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DnaAcos Hyperinitiates by Circumventing Regulatory Pathways that Control the Frequency of Initiation in *Escherichia coli*

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Summary

Mutants of *dnaAcos* are inviable at 30°C because DnaAcos hyperinitiates, leading to new replication forks that apparently collide from behind with stalled forks, thereby generating lethal double strand breaks. By comparison, an elevated level of DnaA also induces extra initiations, but lethality occurs only in strains defective in repairing double strand breaks. To explore the model that the chromosomal level of DnaAcos, or the increased abundance of DnaA increases initiation frequency by respectively escaping or overcoming pathways that control initiation, we developed a genetic selection and identified *seqA*, *datA*, *dnaN* and *hda*, which function in pathways that either act at *oriC*, or modulate DnaA activity. To assess each pathway's relative effectiveness, we used genetically inactivated strains, and quantified initiation frequency after elevating the level of DnaA. The results indicate that the *hda*-dependent pathway has a stronger effect on initiation than pathways involving *seqA* and *datA*. Testing the model that DnaAcos overinitiates because it fails to respond to one or more regulatory mechanisms, we show that *dnaAcos* is unresponsive to *hda* and *dnaN*, which encodes the β clamp, and also *datA*, a locus proposed to titer excess DnaA. These results explain how DnaAcos hyperinitiates to interfere with viability.

Keywords

DnaA; Hda; β clamp; SeqA; *datA*; initiation

Introduction

Chromosomal DNA replication in free-living organisms is a highly regulated event that is coordinated with the cell cycle. In *E. coli*, DnaA protein initiates chromosomal DNA replication by orchestrating a step-wise process that leads to the assembly of two replisomes for bidirectional fork movement from *oriC*, the chromosomal origin (reviewed in Kaguni, 2006). First, DnaA protein forms a unique DnaA-*oriC* complex by recognizing 9-mer motifs termed DnaA boxes, other sequences named I-sites, and possibly the AT-rich region carrying three 13-mer motifs near the left boundary of *oriC* (reviewed in Leonard and Grimwade, 2005). Assisted by HU protein or integration host factor (Hwang and Kornberg, 1992; Skarstad *et al.*, 1990; Chodavarapu *et al.*, 2008), DnaA complexed to ATP then unwinds the AT-rich region (Bramhill and Kornberg, 1988), and loads two DnaB helicase molecules on each separated strand of *oriC* to form the prepriming complex (Fang *et al.*, 1999; Carr and Kaguni, 2001). Next, primase interacts with DnaB to synthesize primers that DNA polymerase III holoenzyme extends. The complex of primase, DnaB helicase and the

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DNA polymerase constitute the replisome at each replication fork in the duplication of the bacterial genome.

Several separate mechanisms have been suggested to regulate the frequency of initiation (reviewed in Kaguni, 2006; Nielsen and Lobner-Olesen, 2008). First, DnaA protein forms a unique DnaA-*oriC* complex by recognizing 9-mer motifs termed DnaA). In preparation for a cycle of DNA replication, *dnaA* expression during cell growth leads to a critical level that induces initiation at a particular cell mass (Hansen *et al.*, 1991). In support, increased *dnaA* expression via a regulated promoter causes initiation at an earlier time in the cell cycle (Atlung *et al.*, 1987; Lobner-Olesen *et al.*, 1989). Following initiation, three separate pathways are proposed to inhibit re-initiation. One involves the preferential binding of SeqA to hemi-methylated GATC sequences in *oriC* (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994; Olsson *et al.*, 2003; Nievera *et al.*, 2006). After duplication of *oriC*, which has an unusual abundance of 11 GATC sequences recognized by DNA adenine methylase, the binding of SeqA to the resultant hemi-methylated DNA is thought to occlude *oriC* from DnaA and other proteins needed for replisome assembly. When DNA adenine methylase converts the hemi-methylated sites in *oriC* to the fully methylated form, another cycle of initiation can follow after SeqA dissociates from *oriC*. The second pathway involves the *datA* locus, which contains four to five DnaA boxes that are proposed to titrate DnaA when its abundance surpasses the level needed for a new cycle of initiation (Kitagawa *et al.*, 1998). The third pathway involves Hda and the β clamp of DNA polymerase III holoenzyme (Kato and Katayama, 2001). Hda in a complex with the β clamp stimulates the hydrolysis of ATP bound to DnaA in a process named the “regulatory inactivation of DnaA” (reviewed in Katayama, 2001). Because DnaA complexed to ATP is active in initiation, but DnaA bound to ADP is relatively inactive, the change in the nucleotide-bound state of DnaA by Hda and the β clamp is suggested to control DnaA activity in initiation.

The pathways described above are based in part on indirect estimates of the frequency of initiation by flow cytometry. This method measures the number of completed chromosomes in individual cells after treatment with rifampicin and cephalexin (Boye and Lobner-Olesen, 1990; Skarstad *et al.*, 1995). Rifampicin blocks initiation but ongoing replication forks continue to completion. Cephalexin inhibits cell division. Thus, the number of chromosomes in a cell reflects the number of origins present when the antibiotics were added. Compared with wild type cells, the increased number of chromosomes in cells lacking SeqA, *datA*, or Hda led to the interpretation that these mutants overinitiate (Lu *et al.*, 1994; Kitagawa *et al.*, 1996; Camara *et al.*, 2003). Subsequently, flow cytometry analysis of Δ*datA* cells revealed that rifampicin induces extra initiations (Morigen *et al.*, 2005), suggesting that *datA* does not normally control initiation frequency. In support, microarray and Southern blot analysis to measure the *oriC*-to- terminus ratio, which directly reflects initiation frequency, revealed a value for the Δ*datA* mutant that was essentially indistinguishable from wild type cells (Camara *et al.*, 2005). These observations controvert the importance of *datA* in limiting initiation to once per cell cycle.

Other studies question the role of SeqA in regulating initiation. Compared with wild type cells, only a 1.17-fold increase in the origins per cell was measured by flow cytometry when the ability of SeqA to sequester *oriC* was impaired by mutation of sites that are normally bound by SeqA (Bach and Skarstad, 2004). In addition, microarray and Southern blot analysis showed that the *oriC*-to-terminus ratio of a Δ*seqA* mutant was similar to the isogenic *seqA*$^{+}$ strain (Camara *et al.*, 2005). These results suggest that the sequestration of *oriC* by SeqA is not a major mechanism to regulate initiation.

Using a density-shift method to measure the interval between initiations, other investigators studied a Δ*hda* mutant that also encoded a mutation named *hsm-1*, which improved the

growth rate of the mutant but by itself is thought not to affect other cell cycle parameters such as doubling time, cell size, DNA content or initiation synchrony (Riber *et al.*, 2006). Exponentially growing Δ*hda* cells were rarely found to initiate more than once per cell cycle. In support, real-time PCR analysis to measure the *oriC*-to-terminus ratio showed that the frequency of initiation in a Δ*hda* mutant was comparable to an isogenic *hda*$^+$ strain. These results contradict those from microarray and Southern blot analysis, showing a two-fold increased *oriC*-to-terminus ratio for a similar Δ*hda* strain relative to a wild type strain (Camara *et al.*, 2005), and do not support the model that the conversion of DnaA-ATP to DnaA-ADP by Hda and the β clamp controls the initiation process.

Isolated as an intragenic suppressor of a *dnaA46*(Ts) mutant, the *dnaAcos* allele at 30°C induces excessive initiation, which causes lethality (Kellenberger-Gujer *et al.*, 1978). Controlled by a regulated promoter in a multicopy plasmid, induced *dnaAcos* expression is also toxic (Simmons and Kaguni, 2003), but elevated *dnaA*$^+$ expression only interferes with viability in strains that cannot repair double strand breaks (Simmons *et al.*, 2004). These observations suggest either that DnaAcos is enzymatically more active in initiation, or that the mutant protein fails to respond to regulatory pathways that control in initiation. Because a mutant DnaA carrying an A184V substitution that is responsible for the lethal phenotype of *dnaAcos* mutants (Carr and Kaguni, 1996; Simmons and Kaguni, 2003), or DnaAcos (Katayama and Kornberg, 1994) is not more active in *oriC* plasmid replication at 30°C in vitro than DnaA$^+$, the first possibility seems unlikely. Supporting the second possibility, DnaAcos is insensitive to IdaA, a partially purified cellular factor later identified as the β clamp, which acts with Hda to inactivate DnaA$^+$ in initiation (Katayama and Kornberg, 1994; Katayama and Crooke, 1995; Katayama *et al.*, 1998).

As *dnaAcos* mutants are not viable at 30°C because they hyperinitiate (Kellenberger-Gujer *et al.*, 1978), the conundrum is that null mutants of *hda*, *seqA*, and *datA* should be inviable if the respective loci are critical in regulating initiation frequency, yet these mutants survive. These observations raise the possibility that the conditions have not been identified under which Hda, *datA* and SeqA are physiologically important in controlling initiation.

In preceding work (Simmons *et al.*, 2004), we showed that the induced expression of *dnaA* causes more frequent initiations, which lead to an accumulation of double strand breaks in a strain defective in double-strand break repair. Presumably, the new forks collide from behind with stalled and collapsed replication forks, causing inviability from the failure to repair the double strand breaks. These results also suggest that an elevated level of wild type DnaA causes overinitiation by either surpassing or circumventing regulatory pathways that control the frequency of initiation. If so, increasing the copy number of a gene encoding a critical regulatory factor may suppress the lethal effect. To test this model, we prepared a library of chromosomal DNA fragments carried in a multicopy plasmid, selected for plasmids that suppressed the lethal effect caused by the induced expression of wild type *dnaA*, and obtained plasmids carrying *seqA*, *datA*, *dnaN* and *hda*. We also show genetically that *hda* functions with *dnaN*, which encodes the β clamp of DNA polymerase III holoenzyme, to control the frequency of initiation dependent on wild type DnaA, and that this pathway appears to be more critical in regulating initiation frequency than those involving *datA* or the sequestration of hemi-methylated *oriC* by SeqA. Finally, we show that DnaAcos fails to respond to Hda and the β clamp, and overcomes the *datA* pathway, providing an understanding of how *dnaAcos* hyperinitiates.

Results

The growth interference caused by elevated *dnaA* expression requires initiation from *oriC* and a deficiency in DNA repair

Using a plasmid that carries *dnaA* under control of the *araBAD* promoter (pDS596) and measuring the frequency of colony formation (Table 2), we confirmed previous results that the elevated expression of *dnaA* reduces viability of a *recB* mutant (SK002) but not of an isogenic *recB*$^+$ strain (MC1061) (Simmons *et al.*, 2004). As controls, we examined pBR322 and a plasmid-borne *dnaA* allele (*T435M*) that substitutes methionine for threonine at residue 435. The substitution disrupts recognition of the DnaA box motif and impairs DnaA function in initiation of an *oriC* plasmid (Sutton and Kaguni, 1997). Under induced conditions, this *dnaA* allele and the empty vector had essentially no effect on viability of the *recB* mutant, indicating that the growth interference requires wild type *dnaA* function.

We also showed that induced *dnaA* expression similarly interfered with viability of *recA* mutants (*E. coli* XL1-Blue (*recA1*) or MF0804 (Δ(*recA-srl*)::Tn*10*) but not of an isogenic *recA*$^+$ strain (C600). With a null *recA* strain lacking a functional *oriC* locus (MF0805 (Δ*oriC* Δ(*recA-srl*)::Tn*10*), induced *dnaA* expression did not affect viability, indicating that the lethal effect caused by the increased level of DnaA requires initiation from *oriC* (Table 2B). These results support the model that extra initiations lead to replication forks that collide from behind with stalled replication forks, followed by their collapse (Simmons *et al.*, 2004). In the absence of RecA or RecBCD function, the failure to repair the double strand breaks leads to inviability.

Isolation of genes and loci that negatively regulate initiation when present in a multicopy plasmid

Because extra initiations interfere with the viability of strains disabled in the repair of double strand breaks, we speculated that increasing the copies of a gene that negatively regulates initiation may suppress the lethal effect caused by increased *dnaA* expression. If so, *dnaAcos* may initiate excessively because it fails to respond to one or more regulators. Based on this reasoning, we constructed a plasmid library by partially digesting chromosomal DNA from *E. coli* MV1193 (Δ(*recA-srl*)::Tn*10*) with Sau3A endonuclease, and inserting DNA fragments (1–3 kb) into the single BamH1 site of pACYC184. DNA from a *recA* deletion mutant was used because plasmids carrying *recA*$^+$ would have complemented the *recA* mutation of the transformed strain (XL1-Blue (*recA1*)). Following electroporation with the ligation mixture and pDS596, transformants were selected on media supplemented with arabinose and the appropriate antibiotics. The pACYC184 derivatives were isolated from the transformants and re-introduced with the *dnaA* plasmid into *recA* and *recB* mutants to confirm their ability to suppress the toxicity causede by induced *dnaA* expression (Table 3). DNA sequence analysis of the chromosomal DNA fragments carried in pACYC184 revealed that eleven plasmids encode proteins or carry DNA sequences that are proposed to regulate initiation frequency (Figure 1).

We obtained one plasmid carrying *seqA* or *hda*, and three encoding *dnaN* (Figure 1). The separate isolation of the *hda* and *dnaN* plasmids suggests that the respective proteins act independently, which contrasts with biochemical studies showing that both Hda and the β clamp are required to stimulate the hydrolysis of ATP bound to DnaA (Kato and Katayama, 2001). We show below that *hda* and *dnaN* do not act separately. On arabinose-supplemented media, colonies of the *recA* mutant carrying the *hda* plasmid were noticeably larger than those obtained with plasmids bearing *seqA*, *dnaN*, or *datA* (data not shown), suggesting that *hda* suppresses more effectively than *seqA*, *dnaN* or *datA*. In contrast, the *seqA* plasmid

(pMMF57) failed to suppress the lethal effect caused by elevated *dnaA* expression in the *recB* strain (SK002); we suggest a possible explanation in the *Discussion*.

The isolation of pACYC184 derivatives carrying the *datA* locus supports the importance of this site (Kitagawa *et al.*, 1998). Apparently following induced expression, excess DnaA oligomerizes at *datA* in the multicopy plasmid to reduce the frequency of initiations. We note that the plasmids carrying *datA* do not contain the DnaA box within *yjeS*, suggesting that this sequence is not part of the *datA* locus (see Figure 1). This observation supports a previous study, which showed that mutations in this DnaA box reduced DnaA binding but did not affect the frequency of initiation (Ogawa *et al.*, 2002).

Multicopy plasmids carrying *oriC* and the adjacent *mioC* gene mitigate the toxicity caused by extra initiations

DnaA transcriptionally regulates the *mioC* gene, which is located to the right of *oriC* on the genetic map, by binding to specific sites in the *mioC* promoter region (Bogan and Helmstetter, 1996; Hansen *et al.*, 2007). As *oriC* contains DnaA boxes at which DnaA oligomerizes (Simmons *et al*., 2003; Felczak *et al*., 2005), multicopy plasmids that carry *oriC* or *mioC*, like those that bear *datA*, should titrate excess DnaA to offset the lethal effect caused by DnaA overproduction. As we failed to isolate plasmids carrying *oriC* or *mioC*, we constructed plasmids carrying these loci either separately or together (Figure 2A), and showed that they suppressed the growth interference caused by elevated *dnaA* expression (Figure 2B). However, colony size for the strain carrying plasmids encoding only *oriC* or *mioC* was minute on media containing arabinose relative to media lacking the inducer, so we may have overlooked transformants carrying *oriC* or *mioC* in selecting for multicopy suppressors. In contrast, the colonies for the strain carrying the plasmid with both *oriC* and *mioC* were comparable with those carrying the *dnaN* or *datA* plasmid on media containing the inducer (data not shown), which were only about four-fold smaller compared with media lacking arabinose (Figure 2B). Apparently, separate plasmids carrying *oriC* or the *mioC* promoter region are less effective in titrating the excess DnaA than a plasmid encoding both loci. Because we constructed the plasmid library with chromosomal DNA that had been partially digested with Sau3A endonuclease, which has fifteen sites in the region from *oriC* to the *mioC* promoter region, we believe that cleavage lowered the probability of constructing the corresponding plasmids.

To summarize at this point, the results strongly suggest that the increased abundance of SeqA, Hda, or DnaN, or more copies of either *datA*, or *oriC* joined to *mioC* diminish the frequency of initiations that would otherwise occur from elevated *dnaA* expression.

Comparison of pathways that inhibit re-initiation

If *seqA*, *hda*, or *datA* acts to regulate the frequency of initiation, the chromosomally encoded level of DnaA protein may initiate DNA replication in an unregulated manner in the respective null mutants, leading to the accumulation of lethal double strand breaks. In refute, these null mutants are viable, although a recent study described that a Δ*hda::cat* strain is severely growth-impaired, and acquires ameliorating suppressors (Riber *et al.*, 2006). Furthermore, genetic inactivation of *datA* or *seqA* has little if any measurable effect on initiation frequency measured by Southern blotting and microarray analysis, and deletion of *hda* only increased initiations by two-fold (Camara *et al.*, 2005).

Because of the apparent discrepancy between our results, which show that all three pathways are important in regulating initiation caused by an elevated level of DnaA, and those cited above (Camara *et al.*, 2005), we examined the effect of induced *dnaA* expression on the frequency of colony formation in isogenic repair-proficient strains lacking *hda*, *seqA*,

or *datA* as well as a strain carrying null mutations in both *seqA* and *datA*. As shown in Table 4, plating efficiency was unaffected when *seqA* or *datA* function was absent. In the strain lacking both *seqA* and *datA*, plating efficiency was only slightly reduced relative to the wild type control, suggesting that other mechanisms reduce the frequency of initiation. In contrast, reduced viability was observed with the Δ*hda* mutant, confirming previous observations (Riber *et al.*, 2006) and suggesting that initiation occurs more frequently in the *hda* null strain when *dnaA* expression was induced. Evidently, the repair capacity of the cell is insufficient in processing the increased level of double strand breaks.

The results of Table 4 also indicate the relative importance of *hda* and presumably also *dnaN* compared with other pathways in regulating initiation frequency. To support this conclusion, we quantified the abundance of *oriC* and *relE*, which is in the terminus region, by real-time PCR analysis with bacterial DNA isolated from cultures of the respective mutants or these strains bearing the *dnaA* expression plasmid (pDS596) under induced and uninduced conditions. With DNA from the plasmid-free cultures grown in media supplemented with glucose or arabinose, the respective *oriC*-to-*relE* ratio was 1.8, or 2.3 for the wild type strain (Figure 3A). The ratios for the Δ*datA* strain were similar to those for the wild type strain, in agreement with a previous study (Camara *et al.*, 2005). For the Δ*seqA* strain, ratios of 2.7 and 3.0 indicate slightly more frequent initiations compared with the wild type control. With the Δ*datA* Δ*seqA* mutant, which we constructed to measure the effect of the hda^+ gene alone or with other unidentified factors that regulate the frequency of initiation, the ratios were similar to the ratio measured for the Δ*seqA* mutant. In comparison, we respectively measured ratios of about 6 in the Δ*hda* mutant grown in synthetic media containing either glucose or arabinose, which is about three-fold greater than the wild type control. Previously, Camara *et al*. measured a lower ratio of 4.1 by Southern blotting for the same Δ*hda::tet*^{R} strain grown in rich media (Camara *et al.*, 2005). We do not know the reason for this difference.

We then measured the frequency of initiation in these strains carrying *dnaA* expression plasmid (pDS596) (Figure 3B). As a control, we verified that DnaA protein was induced to similar levels relative to the abundance of ribosomal protein L2 in portions of the same cultures that were analyzed by quantitative PCR (Figure 3C). With the uninduced cultures, the *oriC*-to-*relE* ratios were comparable to those values obtained with the plasmid-free strains (Figure 3A). When *dnaA* expression was induced, we again observed the highest ratio (7.3) for the Δ*hda* strain compared with lower values for Δ*datA*, Δ*seqA*, and Δ*datA* Δ*seqA* mutants (4.7, 4.3, and 4.9 respectively). These latter ratios were slightly higher than the ratio obtained for the wild type strain (3.6) under similar conditions. Together, these results (Figure 3A, 3B) show that *hda* has a greatest effect on initiation compared with the lesser effect of *seqA* and *datA*.

Genetically, *hda* and *dnaN* act in the same regulatory pathway

The separate isolation of plasmids encoding *hda* or *dnaN* suggests two possibilities. One is that their increased gene dosage compensates for the chromosomally encoded level of the β clamp (Burgers *et al.*, 1981; Leu *et al.*, 2000; Sutton *et al.*, 2005), or Hda (Su'etsugu *et al.*, 2005), which is insufficient to reduce initiations caused by the increased level of DnaA. The second possibility is that Hda and the β clamp act independently, which contrasts with findings from biochemical studies that Hda and the β clamp are both required to stimulate the hydrolysis of ATP bound to DnaA (Kato and Katayama, 2001). The inviability of the Δ*hda* strain when *dnaA* expression was elevated provides an assay to test genetically if *dnaN* and *hda* act in separate pathways. We observed that the plasmid encoding *hda* suppressed the harmful effect of extra initiations whereas the *dnaN* plasmid did not (Table 5). In contrast, we showed that the *dnaN* plasmid suppressed the lethal effect caused by elevated

dnaA expression in a *recA* or *recB* strain that also encoded the *hda*$^+$ gene (Table 3). These results support the conclusion that *hda* and *dnaN* function in the same regulatory pathway.

Surplus DnaAcos induces overinitiation at 30, 37 and 42°C, which is toxic, whereas excess DnaA causes extra initiation, which is lethal in a strain defective in DNA repair at 37 and 42°C but not at 30°C

We confirmed previous observations that repair-proficient strains encoding the chromosomal level of DnaAcos are viable at 42°C, but grow poorly if at all at 37 or 30°C (Table 6) (Kellenberger-Gujer *et al.*, 1978). This poor viability, which is exacerbated by a *recA* mutation, has been attributed to excessive initiation whereas the level of initiation at 42°C is apparently tolerable. In contrast, induced expression of *dnaAcos* reduces viability at all temperatures regardless of the strain's proficiency in DNA repair presumably because excess initiation leads to the accumulation of lethal double strand breaks (Table 7). By comparison, induced *dnaA*$^+$ expression at 37 and 42°C decreased the viability of a *recA* mutant but not of an isogenic *recA*$^+$ strain. Interestingly, an increased level of DnaA at 30°C did not affect viability of the *recA* mutant. Similar results were obtained when *dnaA*$^+$ expression was induced in a *recB* mutant (data not shown).

To correlate the reduced viability or lack thereof by *dnaA*$^+$ at 30°C in Table 7 to the level of initiation, we examined strains carrying the plasmids encoding the *dnaA*$^+$ (pDS596) and *dnaAcos* allele (pLS120), and the plasmid-free strain (MC1061) at 30, 37, or 42°C under induced and non-induced conditions. We used a repair-proficient strain because DNA repair should not affect initiation. Using genomic DNA prepared from culture samples removed at the indicated times, we measured initiation frequency by calculating the ratio of *oriC* to *relE* by real-time PCR analysis (Figure 4). Compared with the uninduced controls, elevated DnaAcos caused more frequent initiation at 30, 37 and 42°C. Initiation frequency, which increased with time after induced expression, was slightly greater than those obtained when the *dnaA*$^+$ gene was induced at 37°C, and were substantially higher at 42°C. However, we observed only a small increase in initiation following induced *dnaA*$^+$ expression at 30°C, which correlates with the lack of effect of increased *dnaA*$^+$ expression on viability at this temperature (Table 7). By quantitative immunoblot analysis with part of the same culture samples analyzed in Figure 4, we confirmed similar levels of DnaA$^+$ and DnaAcos after induction at all temperatures examined (data not shown), so a lower level of DnaA$^+$ does not cause the reduced initiation frequency at 30°C. These results suggest that DnaA$^+$ is less active at 30°C despite its overproduction. In contrast, DnaAcos is considerably more active than DnaA$^+$ at this temperature and at 42°C. At 37°C, although initiation by DnaAcos relative to wild type DnaA was only slightly more frequent, this mutant DnaA promotes appreciably more double strand breaks to account for its toxicity (Simmons *et al.*, 2004). Perhaps DnaAcos resists the effect of a regulatory factor(s) at 30°C whereas wild type DnaA is sensitive.

DnaAcos fails to respond to Hda and the *β* clamp, which leads to excessive initiation

Because overexpression of wild type DnaA interferes with viability in *recA* and *recB* strains, and multicopy plasmids bearing *hda*, *dnaN*, *seqA* or *datA* suppress this lethal effect, we hypothesized that DnaAcos initiates excessively because it fails to respond to one or more of these regulatory pathways. If so, one or several of the plasmids described above may fail to suppress the lethal effect caused by elevated *dnaAcos* expression. Table 8 summarizes the results of an experiment to test this hypothesis. As a control, we observed that the induced expression of *dnaA*$^+$ at 30, 37, or 42°C did not reduce viability of a wild type strain carrying either pACYC184, or plasmids encoding *hda*, *seqA*, *datA* and *dnaN*. In comparison, induced *dnaA*$^+$ expression was toxic in an isogenic *recA* mutant carrying pACYC184 at 37 and 42°C, but not at 30°C, in agreement with the results of Table 7. Except for the *seqA* plasmid

at 30°C, the remaining plasmids in the *recA* mutant suppressed the toxicity associated with increased *dnaA*$^+$ expression. As increased *dnaA*$^+$ expression is not lethal at 30°C, we did not expect to see a reduction in viability by the *seqA* plasmid at this temperature. We do not understand the basis of this observation.

When we examined if these plasmids were able to neutralize the toxicity associated with increased *dnaAcos* expression, only the *seqA* plasmid was active regardless of the strain's proficiency in DNA repair. In a comparable experiment, only the *seqA* plasmid suppressed the cold sensitivity of a *dnaAcos* mutant at 30°C (data not shown), in agreement with previous observations (Lu *et al.*, 1994). The results of Table 8 strongly suggest that DnaAcos fails to respond to Hda and the β clamp, and is the first genetic evidence to explain its hyperinitiation activity. The *datA* plasmid also failed to suppress the lethal effect caused by *dnaAcos* expression. Because the DNA binding domain of DnaAcos is unaffected by mutation, molecules of DnaAcos not bound to *datA* apparently are sufficient to cause excess initiation.

Discussion

Multicopy plasmids carrying *hda, datA, seqA*, or the *oriC-mioC* region alleviate the decrease in viability caused by the elevated expression of *dnaA*$^+$ in *recA* and *recB* mutants

We developed a genetic method designed to select multicopy suppressors that decrease the frequency of initiation when dnaA expression is elevated. The isolation of hda, dnaN, datA, and seqA as multicopy suppressors suggest that these loci regulate initiation, in support of other studies (Katayama, 2001; Kitagawa et al., 1998; Lu et al., 1994; von Freiesleben et al., 1994). We also showed that a multicopy plasmid carrying the oriC-mioC region neutralized the lethal effect caused by induced dnaA expression, and suggest that DnaA when in excess binds to this DNA.

We isolated other plasmids carrying *cpdA*, *cspC*, *ydaC*, and *traR*. The plasmid bearing *cpdA*, which encodes cAMP phosphodiesterase, may suppress by lowering the cAMP level to restrict the activity of catabolite activator protein. If so, reduced *dnaA* expression controlled by the *araBAD* promoter may lower the frequency of initiation. However, real-time PCR analysis shows similar frequencies of initiation in arabinose-induced cells carrying both the *dnaA* expression plasmid and the *cpdA* plasmid, or plasmids bearing *cspC*, *ydaC*, or *traR*, or with the empty vector (Felczak and Kaguni, unpublished results). In addition, quantitative immunoblot analysis showed similar levels of DnaA overproduction. Thus, we do not know how these genes act to suppress lethality.

DnaAcos overinitiates because it does not respond to Hda and the β clamp

At the onset of this work, we considered selecting for multicopy suppressors of *dnaAcos*. Had we done so, we would not have identified *datA*, *hda*, or *dnaN*, which control the activity of DnaA$^+$ but not DnaAcos. Indeed, other laboratories have isolated suppressors of *dnaAcos* that act indirectly. In one study, a multicopy plasmid carrying *cedA*, which is proposed to stimulate cell division instead of inhibiting DnaAcos, was isolated based on suppression of the cold-sensitive phenotype of *dnaAcos* (Katayama *et al.*, 1997). In another report, mutations in *holC* encoding the χ subunit of DNA polymerase III holoenzyme, or *ndk* (nucleoside diphosphate kinase) were isolated by apparently reducing the rate of replication fork movement (Nordman *et al.*, 2007). In a third study, the *seqA* gene carried in a multicopy plasmid suppressed *dnaAcos* (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994). In our multicopy suppressor approach, we showed that *datA*, *hda* and *dnaN* fail to regulate initiation caused by *dnaAcos*, thus providing a biochemical understanding of how this *dnaA* allele hyperinitiates.

Previously, we showed that the A184V substitution is responsible for the *dnaAcos* phenotype (Simmons and Kaguni, 2003), which severely reduces the affinity for ATP (Carr and Kaguni, 1996). If the concentration of free ATP in vivo is in the range of 0.1 mM (Schneider and Gourse, 2004), a substantial fraction of DnaAcos may be bound to ATP in vivo. Thus, the failure of Hda and the β clamp to stimulate the hydrolysis of ATP bound to DnaAcos maintains this protein in the active state for initiation to explain why DnaAcos hyperinitiates.

Possible mechanisms of suppression by *seqA* in a multicopy plasmid

Considering other studies of SeqA, several mechanisms may explain the ability of a multicopy *seqA* plasmid to suppress the inviability caused by oversupply of wild type DnaA or DnaAcos. As SeqA stimulates the relaxation activity of topoisomerase IV by interacting with its ParC subunit (Kang *et al.*, 2003), one possibility is that an elevated level of SeqA may reduce the superhelical density of the chromosomal domain containing *oriC.* Because a supercoiled plasmid is required for in vitro *oriC* plasmid replication (Funnell *et al.*, 1986), less frequent initiations may follow. Alternatively, the increased level of SeqA may inhibit re-initiation by prolonging the sequestered hemi-methylated state of *oriC* (Bach *et al.*, 2003), and by interfering with the binding of DnaA or DnaAcos to low affinity sites within *oriC*, which is necessary for initiation (Nievera *et al.*, 2006). In support, we noticed that the original transformant of *E. coli* XL1-Blue that was genetically selected and discovered to harbor the *seqA* plasmid (pMMF57) grew very poorly in both liquid and solid media, but induced *dnaA* expression alleviated this effect. These results, which were reproducible on re-transformation of this strain with the plasmids encoding *seqA* and *dnaA* (pDS596), suggest that the relative ratio of SeqA to DnaA determines if initiation has been perturbed, interfering with viability when SeqA is either in excess or sparse relative to active DnaA. However, these observations are strain-specific because they were not observed with the *recA::cam*^{R} derivative of MC1061 (Table 8). In addition, increased *dnaA*$^{+}$ expression in this strain was not lethal at 30°C, yet the *seqA* plasmid unexpectedly interfered with viability when DnaA was overproduced at this temperature.

Mutations in *recA* and *recB* compromise the repair of double strand breaks, which can form regressed replication forks when nascent strands anneal to form a four-strand Holliday junction. In specific replication mutants that require *recB* and not *recA* for viability, the exonuclease of RecBCD is proposed to degrade the duplex end of a regressed replication fork to restore a normal fork structure (Michel *et al.*, 2001). That the *seqA* plasmid was unable to suppress the toxic effect caused by surplus DnaA in the *recB* mutant suggests the requirement for RecB to process regressed replication forks that arise after overinitiation. This observation was reproducible in derivatives of MC1061 carrying the *recB268*::Tn*10* and Δ*recB745::kan* alleles (Baba *et al.*, 2006). As SeqA binds to hemimethylated DNA at the replication fork, appearing as discrete foci that co-localize with replication proteins (Onogi *et al.*, 1999; Brendler *et al.*, 2000), the increased SeqA level may strengthen the stability of these regressed forks.

Loci that are bound by DnaA with high affinity

DnaA is described to bind with high affinity to *oriC*, *datA*, and to four other chromosomal sites (*appY*, *mutH*, *narU*, and *ydfI*) (Roth and Messer, 1998). Except for *datA*, at which IHF aids DnaA binding (Nozaki *et al.*, 2009), we failed to isolate the other DNAs. We speculate that our failure to obtain plasmids carrying the *oriC-mioC* region likely originates from using Sau3A endonuclease to construct the library, which under-represented the *oriC-mioC* region due to its unusual abundance of Sau3A cleavage sites (Figure 2A). However, the other DNAs (*appY*, *mutH*, *narU*, and *ydfI*) have only a few Sau3A sites, so the failure to

isolate these loci suggests that these DNAs may not sequester a sufficient amount of DnaA when it is overproduced.

hda, dnaN, seqA, and *datA* each affect the frequency of initiation at *oriC* when *dnaA* expression is elevated

One study compared the frequency of initiation in strains lacking *hda*, *seqA* or *datA* (Camara *et al*., 2005). Measuring the ratio of *oriC* to a site in the terminus region by Southern blotting and microarray analysis, only the Δ*hda::tet*^{R} strain initiated more frequently, which was just two-fold higher than an isogenic wild type strain. In contrast, a separate study showed that the frequency of initiation in an independently constructed Δ*hda::cat* strain was comparable with an *hda*$^{+}$ strain (Riber *et al*., 2006). The conclusion from the first study that Hda and presumably also the β clamp regulate the activity of DnaA contrasts with that of the second study that Hda is not the predominant factor controlling initiation. Because DnaA complexed to ATP represses *dnaA* expression, and Hda with the β clamp stimulates the hydrolysis of ATP bound to DnaA (Katayama *et al*., 1998), the authors of the second study suggested that the effect of Hda on *dnaA* expression, coordinated with its role in modulating the nucleotide bound state of DnaA, regulates initiation (Riber *et al*., 2006). Our analysis of strains lacking *hda*, *datA* or *seqA* shows the importance of *hda* and likely also *dnaN* over the two other pathways regardless of whether *dnaA* is expressed from either the bacterial chromosome or an arabinose-inducible promoter in a multicopy plasmid (Figure 3). The *seqA* and *datA* pathways also contribute to reducing the frequency of initiation under conditions of elevated *dnaA* expression as shown by RT-PCR (Figure 3B) and by selection of these loci as multicopy suppressors (Table 3).

RecA is necessary to maintain viability when the level of DnaA is elevated

In a preceding study (Simmons *et al*., 2004), we stated that induced *dnaA* expression did not affect the viability of a strain carrying the Δ(*recA-srl*)::Tn*10* mutation. Whereas this result suggests that RecA is not required for the repair of double strand breaks, other laboratories have shown that RecA is necessary (reviewed in Michel *et al*., 2004). We now find that RecA is needed to maintain viability when *dnaA* expression is induced based on the decreased frequency of colony formation in strains carrying either *recA1* or Δ(*recA-srl*)::Tn*10* mutations (Table 2). Our earlier observations appear to stem from how a strain bearing a *dnaA* plasmid was handled. Whereas a newly transformed *recA* mutant (MF0804) showed a decrease of $>3 \times 10^{4}$-fold when *dnaA* expression was induced (Table 2), we observed only a three-fold reduction after the strain was stored in the cold for a few days (Felczak & Kaguni, unpublished results). The latter result correlates with a greatly reduced level of *dnaA* expression. We conclude that when extra initiations lead to double-strand breaks, RecA is necessary to maintain viability.

Experimental procedures

Bacteriological methods, strains and plasmids

Bacteriological methods including P1 transduction to construct the indicated strains in Table 1 were performed essentially as described (Miller, 1992). Bacterial strains and their plasmid-containing derivatives (Table 1, Figure 1 and 2) were grown as indicated in Luria-Bertani (LB) media or M9 media supplemented with 0.4% casamino acids, 1% (w/v) glucose or 0.5% (w/v) L-arabinose, and the appropriate antibiotics (100 μg/ml ampicillin, 35 μg/ml chloramphenicol, 50 μg/ml kanamycin, 100 μg/ml rifampicin, and/or 15 μg/ml tetracycline). Bacterial transformation was done by electroporation with 0.1 cm cuvettes, 40 μl of electrocompetent cells and 10 ng of plasmid DNA at 1.7 KV in a Bio-Rad Gene Pulser. To measure the effect of induced *dnaA* expression on viability, various dilutions of the electroporated samples were plated on media containing the appropriate antibiotic, and

either lacking or supplemented with L-arabinose (0.5% w/v). Unless noted, the frequency of colony formation was measured after overnight incubation at 37°C.

Construction of an *E. coli* genomic library

Bacterial DNA (25 μg), purified from MV1193 (Δ(*recA-srl*)::Tn*10*) as described (Neumann *et al*., 1992), was incubated with 4 units of Sau3A endonuclease at 37°C. From 3–8 min, portions were removed at one min time intervals, Na_2-EDTA was added to a final concentration of 20 mM, followed by incubation for 20 min at 65°C. Gel-purified DNA fragments ranging from 1–3 kb in size (1 μg) were ligated with T4 DNA ligase at a 1:1 molar ratio to pACYC184 DNA that had been linearized with BamH1 endonuclease and treated with calf intestinal phosphatase. The ligation mixture was purified using a PCR-purification kit (Qiagen), concentrated by lyophilization, and electroporated with 20 ng of pDS596 into highly electrocompetent *E. coli* XL1-Blue cells (60 μl, Stratagene). After incubation for 1 hr in SOC media at 37°C with aeration, most of the transformation mixture was plated on antibiotic-supplemented media containing L-arabinose to select for pACYC184 derivatives that suppressed the lethal effect caused by elevated *dnaA* expression. A smaller portion of the transformation mixture was plated on antibiotic-supplemented media lacking the inducer to calculate the number of transformants expected if the former portion of the mixture had been plated on media lacking arabinose. This value of 6.7×10^4 exceeds the 1.1×10^4 transformants necessary to represent the entire *E. coli* genome with a probability of 99%.

Individual transformants from the plates supplemented with arabinose were then grown overnight in LB media supplemented with ampicillin, chloramphenicol and arabinose. To confirm their ability to suppress the inviability caused by elevated *dnaA* expression, the pACYC184 derivates were gel-purified and electroporated with exogenously added pDS596 into a *recA* mutant (XL1-Blue). At this stage, we excluded pACYC184 derivatives that were originally obtained because each resided with a *dnaA* plasmid encoding a spontaneous inactivating mutation either in the regulatory *araBAD* promoter region, which will fail to express *dnaA* at an elevated level, or in the *dnaA* coding region. The remaining pACYC184 derivatives were then co-transformed to a *recB* strain (SK002) with supplementary pDS596 to establish that suppression can occur when a different step is blocked in the pathway to repair double strand breaks. The genes contained in these plasmids were identified by DNA sequence analysis.

Construction of pACYC184 derivatives carrying *oriC* and/or *mioC*

The plasmid pTSO182 was digested with SalI endonuclease to obtain a 1015 bp DNA fragment containing *oriC* and the flanking *mioC* gene, or with both SalI and XhoI endonuclease to obtain a 461 bp DNA containing *oriC*, and a 554 bp DNA carrying *mioC*. Each gel-purified DNA fragment was inserted into the Sal1 site of pACYC184. Because insertion of DNA into the SalI site of pACYC184 should inactivate the tetracycline resistance gene, chloramphenicol-resistant transformants were screened by replica plating on LB media containing both chloramphenicol and tetracycline to identify the appropriate isolates. Restriction analysis confirmed the expected structure of the plasmids carrying both *oriC* and *mioC* or the separated loci.

Real-time PCR analysis

E. coli MG1655 or MC1061 or its their isogenic derivatives (see Table 1) were grown with aeration in M9 or LB media supplemented with 1 % glucose (w/v) at 37°C. For strains bearing the plasmid with *dnaA* under *araBAD* promoter control (pDS596), the media also contained 100 μg/ml ampicillin. At a turbidity of about 0.25 OD (595 nm), the cells were collected by centrifugation at 18,000 rpm for 1 min in a Beckman F0650 rotor at 20°C, and

immediately resuspended in the pre-warmed media described above, but lacking glucose. Glucose or L-arabinose was then added to a final concentration of 1 % or 0.5%, respectively, to equal portions of the resuspended cells, and the cultures were grown with aeration at 30, 37 or 42 °C. Genomic DNA was isolated from the samples removed at the times indicated as described (Simmons *et al.*, 2004), and real-time PCR analysis was performed following the manufacturer's instructions with 5 ng of genomic DNA, SYBR Green PCR master mix (Applied Biosystems), and primers to amplify 100 base pair fragments in a 25 μl reaction volume. The oligonucleotide primers to amplify *oriC* and *relE* respectively are GAGATCTGTTCTATTGTGATCTCTTATTAGGAT paired with ACAGTTAATGATCCTTTCCAGGTTGT, and AGACCGGAGCTTAATCTTGTAACAA paired with ACAGTTGAAAA AGAAGCTGGTTGA. Using an ABI 7500 system, the instrument's default settings and the SDS software package, four identical PCR reactions for each sample was analyzed to quantify the abundance of *oriC* or *relE* relative to a standard curve prepared with genomic DNA isolated from a stationary phase culture of MG1655.

Immunoblot analysis

Immmunoblot analysis of whole cell lysates was performed with portions of the cultures analyzed in Figure 3B in parallel with the indicated amounts of purified DnaA or L2 ribosomal protein. After SDS-polyacrylamide gel electrophoresis and electrophoretic transfer, the membranes (Protran, Schleicher & Schuell) were incubated with rabbit antiserum that specifically recognizes one or more epitopes within amino acids 370 to 467 of the C-terminal region of DnaA protein. The same membrane was then stripped and reprobed with a rabbit antiserum against ribosomal protein L2. Detection of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (BioRad) in immune complexes was by chemiluminescence (Supersignal, Pierce) with a Kodak 4000R system.

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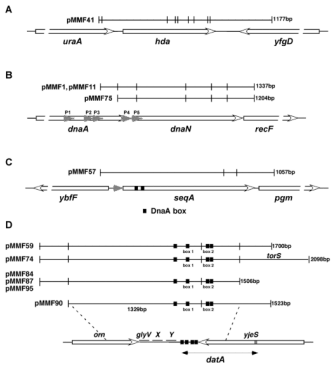

Figure 1. Chromosomal DNA fragments carrying genes and loci that regulate the frequency of initiation

In each panel, the horizontal lines represent the chromosomal DNAs carried by the indicated pACYC184 derivatives; the intersecting short vertical lines denote Sau3A recognition sites. Below the respective chromosomal DNA fragments, the horizontal boxes with arrows show each protein-coding gene and its polarity of transcription. The two *dnaN* plasmids and three *datA* plasmids carrying the same chromosomal DNA fragment were obtained from independent transformants. The orientation of *hda* (panel A), *dnaN* (panel B), and *seqA* (panel C) is counterclockwise relative to transcription of the *tet* gene encoded by pACYC184. Fragments containing *datA* (panel D) were obtained in both orientations. In panel D, *glyV*, *glyX*, and *glyY* correspond to tRNA genes. The shaded arrows and the filled boxes indicate promoters and DnaA boxes, respectively. The shaded box in *yjeS* represents the nonessential DnaA box (see *Results*). The plasmid named pMMF74 carries part of *torS*, which was joined adventitiously to the DNA carrying *datA*.

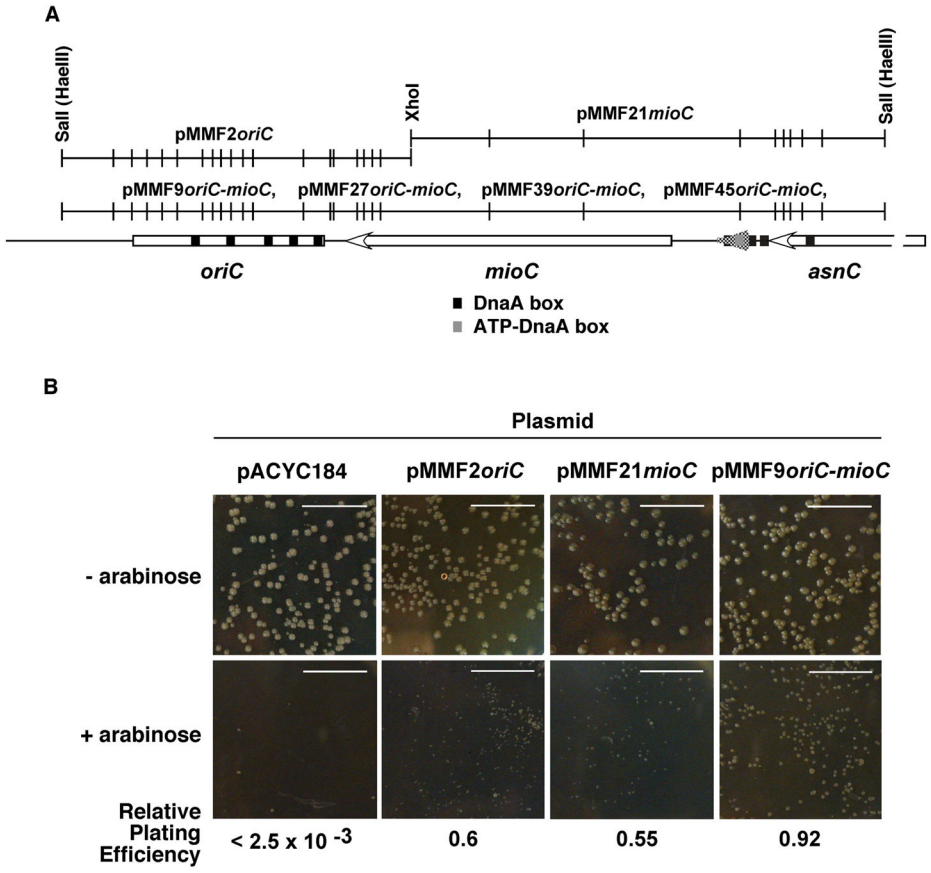

Figure 2. Derivatives of pACYC184 carrying *oriC* and/or *mioC*

The horizontal lines in panel A denote the DNA fragments in the respective plasmids, and the vertical lines are Sau3A recognition sites. The horizontal boxes with arrows represent *mioC* and *asnC*, which are located to the right of *oriC* on the genetic map. The filled boxes represent DnaA boxes, and the shaded arrow corresponds to the *mioC* promoter. Panel B shows colonies of *E. coli* XL1-Blue (*recA1*) carrying pDS596 and the indicated recombinant plasmids after overnight incubation at 37°C, and the relative plating efficiency of this strain bearing both plasmids on antibiotic-supplemented LB media with or without L-arabinose (0.5% w/v). Essentially the same relative plating efficiency was also observed with pMMF27*oriC-mioC*, pMMF39*oriC-mioC* and pMMF45*oriC-mioC*.

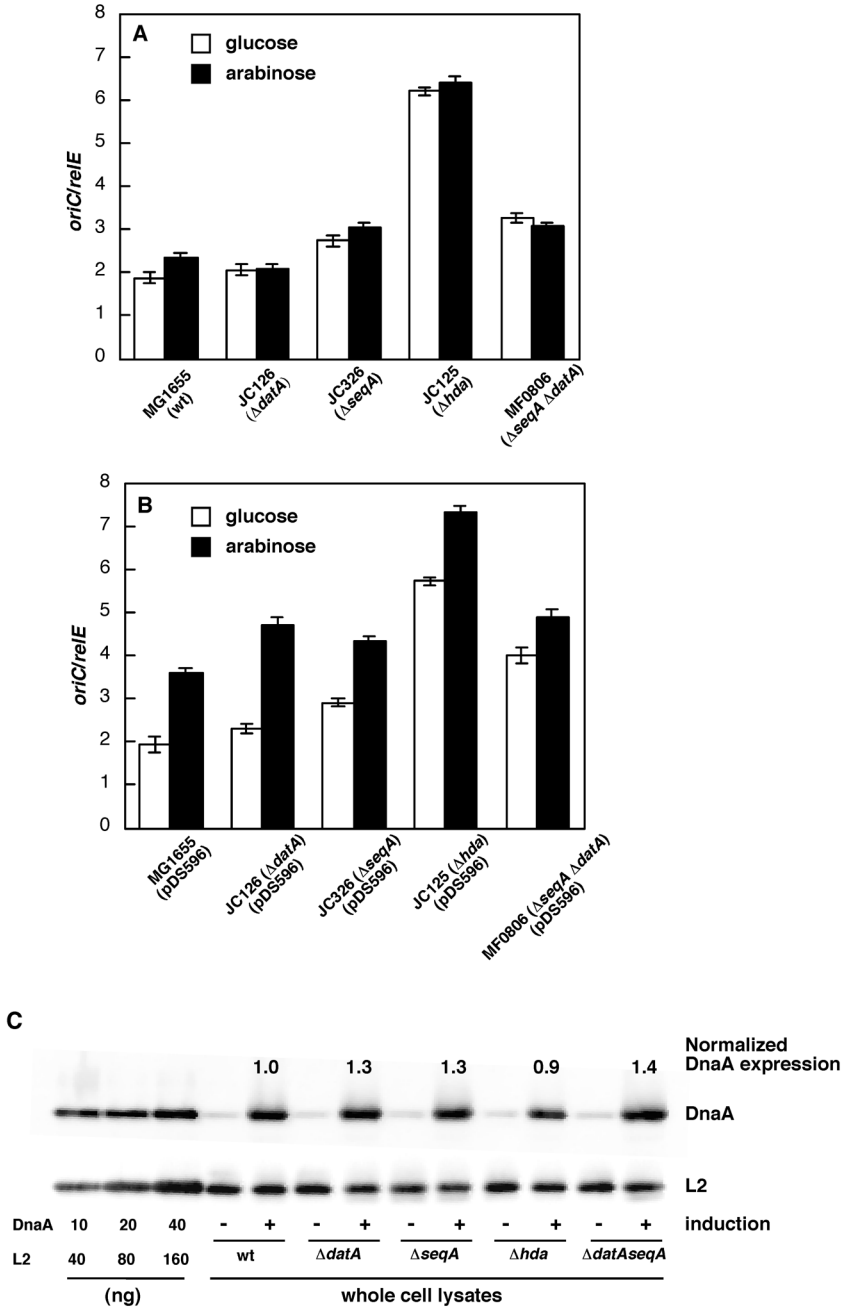

Figure 3. Quantitative PCR analysis of initiation frequency in mutants that are defective in regulating initiation from *oriC*

Real-time PCR analysis was performed as described in *Experimental procedures* to quantify the amounts of *oriC* or *relE*. Panel A and B summarize the analysis of the indicated strains, which were grown at 37°C in M9 media supplemented with glucose (1% w/v) and casamino acids (0.4% w/v) to a turbidity of about 0.25 OD (595 nm), and then transferred into prewarmed M9 media supplemented with casamino acids and glucose (1% w/v) or L-arabinose (0.5% w/v) followed by incubation with aeration at 37°C. Using culture samples removed at 1 hr, genomic DNA was then prepared for real-time PCR analysis. Because interplate variation is a major source of inaccuracy in quantifying the absolute amount of a

target sequence, we used one PCR plate to determine the abundance of *oriC* in the experimental samples and in increasing amounts of bacterial DNA from a stationary phase culture that was used to prepare a standard curve. On a separate plate, we measured the abundance of *relE* in the same DNA samples, and then calculated the ratio of *oriC* to *relE*. In Panel A and B, the *oriC*/*relE* ratios and the standard deviation calculated from 3 independent measurements are summarized in Table S1 and S2 of *Supporting information.* In panel C, whole cell lysates from the cultures analyzed in Figure 3B were examined by immunoblotting as described in *Experimental procedures* with rabbit antiserum that specifically recognizes DnaA. The lanes marked with the "+" or "−" symbols correspond to culture samples (0.02 OD at 595 nm) that either were induced to express DnaA protein, or were mock-treated, respectively. The same membrane was stripped and then incubated with antiserum that specifically recognizes ribosomal protein L2. After quantifying the amount of DnaA and ribosomal protein L2 by densitometry, the ratio of these proteins was normalized relative to the ratio calculated for MG1655, which was set at 1. In each panel, the indicated amounts of purified DnaA and L2 were analyzed in parallel.

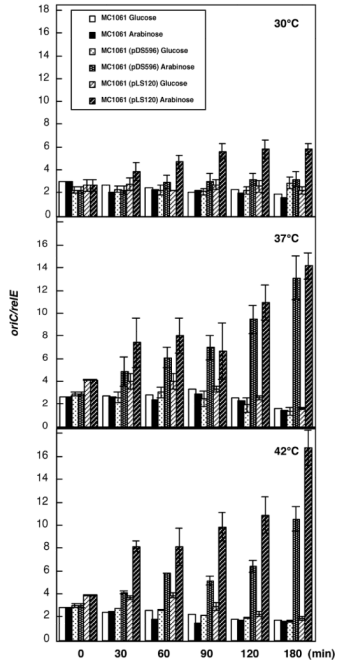

Figure 4. Elevated *dnaAcos* expression induces excessive initiation at 30, 37 and 42°C whereas increased *dnaA*$^+$ expression stimulates extra initiation at 37 and 42°C, but only modestly increases initiation at 30°C

E. coli MC1061 with and without a plasmid were grown at the respective temperatures in LB media supplemented with glucose (1% w/v) to an O.D. of about 0.25 (595 nm). The cells were then collected by centrifugation, resuspended in pre-warmed LB media supplemented with either glucose (1 % w/v) or arabinose (0.5% w/v), and samples were removed for real-time PCR analysis at the indicated times. For each of the three identical DNA samples for a specific time point for the strain bearing the *dnaA* plasmids, we quantified the abundance of these loci in quadruplicate as described in Figure 3 and *Experimental procedures*. The *oriC*/*relE* ratios are summarized in Table S3 of *Supporting information.*

Table 1

E. coli strains and plasmids

Strain	Genotype	Reference or source[a]
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K- m_{K+}) *sup E44 relA1 lac* [*F'*::Tn*10 proAB*$^+$ *lacI*^{q}*lacZ*Δ*M15*]	laboratory stock
MC1061	*araD139* Δ(*ara, leu*)*7697* Δ*lacX74 galU galK rpsL hsdR2* (r_K^- m_K^+) *mcrB1*	laboratory stock
JJC315	*leu6 his4 argE3 lacY*1 *galK*2 *ara*14 *xyl*5 *mtl*1 *tsx*33*rpsL*31 *supE*44 *hsdR recB*268::Tn*10*	Mitchel *et al*., 1997
SK002	MC1061*recB268*::Tn*10*	P1 (JJC315) × MC1061 → tet^R laboratory stock
MV1193	Δ*(lac-proAB) rpsL thi endA sbcB15 hsdR4* Δ(*recA -srl*)*306*::Tn*10* [*F' traD36 proAB lacI*^{q} *lacZ*Δ*M15*]	laboratory stock
C600	*thr-1 leuB6 lacY1 tonA1 supE44 thi-1*	laboratory stock
LS1062	MC1061 Δ*oriC*::pKN1562(clockwise) *kan*^{R} Δ*dnaA*850::Tn*10*	(Simmons and Kaguni, 2003)
MF0802	C600 Δ*oriC*::pKN1562(clockwise) *kan*^{R}	P1 (LS1062) × C600 → kan^R; this work
MF0804	C600 Δ(*recA-srl*)*306*::Tn*10*	P1 (MV1193) × C600 → tet^R; this work
MF0805	C600 Δ*oriC*::pKN1562 (clockwise) *kan*^{R} Δ(*recA- srl*)*306*::Tn*10*	P1 (MV1193) × MF0802 → tet^R; this work
MG1655	F$^-$ λ$^-$ *rph-1*	laboratory stock
JC125	MG1655 Δ*hda::tet*^{R}	(Camara *et al*., 2005)
JC326	MG1655 Δ*seqA::tet*^{R}	(Camara *et al*., 2005)
JC126	MG1655 Δ*datA::kan*^{R}	(Camara *et al*., 2005)
MF0806	MG1655 Δ*datA::kan*^{R} Δ*seqA::tet*^{R}	P1 (JC326) × JC126 → tet^R; this work
KO1125	*ilvO trpB9700 dnaAcos tna::*Tn*10*	(Nordman *et al*., 2007)
GW8026	*lacAU169 tif-1 sfiAll his-4 lexA300* (*Def*)*::spc* Δ(*recA-srlR*)306:: *cat*	G. C. Walker
MF0807	MC1061 *tna*::Tn10	P1 (KO1125) × MC1061 → tet^R; and cold resistance, this work
MF0808	MC1061 *dnaAcos tna* ::Tn10	P1 (KO1125) × MC1061 → tet^R; and cold sensitivity, this work
MF0809	MC1061 *tna*::Tn*10 recA::cam*	P1 (GW8026) × MF0807 → cam^R; this work
MF0810	MC1061 *dnaAcos tna* ::Tn*10 recA::cam*	P1 (GW8026) × MF0808 → cam^R; this work
Plasmid	**Properties**	**Reference or source**
pACYC184	*cat*^{R}; *tet*^{R}	(Chang and Cohen, 1978)
pBR322	*tet*^{R}; *amp*^{R}	(Bolivar *et al*., 1977)
pTSO182	*amp*^{R}, *oriC* and *mioC* in pBR322	(Oka *et al*., 1980)
pDS596	*amp*^{R}*dnaA* p_{araBAD};	(Hwang and Kaguni, 1988)
pLST435M	*amp*^{R}; *dnaAT435M*p_{araBAD}	(Simmons *et al*., 2004)
pLS120	*dnaAcos* p_{araBAD}	(Simmons *et al*., 2004)

[a]The arrow indicates the method of selection after transduction with P1 bacteriophage.

Table 2

Upon induced *dnaA*$^+$ expression the decreased viability of strains defective in DNA repair requires initiation from oriC.

	Strain	Relevant Genotype	Plasmid-Borne *dnaA* Allele	Relative Plating Efficiency[a]
A	MC1061 (pBR322)	*rec*$^+$ *oriC*$^+$	None	1.0
	MC1061 (pDS596)	*rec*$^+$ *oriC*$^+$	*dnaA*$^+$	0.9
	SK002 (pDS596)	*recB*::Tn*10 oriC*$^+$	*dnaA*$^+$	8×10^{-5}
	SK002 (pLST435M)	*recB*::Tn*10 oriC*$^+$	*T435M*	0.92
B	C600 (pDS596)	*rec*$^+$ *oriC*$^+$	*dnaA*$^+$	1.2
	XL1-Blue (pDS596)	*recA1 oriC*$^+$	*dnaA*$^+$	$<0.8 \times 10^{-4}$
	MF0804 (pDS596)	Δ(*recA-srl*)::Tn*10 oriC*$^+$	*dnaA*$^+$	4×10^{-3}
	MF0805 (pDS596)	Δ(*recA-srl*)::Tn*10* Δ*oriC*::pKN1562	*dnaA*$^+$	0.95

[a] Plasmids were electroporated into the indicated strains, and various dilutions were then plated on LB media supplemented with 100 μg/ml ampicillin with or without L-arabinose (0.5% w/v). Overnight incubation was at 37°C. Transformation efficiencies in the absence of inducer ranged from 1–3.7 × 10^6 per μg of plasmid DNA for the various strains. For all *rec*$^+$ strains, colony size was slightly smaller on media supplemented with arabinose compared with media lacking the inducer. The relative plating efficiency is the ratio of the number of colonies observed upon induced *dnaA* expression divided by the number of colonies in the absence of inducer.

Table 3

The *seqA, hda and dnaN* genes, and the *datA* locus carried in multicopy plasmids suppress the growth interference caused by the elevated expression of *dnaA*$^+$ in repair-defective strains

Plasmid	Locus	Map Position (minutes)	Relative Plating Efficiency[a]	
			XL1-Blue (*recA1*)	SK002 (*recB*::Tn*10*)
pACYC184	-	-	$< 0.8 \times 10^{-3}$	$< 1.7 \times 10^{-3}$
pMMF57	*seqA*	15.3	1.8[b]	$< 2.9 \times 10^{-3}$
pMMF41	*hda*	56.4	1.2	0.8
pMMF75	*dnaN*	83.6	0.8	0.9
pMMF1	*dnaN*	83.6	0.8	0.9
pMMF11	*dnaN*	83.6	0.8	0.9
pMMF59	*datA*	94.7	1.1	0.9
pMMF74	*datA torS*	94.7, 22.7	1.1	0.9
pMMF84	*datA*	94.7	1.1	1.0
pMMF87	*datA*	94.7	1.1	1.0
pMMF95	*datA*	94.7	0.9	0.9
pMMF90	*datA*	94.7	1.1	1.0

[a] The relative plating efficiency of the indicated strains after co-electroporation with pDS596 and the indicated plasmid is defined in Table 2.

[b] After overnight incubation at 37°C, colony size was two-fold larger on antibiotic-supplemented LB plates containing L-arabinose (0.5% w/v) compared with this media lacking L-arabinose.

Table 4

Induced *dnaA*$^{+}$ expression reduces the viability of a *Δhda::tet*^{R} mutant.

Strain	Relevant Genotype	Relative Plating Efficiency[a]
MG1655	wild type	1.0
JC125	Δ*hda*	8×10^{-4}
JC326	Δ*seqA*	1.0
JC126	Δ*datA*	1.0
MF0806	Δ*seqA* Δ*datA*	0.9

[a]The relative plating efficiency of the indicated strains after electroporation with pDS596 is defined in Table 2.

Table 5

A plasmid carrying *dnaN* in a Δ*hda*::*tet*^{R} strain fails to suppress the inviability caused by induced *dnaA*$^{+}$ expression.

Plasmid	Plasmid-borne Gene	Relative Plating Efficiency[a]
pACYC184	-	5×10^{-3}
pMMF41	*hda*	1.2
pMMF57	*seqA*	0.9
pMMF84	*datA*	0.2
pMMF1	*dnaN*	$<1 \times 10^{-3}$

[a]The relative plating efficiency of JC125 (Δ*hda*::*tet*^{R}) after electroporation with pDS596 and the indicated plasmid is defined in Table 2.

Table 6

The inability to repair double strand breaks exacerbates the cold-sensitive phenotype of a *dnaAcos* mutant.

Strain	Relevant Genotype	Relative Plating Efficiency [a]		
		30°C	37°C	42°C
MF0807	wild type	1.0	0.9	1.0
MF0809	*recA::cam*^{R}	0.9	1.0	1.0
MF0808	*dnaAcos*	2.6×10^{-3}	4.4×10^{-3}	1.0
MF0810	*dnaAcos recA::cam*^{R}	$<1.4\times10^{-5}$	1.4×10^{-4}	1.0

[a] Isogenic strains grown at 42°C were plated on LB media at the indicated temperatures. The relative plating efficiency is the ratio of colonies obtained at 30 and 37°C relative to 42°C, normalized to a value of 1.

Table 7

Surplus DnaAcos causes lethality at 30, 37 and 42°C whereas excess DnaA is toxic only at 37 and 42°C but not at 30°C in a repair-defective strain.

Strain	Relevant Genotype	Plasmid	Relative Plating Efficiency [a]		
			30°C	37°C	42°C
MF0807	wild type	pDS596 (*dnaA*$^+$)	0.7	1.1	1.3
MF0809	*recA::cam*^{R}	pDS596 (*dnaA*$^+$)	1.0	3×10^{-3}	1×10^{-2}
MF0807	wild type	pLS120 (*dnaAcos*)	2.9×10^{-3}	2.2×10^{-3}	2.6×10^{-3}
MF0809	*recA::cam*^{R}	pLS120 (*dnaAcos*)	4×10^{-3}	2.7×10^{-3}	3×10^{-3}

[a]Isogenic strains transformed with the respective plasmids were plated at the indicated temperatures on antibiotic-supplemented LB media containing 0.5% arabinose or lacking the inducer. The relative plating efficiency is defined in Table 2.

Table 8

Plasmids encoding *hda*, *dnaN*, *datA* and *seqA* suppress the lethality caused by oversupply of wild type DnaA whereas DnaAcos responds only to the plasmid encoding *seqA*.

Strain	Relevant Genotype	Co-transformed Plasmids		Relative Plating Efficiency [a]		
		dnaA plasmid	pACYC184 or derivative	30°C	37°C	42°C
MF0807	wild type	pDS596 (*dnaA*$^+$)	pACYC184	1.1	0.9	0.9
			pMMF41 (*hda*)	1.0	1.0	0.9
			pMMF57 (*seqA*)	1.0	1.0	0.8
			pMMF84 (*datA*)	1.4	1.0	0.9
			pMMF1 (*dnaN*)	1.0	0.7	1.0
MF0809	*recA::cam*^{R}	pDS596 (*dnaA*$^+$)	pACYC184	0.8	8×10^{-4}	8×10^{-4}
			pMMF41 (*hda*)	1.3	1.3	0.9
			pMMF57 (*seqA*)	2×10^{-3}	0.4	0.5
			pMMF84 (*datA*)	1.1	1.0	0.8
			pMMF1 (*dnaN*)	1.1	1.0	0.7
MF0807	wild type	pLS120 (*dnaAcos*)	pACYC184	7×10^{-4}	1.3×10^{-3}	1.4×10^{-3}
			pMMF41 (*hda*)	1.7×10^{-3}	1.7×10^{-3}	1.7×10^{-3}
			pMMF57 (*seqA*)	1.1	0.8	0.9
			pMMF84 (*datA*)	1.8×10^{-3}	3.6×10^{-3}	3.3×10^{-3}
			pMMF1 (*dnaN*)	1.5×10^{-3}	1.7×10^{-3}	1.5×10^{-3}
MF0809	*recA::cam*^{R}	pLS120 (*dnaAcos*)	pACYC184	1.9×10^{-3}	8×10^{-4}	8×10^{-4}
			pMMF41 (*hda*)	3×10^{-3}	1.5×10^{-3}	1.3×10^{-3}
			pMMF57 (*seqA*)	0.9	0.2	0.3
			pMMF84 (*datA*)	2.1×10^{-3}	1.5×10^{-3}	1.8×10^{-3}
			pMMF1 (*dnaN*)	3.6×10^{-4}	2.2×10^{-3}	2.5×10^{-3}

[a] Isogenic strains were co-transformed with the respective *dnaA* plasmids and either pACYC184 or its derivatives. The relative plating efficiency is the ratio of colonies obtained on antibiotic-supplemented LB media containing 0.5% arabinose or lacking the inducer.