)} selecting for MLS resistance on

rich medium. After purification, this intermediate strain was transformed with DNA from a *ΔyabA*::*spc* mutant (CAL2055), selecting for spectinomycin-resistance on minimal medium agar plates.

MEA360 {*ycgO*::Pspank-*dnaC*-*cat*} was constructed by transforming linearized pMEA358 (cut with DraIII) into AG174. pMEA358 is a derivative of pBOSE1404 that places the open reading frame (and ribosome binding site) of *dnaC* under the IPTG-inducible promoter Pspank. pBOSE1404 is a plasmid that introduces *cat* (chloramphenicol resistance) and Pspank at *ycgO* (*ycgO*-*cat*-*lacI*-Pspank-*ycgO*) by double crossover. oMEA260 (5′-TTGTGAGCGG ATAACAATTA AATGAGAGGA CGGTGCTTAG C-3′) and oMEA261 (5′-GAATTAGCTT GCATGCGGAT CTTTCGAATG AAAAAAACCC CAAGAG-3′) were used to amplify a 1420 bp fragment (AG174 as the template) that includes the open reading frame of *dnaC*. Using isothermal assembly, this fragment was introduced into pBOSE1404 cut with NheI and HindIII, generating pMEA358. MEA361 was constructed by replacing the open reading frame of *dnaC* with a kanamycin resistance cassette (*kan*) by using linear Gibson isothermal assembly fragments containing ~1 kb of flanking homology transformed into MEA360, selecting on kanamycin +1 mM IPTG to induce expression of the essential *dnaC*.

4.3 | Suppressor screen

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

4.4 | Genome sequencing

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

4.5 | qPCR to determine *ori/ter* ratio

Cultures were grown to mid-exponential phase and diluted back to OD 0.05 and grown to mid-exponential phase (OD 0.2–0.4)

in defined minimal medium at 37°C. Cells were harvested in ice-cold methanol (1:1 ratio) and pelleted. Genomic DNA was isolated using Qiagen DNeasy kit with 40 µg/ml lysozyme. The copy number of the origin (*ori*) and terminus (*ter*) were quantified by qPCR to generate an *ori/ter* ratio. qPCR was done using SSoAdvanced SYBR master mix and CFX96 Touch Real-Time PCR system (Bio-Rad). Primers used to quantify the origin region were oMEA316 (5′-TTGCCGCAGATTGAAGAG-3′) and oMEA317 (5′-AGGTGGACACTGCAAATAC-3′). Primers used to quantify the terminus region were oMEA318 (5′-CGCGCTGACTCTGATATTATG-3′) and oMEA319 (5′-CAAAGAGGAGCTGCTGTAAC-3′). qPCR data were quantified using the Pfaffl method (Pfaffl, 2001) and origin-to-terminus ratios were determined by dividing the number of copies of the origin by the number of copies quantified at the terminus. Ratios were normalized to the origin-to-terminus ratio of a temperature sensitive mutant, *dnaB134* (KPL69), that was shifted to the non-permissive temperature, which allows ongoing replication to be completed but does not allow new initiation, resulting in 1:1 ratio of the origin: terminus.

4.6 | RT-qPCR

Cultures were grown to mid-exponential phase and diluted back to OD 0.05 and grown to mid-exponential phase (OD 0.2–0.4) in defined minimal medium at 37°C. Cells were harvested in ice-cold methanol (1:1 ratio) and pelleted. RNA was isolated using Qiagen RNeasy PLUS kit with 10 mg/ml lysozyme. iScript Supermix (Bio-Rad) was used for reverse transcriptase reactions to generate cDNA. RNA was degraded by adding 75% volume of 0.1 M NaOH and incubating at 70°C 10 min, followed by neutralizing the reaction with adding 75% of the original volume 0.1 M HCl. qPCR was done using SSoAdvanced SYBR master mix and CFX96 Touch Real-Time PCR system (Bio-Rad). Primers used to quantify *dnaC* were oMEA126 (5′-AGCTGCAAGTCCCTGTTATC-3′) and oMEA127 (5′-CCTGCTCGATACTTCCTGATTC-3′). Primers used to quantify *dinC* were oMEA207 (5′-ACCAAGATACACCTCCAGAAAG-3′) and oMEA208 (5′-AACCTGAAGTCGGAACCATC-3′). Primers used to quantify *sigA* were oMEA252 (5′-ATACCGGCTCTTGAGCAATC-3′) and oMEA253 (5′-ACTTAGGCAGAGAACCAACAC-3′). Primers used to quantify *gryA* were oMEA128 (5′-TGGAGCATTACCTTGACCATC-3′) and oMEA129 (5′-AGCTCTCGCTTCTGCTTTAC-3′).

4.7 | Western blots to measure protein levels

Cultures were grown to mid-exponential phase and diluted back to OD 0.05 and grown to mid-exponential phase (OD 0.2–0.4) in defined minimal medium or LB medium (as indicated) at 37°C. Exponentially growing cells were lysed with lysozyme and a protease inhibitor cocktail (Sigma-Aldrich, P8849) at 37°C. Lysates were run on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane using the Trans-blot SD semi-dry transfer

cell (Bio-Rad). To represent the same number of cells in each lane, the amounts loaded in each lane were normalized to culture OD. All the steps were performed at room temperature. According to the manufacturer's instructions, blots were blocked with Odyssey Blocking Buffer for 1 hr and incubated with primary antibody (rabbit anti-DnaC antibody 1:10,000 in Odyssey Blocking Buffer +0.2% Tween) for 1 hr. The primary antibody to DnaC was affinity purified rabbit polyclonal antibody (Covance) made against purified DnaC-His6. Blots were washed with PBST (phosphate-buffered saline +0.2% Tween) for at least 5 ×5 min and then incubated for 30 min with secondary antibody (LiCor dye 800 goat anti-rabbit 1:10,000 in Odyssey Blocking Buffer +0.2% Tween). Blots were imaged and quantitated on a LiCor scanner. Dilutions of AG174 lysates were used to generate a standard curve and determine the linear range of the fluorescence signal. Verification of the antibodies to DnaC (DNA helicase) was previously described (Smits et al., 2010).

4.8 | ^{3}H-thymidine incorporation to measure DNA synthesis

Cells were grown at 37°C and were assayed at 3 to 5 time-points between OD 0.2 and 0.5. Incorporation per OD was found to be linear over this range. A 250 µl aliquot was pulse labeled with 10 µl ^{3}H-thymidine (6.7 Ci/mmol; 1 mCi/ml). After 1 min, label was "chased" by adding 50 µl 10 mM unlabeled thymidine and incubating for an additional 1 min. An equal volume ice-cold 20% trichloroacetic acid (TCA) was added, and the samples were incubated on ice for 30–60 min. A 350 µl aliquot was vacuum-filtered on glass-fiber filters (24 mm GF/A, Whatman) and washed with 25 ml of ice-cold 5% TCA, followed by 2 ml 100% ethanol. The amount of radioactivity that had been incorporated into nucleic acid was determined by scintillation counting of the dried filters. A time course was performed to confirm that the 1 min pulse was in the linear range of the assay.

4.9 | Mass spectrometry to measure nucleotide pools

Cultures were grown in minimal glucose medium to OD 0.45–0.55, transferred to 50 ml conical tubes, and quickly chilled to 10°C by swirling in liquid nitrogen. Cells from 5 ml of the chilled culture were collected onto a 25 mm MilliPore Type HAWP 0.45 µM filter using a filtration apparatus chilled to 4°C. The filter was immediately placed in a tube containing 1 ml ice-cold extraction buffer (40% methanol, 40% acetonitrile, 20% water with 0.1 M formic acid, and 500 nM each of 17 isotopically labeled amino acids), and quickly frozen in liquid nitrogen. After removing from liquid nitrogen, 87 µl 15% ammonium bicarbonate was added to each tube, and vortexed and shaken vigorously to disrupt the cells. After 5 min centrifugation at 16,100*g* at 4°C, the supernatant was removed and

dried under vacuum. LC–MS profiling and analysis was performed by the Whitehead Institute Proteomics Core Facility essentially as described (Kanarek et al., 2018). Raw LC–MS data were analyzed using MZMine 2 (Pluskal et al., 2010). The dTMP peak areas were corrected for small differences in the ODs of cells processed and normalized to the glutamate internal standard.

AUTHOR CONTRIBUTIONS

MEA, JLS, and ADG conceived and designed the study. MEA and JLS acquired data. MEA, JLS, and ADG analyzed and interpreted data, and wrote and edited the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Polyclonal antibodies to purified DNA helicase were raised in rabbits as a custom service of Covance.

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