Regulating DNA Replication in Bacteria

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The replication origin and the initiator protein DnaA are the main targets for regulation of chromosome replication in bacteria. The origin bears multiple DnaA binding sites, while DnaA contains ATP/ADP-binding and DNA-binding domains. When enough ATP-DnaA has accumulated in the cell, an active initiation complex can be formed at the origin resulting in strand opening and recruitment of the replicative helicase. In *Escherichia coli, oriC* activity is directly regulated by DNA methylation and specific *oriC*-binding proteins. DnaA activity is regulated by proteins that stimulate ATP-DnaA hydrolysis, yielding inactive ADP-DnaA in a replication-coupled negative-feedback manner, and by DnaA-binding DNA elements that control the subcellular localization of DnaA or stimulate the ADP-to-ATP exchange of the DnaA-bound nucleotide. Regulation of *dnaA* gene expression is also important for initiation. The principle of replication-coupled negative regulation of DnaA found in *E. coli* is conserved in eukaryotes as well as in bacteria. Regulations by *oriC*-binding proteins and *dnaA* gene expression are also conserved in bacteria.

Bacteria typically contain few chromosomes, each carrying a defined origin of replication (Messer 2002). The model bacteria referred to in this work *(Escherichia coli, Bacillus subtilis, Caulobacter crescentus*, and *Helicobacter pylori*) all have a single circular chromosome that is replicated bidirectionally from the origin. Some bacteria, for instance *E. coli* and *B. subtilis,* grow with overlapping replication cycles in rich media (Fig. 1) (Kornberg and Baker 1992; Helmstetter 1996). This allows for cell doubling times that are shorter than the replication phase, and requires replication initiation to occur at 2, 4, or 8 origins, depending on the growth rate. Replication timing is maintained such that initiation occurs simultaneously at all origins once per generation (Skarstad et al. 1986).

The key protein responsible for DNA strand opening at the origin and for the recruitment of replisome components is the initiator protein, DnaA (Kornberg and Baker 1992; Messer 2002; Duderstadt and Berger 2008; Ozaki and Katayama 2009; Kaguni 2011; Leonard and Grimwade 2011). DnaA is an AAA^+ type protein that binds ATP and ADP with high affinity. DnaA binds to high- and low-affinity sites in *oriC* and forms an oligomeric structure (Fig. 2) (Grimwade et al. 2000; Kawakami et al. 2005;

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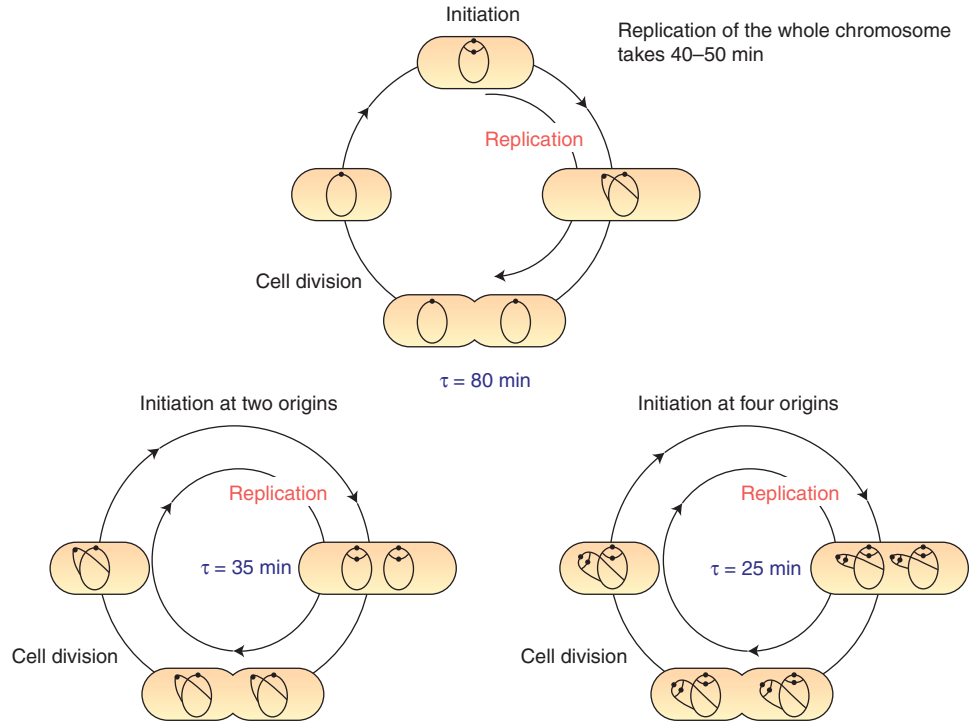

Figure 1. The replication cycle of the *E. coli* chromosome in slowly and rapidly growing cells. The chromosomal replication cycle and the cell division cycle in *E. coli* are shown. When cells are growing slowly (in this example, the doubling time τ is 80 min), cell division occurs after replication of the sister chromosomes is completed. When cells are growing rapidly (i.e., τ is 35 or 25 min), replication initiation simultaneously occurs at each *oriC* on the partially replicated chromosomes, and cell division occurs after the previous round of replication has been completed. Closed circles, *oriC*.

Erzberger et al. 2006; Kawakami and Katayama 2010) that involves two types of DnaA–DNA interactions, one with double-stranded and one with single-stranded DNA (Speck and Messer 2001; Fujikawa et al. 2003; Ozaki et al. 2008; Duderstadt et al. 2011; Ozaki and Katayama 2012). Only the ATP-bound form of DnaA is capable of binding to low-affinity sites (Fig. 2), which is supported by specific inter-DnaA interaction mediated through its AAA^+ domain, resulting in the nucleoprotein structures required for initiation activity (McGarry et al. 2004; Kawakami et al. 2005). Like ATP-DnaA, ADP-DnaA binds to high-affinity 9-mer DnaA boxes through its carboxy-terminal dsDNA binding domain, but forms multimers on *oriC* that are structurally distinct from those of ATP-DnaA. The binding of IHF, another positive regulator of initiation, to *oriC* induces a 180 degree bend in the DNA (Dillon and Dorman 2010) and plays an important role in forming an optimal initiation complex (Hwang and Kornberg 1992; Hiasa and Marians 1994; Cassler et al. 1995; Grimwade et al. 2000; Ryan et al. 2002; Leonard and Grimwade 2005; Ozaki and Katayama 2012). Other nucleoid-associated proteins (HU and Fis) also affect formation of the initiation complex (Fig. 3) (Gille et al. 1991; Hwang and Kornberg 1992; Hiasa and Marians 1994; Cassler et al. 1995; Wold et al. 1996; Margulies and Kaguni 1998; Ryan et al. 2002, 2004). Transcription by RNA polymerase is required for replication initiation (Kornberg and Baker 1992), though the mechanism by which this occurs remains unclear. Transcription in or around *oriC* is thought to facilitate opening of

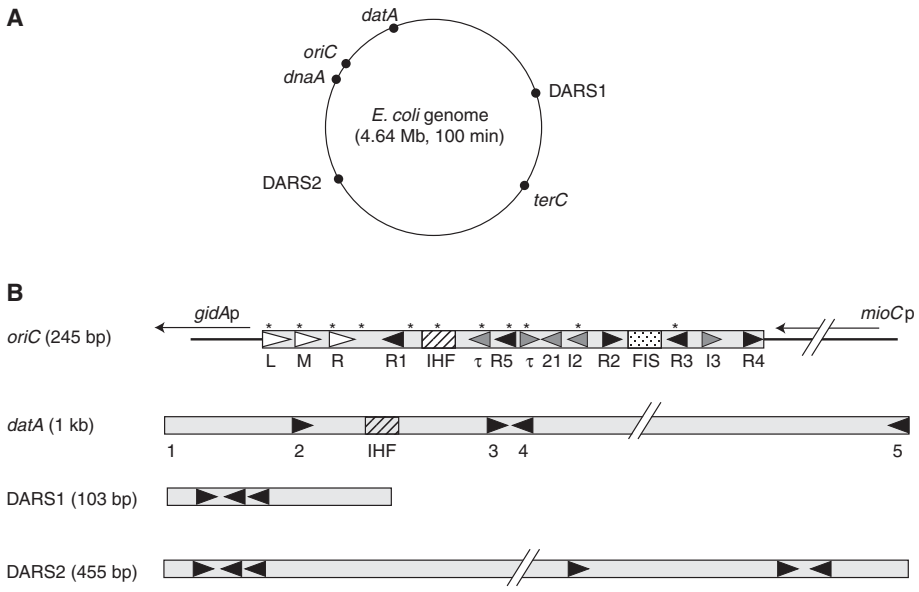

Figure 2. Structures of *oriC*, *datA*, and DARSs. (*A*) The *E. coli* chromosome is shown as a circle and the locations of *oriC* (at the genome map position of 84.6 min), *dnaA* (at 83.6 min), *datA* (at 94.6 min), DARS1 (at 17.5 min), DARS2 (at 64.0 min), and *terC* (around 36 min) are indicated. (*B*) Basic structures of *oriC*, *datA*, and DARSs are schematically shown. Closed triangle, DnaA box (9-nucleotide sequence). Gray triangle, I-sites, and τ-sites (both 6-nucleotide sequence). As for *datA*, DnaA boxes 2 and 3 are most crucial in repression of initiation (Ogawa et al. 2002). For details on low-affinity DnaA binding sites in *datA*, see Hansen et al. (2007). Open triangle, 13-mer AT-rich motif within DUE. IHF, IHF-binding site; FIS, FIS-binding site; *, GATC sequence within *oriC*.

the DNA strands, and may be a prerequisite for initiation in vivo under certain conditions, for instance, when nucleoid-associated proteins partially restrain negative superhelicity, thereby impeding strand opening (Baker and Kornberg 1988; Skarstad et al. 1990; Bates et al. 1997). In some circumstances, transcription through *oriC* may also be inhibitory (Su'etsugu et al. 2003) and for such transcription, the DnaA protein functions as a transcriptional regulator (Weigel and Messer 1997; Flåtten et al. 2009). Thus, DnaA has two roles at *oriC*, one as an initiator and the other as a transcription regulator.

After the DNA strands at *oriC* are separated, the DnaB helicase, bound to the helicase loader DnaC, is recruited to the initiation complex via interactions with *oriC*-bound DnaA (Sutton et al. 1998; Seitz et al. 2000; Messer 2002; Abe et al. 2007; Duderstadt and Berger 2008; Leonard and Grimwade 2010; Kaguni 2011). This stage may involve a reorganization of the DnaA-*oriC* complex and is influenced by the DiaA protein (Fig. 3) (Keyamura et al. 2009). DiaA binds to the amino terminus of DnaA and promotes initiation (Ishida et al. 2004; Keyamura et al. 2007). DiaA may also have a modulating effect on DnaB loading, because it binds to DnaA in the same place as DnaB, and inhibits loading of DnaB in vitro (Keyamura et al. 2009).

Regulation of initiation must fulfill two requirements. It must prevent extra initiation events, and it must ensure sufficient initiation so that one initiation event occurs per generation per origin. Several mechanisms ensure that extra initiation events do not occur (described below), but less is known about the timing of replication initiation, i.e., the rate-limiting steps, and whether the same factor(s) are required under all conditions. The frequency of replication must match the growth rate, otherwise the cellular DNA concentration will be altered. The cellular concentration of DnaA

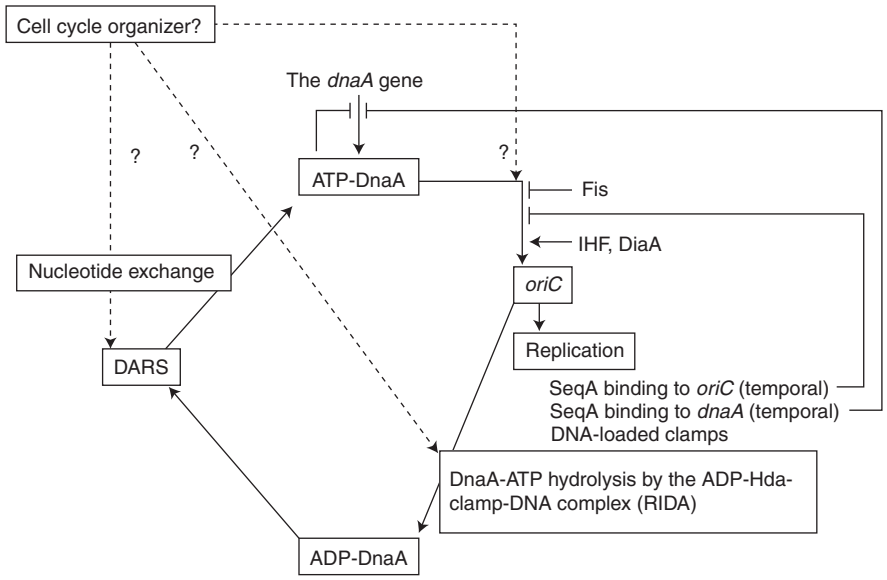

Figure 3. The DnaA cycle and regulation of *oriC* initiation. Transcription of the *dnaA* gene is autoregulated. ATP-DnaA is more active in inhibiting *dnaA* transcription than ADP-DnaA. Also, *dnaA* transcription increases before replication initiation and is repressed after it in a SeqA-dependent manner. Newly synthesized DnaA molecules adopt the ATP form. ATP-DnaA molecules are also generated by nucleotide exchange of ADP-DnaA in a DARS-dependent manner, though the cell cycle-dependent regulation of DARS remains unclear. With the assistance of DiaA and IHF, ATP-DnaA molecules form a multimeric complex on *oriC* in a cooperative manner. The resultant *oriC* complex unwinds DUE and is then engaged in replisome formation. The clamps remain on the synthesized DNA after Okazaki fragments are synthesized and form a complex with ADP-Hda, which interacts with and promotes the hydrolysis of ATP-DnaA (i.e., RIDA). Broken lines indicate expected but unrevealed pathways for cell cycle-coordinated regulation.

protein was found to be constant irrespective of the growth media (Hansen et al. 1991a) and a theoretical model has suggested that the production of DnaA couples growth rate to replication frequency (Hansen et al. 1991b). The ratio of ATP-DnaA to ADP-DnaA varies throughout the cell cycle and peaks right before replication initiation (Kurokawa et al. 1999). It has therefore been suggested that the frequency of initiation is determined by the accumulation of ATP-DnaA at *oriC* during steady-state growth (Donachie and Blakely 2003).

REGULATION AT THE ORIGIN SEQUENCE IN *E. coli*

Origin Sequestration

There are, in principle, two ways to prevent excess initiation of replication, one is to hinder origin usage, and the other is to inactivate the factors that perform initiation. In *E. coli*, origin usage is prevented by a process called origin sequestration that depends on the binding of the SeqA protein to newly replicated origins (Fig. 3; Table 1) (Lu et al. 1994; Slater et al. 1995; Brendler and Austin 1999; Waldminghaus and Skarstad 2009). Lack of SeqA causes untimely extra initiations and asynchrony in initiation of multiple origins (i.e., asynchrony phenotype).

SeqA discriminates between new and old origins by the methylation status of GATC sequences, which are present at high frequency in the *oriC* DNA sequence. The adenines of these sequences are recognized and methylated by Dam methylase. Chromosomal GATC sites remain hemimethylated for about a minute after the replication fork has passed (Campbell and Kleckner 1990). The high frequency of GATC sites in the origin (Fig. 2), however,

Table 1. Regulatory pathways for replication initiation

Regulation in *E. coli*	Target of regulation	Representative regulatory factor	Representative phenotype when regulation is disrupted	Possible analogs in other organisms
Sequestration	*oriC*	SeqA	Extra initiations, asynchrony phenotype	Regulation by Soj in *B. subtilis* Regulation by CtrA in *C. crescentus*
dnaA transcription	*dnaA* gene	DnaA, SeqA	Extra initiations, asynchrony phenotype (when SeqA function is inhibited or *dnaA* is overexpressed)	*dnaA* autoregulation in *B. subtilis* Cell cycle-coupled transcription in *C. crescentus*
Binding of DnaA to sites other than *oriC*	DnaA	*datA*	Extra initiations, changed timing in some growth conditions, asynchrony phenotype	Regulation by DnaA box clusters in *B. subtilis* and *Streptomyces coelicolor*
DnaA reactivation	ADP-DnaA	DARS	Inhibition of initiation, asynchrony phenotype	Unknown, but DARS-homologous sequences are conserved in certain bacterial genomes (Fujimitsu et al. 2009)
Stimulation of DnaA multimerization	ATP-DnaA	DiaA	Inhibition of initiation, asynchrony phenotype	Regulation by HobA in *H. pylori*
DnaA inactivation (RIDA)	ATP-DnaA	Hda	Severe amount of extra initiations, asynchrony phenotype Inhibition of cell division and cell proliferation	Regulation by YabA-clamp complex in *B. subtilis* Regulation by HdaA-clamp complex in *C. crescentus* Regulation by Cul4-DDB1-PCNA (clamp) complex in eukaryotes

results in origin DNA remaining hemimethylated for about a third of a generation after replication (Campbell and Kleckner 1990; Lu et al. 1994; Bach and Skarstad 2005). During this time, the origin is bound by SeqA, which, in addition to preventing GATC methylation by Dam methylase, inhibits replication initiation (Fig. 3; Table 1) (Lu et al. 1994; Boye et al. 1996; Guarne et al. 2002; Fujikawa et al. 2004). Also, DnaA itself contributes to origin sequestration, probably by hindering remethylation by Dam at some of the GATC sites in *oriC* (Lu et al. 1994; Bach et al. 2008). In vitro, SeqA forms fibers of head-to-head dimers (Guarne et al. 2005; Kang et al. 2005; Odsbu et al. 2005). Additionally, the binding of SeqA multimers to *oriC* can alter DNA superhelicity (Torheim et al. 1999) and inhibit ATP-DnaA binding to low-affinity sites (Nievera et al. 2006), both of which can inhibit the initiation reaction (Fig. 3; Table 1) (Lee et al. 2001; Waldminghaus and Skarstad 2009).

Microscopy of fluorescently labeled SeqA indicates that the majority of cellular SeqA forms relatively compact structures with the hemimethylated DNA at replication forks (Hiraga 1998, 2000; Brendler et al. 2000; Molina and Skarstad 2004; Yamazoe et al. 2005; Fossum et al. 2007). These structures are dynamic and trail the replication forks, and always bind to the most newly synthesized DNA (Yamazoe et al. 2005; Waldminghaus et al. 2012). Because extra SeqA foci representing sequestered origins have not been detected, it has been suggested that

sequestered origins bound by SeqA are situated at, or near, the replication fork structures (Molina and Skarstad 2004; Bach and Skarstad 2005; Morigen et al. 2009).

Regulation by Transcription at and near the Origin

Transcription around and at *oriC* can affect replication initiation and may also contribute to regulation of initiation frequency. The *mioC* and *gidA* genes are located at the right and left side of *oriC*, respectively (Fig. 2). The *mioC* gene does not have a strong terminator and its transcripts read through *oriC* (Messer and Weigel 1997). The *gidA* gene is transcribed away from *oriC*. The *mioC* promoter region contains a DnaA box cluster and DnaA binding negatively regulates *mioC* gene transcription (Messer and Weigel 1997; Hansen et al. 2007). Transcription of *mioC* is repressed before replication initiation and is derepressed after it (Theisen et al. 1993; Ogawa and Okazaki 1994; Bogan and Helmstetter 1997). Constitutive transcription of *mioC* impedes initiation (Su'etsugu et al. 2003). The transcriptional pattern of *gidA* is opposite to that of *mioC* (Theisen et al. 1993; Ogawa and Okazaki 1994; Bogan and Helmstetter 1997). Deleting the promoters of these genes does not affect initiation regulation in wild type cells during steady-state growth, although the transcription of these genes can stimulate initiation in cells bearing a mutation impeding initiation (Bates et al. 1997).

REGULATION OF THE DnaA PROTEIN IN *E. coli*

Overview

The production and activity of *E. coli* DnaA protein is regulated in several ways: by transcription, intracellular localization, and conformation (Katayama et al. 2010; Leonard and Grimwade 2010; Kaguni 2011). DnaA protein is stable, but replication cycle-specific *dnaA* gene transcription is important for sustaining well-timed initiation of replication (Bogan and Helmstetter 1997; Riber and Løbner-Olesen 2005). This can be explained by the role of ATP-DnaA in activating initiation (Kurokawa et al. 1999; Nishida et al. 2002) and the idea that newly synthesized DnaA preferentially binds ATP, the cellular level of which is 10-fold higher than that of ADP (Fig. 3).

Also, timely initiation of replication during the cell cycle requires specific chromosomal regions termed DARS (DnaA-reactivating sequence) 1 and DARS2. These regions bind ADP-DnaA molecules and promote the regeneration of ATP-DnaA by nucleotide exchange (Figs. 2 and 3) (Fujimitsu et al. 2009).

It has been estimated that there are 500–2000 DnaA molecules per cell, depending on strain backgrounds and growth rates (Sekimizu et al. 1988; Chiaramello and Zyskind 1989; Hansen et al. 1991a). *oriC* can bind 10–20 DnaA molecules (Messer 2002). A considerable number of DnaA molecules can be titrated at a specific chromosomal locus termed *datA* that is required for repressing rifamcipin-resistant unregulated initiation events (Figs. 2 and 3) (Kitagawa et al. 1998; Morigen et al. 2005).

The ATP-DnaA level fluctuates during the replication cycle, peaking around the time of initiation (Kurokawa et al. 1999). ATP-DnaA hydrolysis is required to reduce ATP-DnaA levels after initiation (Fig. 3). This regulation, termed RIDA (regulatory inactivation of DnaA), is coupled with the action of the DNA polymerase III holoenzyme. RIDA is crucial for regulating initiation so that it occurs only once per generation (Katayama et al.1998; Kato and Katayama 2001; Su'etsugu et al. 2004; Camara et al. 2005).

Regulation of *DnaA* Gene Transcription

The cellular DnaA concentration was found to be constant irrespective of growth medium and the cell cycle (Hansen et al. 1991a). However, the transcription of the *dnaA* gene varies in a replication cycle-dependent manner (Bogan and Helmstetter 1997). The main reason for the fluctuation seems to be that the *dnaA* gene promoter is sequestered by SeqA for almost the same duration as the origin (Fig. 3; Table 1) (Campbell and Kleckner 1990; Lu et al. 1994; Riber and Løbner-Olesen 2005). During sequestration, the promoter is unavailable to the transcription machinery. Because the *dnaA* gene is situated

near *oriC*, this contributes to reducing the production of DnaA and thus the initiation potential (i.e., the ability to initiate replication) soon after new replication forks have been launched (Riber and Løbner-Olesen 2005). The *dnaA* promoter area also contains DnaA boxes and the promoter was found to be capable of autoregulation (Fig. 3; Table 1) (Messer and Weigel 1997; Hansen et al. 2007). Translation of *dnaA* mRNA has not been well characterized, but it is known that the start codon, GTG, functions inefficiently in *E. coli*.

Binding of DnaA to Sites Other than oriC

As mentioned, the DnaA protein functions not only as the initiator but also as a gene regulatory protein. There are about 300 high-affinity DnaA binding sites and a very large number of low-affinity sites around the chromosome (Kitagawa et al. 1996; Roth and Messer 1998; Hansen et al. 2007). As the chromosome is replicated, the DnaA binding sites are duplicated and contribute to titration of DnaA away from *oriC*. The main titration site, *datA*, is situated near *oriC* and is duplicated soon after initiation of replication (Kitagawa et al. 1996, 1998; Ogawa et al. 2002; Morigen et al. 2003, 2005). In vivo studies indicate that the *datA* site binds on average 60 DnaA molecules (Hansen et al. 2007). The *datA* site should therefore contribute to reducing the initiation potential at *oriC* when *oriC* is still in sequestration. The *datA* site is about 1 kb in size and contains five high-affinity DnaA binding sites and about 25 low-affinity sites (Fig. 2; Table 1) (Kitagawa et al. 1996, 1998; Hansen et al. 2007). High-affinity DnaA boxes 2 and 3 are crucial for efficient binding of DnaA to *datA*. It is possible that the DnaA bound to these sites function as a core for further cooperative DnaA binding (Ogawa et al. 2002). The production of DnaA protein as cells grow and the generation of binding sites as the chromosome is replicated, as well as sequestration and RIDA, has been simulated *in silico* (Hansen et al. 1991; Browning et al. 2004; Atlas et al. 2008) and may indicate that initiation occurs as soon as enough ATP-DnaA has accumulated at *oriC*. However, cells with similar numbers of origins and chromosomal DnaA binding sites but different growth conditions have been reported to contain different amounts of DnaA per origin at initiation (Torheim et al. 2000; Flåtten et al. 2009). So, although origin firing as soon as enough ATP-DnaA has accumulated is an attractive model of cell cycle regulation, a complete understanding of regulation encompassing different growth conditions is still lacking even in the well-characterized *E. coli* bacterium.

Regulation of the DnaA Nucleotide Form by DARS and Acidic Phospholipids

E. coli cells can convert ADP-DnaA to ATP-DnaA by nucleotide exchange (Fig. 3) (Kurokawa et al. 1999; Fujimitsu et al. 2009). The DARS1 and DARS2 sequences promote this reaction and are located halfway within the intergenic region between *oriC* and *terC*, to the right and left of *oriC*, respectively (Fig. 2A) (Fujimitsu et al. 2009). A common feature of DARS1 and DARS2 is the presence of a DnaA box cluster, in which three DnaA boxes are similarly oriented and are located at a similar distance (Fig. 2B). Multiple ADP-DnaA molecules can form complexes with DARS, which facilitates the release of ADP from DnaA. The resultant apo-DnaA molecules are likely released from DARS because of reduced complex formation activity, which allows the binding of ATP and DnaA reactivation. Increasing the cellular copy number of DARS1 or DARS2 increases the ATP-DnaA level, inducing extra initiation events, whereas deleting both DARS1 and DARS2 causes a delay in initiation (Fujimitsu et al. 2009). Newly translated DnaA protein binds ATP, providing cells with a basal level of ATP-DnaA. However, this alone is not enough to initiate replication in a timely manner. Thus, the function of DARSs is crucial for timely initiation (Fig. 3; Table 1). Functional regulation of DARS remains to be clarified, except for the fact that each DARS contains a regulatory region in addition to the common sequence bearing the DnaA box cluster (Fujimitsu et al. 2009; Leonard and Grimwade 2009). In addition to DARS, acidic phospholipids such as cardiolipin and phosphatidylglycerol play an important role in the nucleotide

release of ADP-DnaA (Sekimizu and Kornberg 1988), and DnaA is reactivated by exchange of the bound nucleotide in vitro in the presence of *oriC* and ATP (Crooke et al. 1992). Reduction of the acidic phospholipids in vivo can inhibit initiation at *oriC* (Xia and Dowhan 1995), but exactly how acidic phospholipids affect DnaA activity during the cell cycle remains to be elucidated.

Regulation of DnaA Multimer Formation by DiaA

DiaA forms homotetramers and each protomer contains a specific site for binding to DnaA domain I (Fig. 3) (Ishida et al. 2004; Keyamura et al. 2007, 2009). These features allow a single DiaA tetramer to bind multiple molecules of DnaA, which can stimulate cooperative binding of DnaA to *oriC* and the unwinding reaction (Fig. 3; Table 1). The binding of ATP-DnaA to low-affinity binding sites within *oriC* is enhanced by DiaA (Fig. 1) (see Bell and Kaguni 2013; Leonard and Méchali 2013). In *diaA*-disrupted mutant cells, replication initiation is delayed and initiation at sister *oriC* copies occurs asynchronously in rapidly growing cells (Ishida et al. 2004; Keyamura et al. 2007, 2009). These data are consistent with the observation that replication is initiated asynchronously in mutants bearing DnaA box R4-deleted *oriC* (Bates et al. 1995), because the binding of DnaA to high-affinity DnaA box R4 enhances cooperative DnaA binding to low-affinity sites.

After DUE unwinding in *oriC* complexes, DiaA must be released from DnaA (Keyamura et al. 2009). The DiaA-binding site of DnaA domain I is also used to bind DnaB helicase. However, the mechanism of DiaA-DnaA dissociation has not yet been elucidated.

DiaA orthologs are evolutionarily conserved in bacterial species (Keyamura et al. 2007). In addition, the HobA protein of *Helicobacter pylori* (Hp), a member of ε-Proteobacteria, displays functional and structural similarity to DiaA, although there is no significant sequence similarity (Table 1) (Natrajan et al. 2007, 2009; Zakrzewsak-Czerwinska et al. 2007; Zawilak-Pawlik et al. 2007, 2011; Terradot et al. 2010).

Regulation of the DnaA Nucleotide Form by RIDA

After ATP-DnaA promotes replication initiation, it is hydrolyzed in a manner dependent on a complex consisting of ADP-Hda protein and the DNA-loaded clamp (Fig. 4; Table 1) (Katayama et al. 1998; Kato and Katayama 2001; Su'etsugu et al. 2008). The resultant ADP-DnaA is inactive in initiation. This system is termed RIDA (regulatory inactivation of DnaA). RIDA is crucial for DnaA inactivation and thereby effectively supports once-per-generation initiation (Kurokawa et al. 1999; Camara et al. 2005; Riber et al. 2009). The *hda* gene is required for promoting cell proliferation, decreasing cellular ATP-DnaA levels and repressing overinitiation (Kato and Katayama 2001; Fujimitsu et al. 2008; Charbon et al. 2011). Incubation of temperature-sensitive *hda* mutant cells at the restrictive temperature leads to overinitiation of replication and induces inhibition of cell division, producing filamentous cells (Fujimitsu et al. 2008). Inhibition of cell division is thought to be a consequence of checkpoint regulation, but the exact mechanism by which this occurs remains unknown. DnaA AAA^+ sensor II motif Arg-334 is specifically required for ATP-DnaA hydrolysis and expression of a DnaA R334A mutant protein causes overinitiation and inhibition of cell growth in an *oriC*-dependent manner (Table 1) (Nishida et al. 2002).

Hda protein consists of a short amino-terminal region containing the clamp-binding motif and an AAA^+ domain that is homologous to DnaA domain III (Fig. 4) (Dalrymple et al. 2001; Kato and Katayama 2001; Kurz et al. 2004; Su'etsugu et al. 2005; Xu et al. 2009). The Hda clamp-binding motif is commonly present in clamp-binding proteins such as DNA polymerase III core subunit α (Dalrymple et al. 2001). It binds to the hydrophobic pocket of the clamp, which is the same site to which DNA polymerase III subunit α binds (Dalrymple et al. 2001; Kurz et al. 2004; Su'etsugu et al. 2005). The Hda AAA^+ domain specifically binds ADP, but not ATP (Su'etsugu et al. 2008). ADP-Hda is monomeric and active in RIDA, whereas apo-Hda is multimeric and inactive in RIDA (Su'etsugu

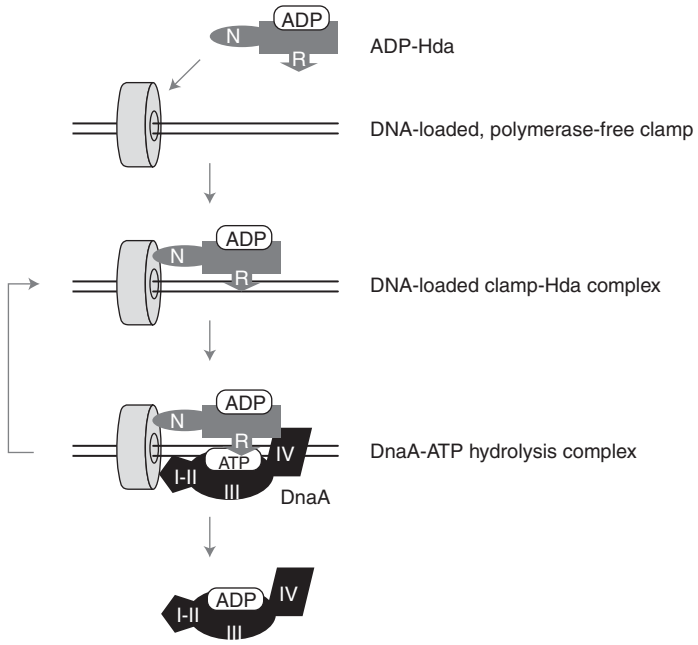

Figure 4. The basic mechanism of RIDA. When DNA polymerase III holoenzyme completes Okazaki fragment synthesis on the lagging strand, the clamp subunit is released from the DNA polymerase III core and remains on the synthesized DNA. ADP-Hda binds to the hydrophobic pocket of the DNA-loaded form of the clamp via the clamp-binding motifs in the amino terminus of Hda. The resultant ADP-Hda-clamp-DNA complex interacts with and promotes the DnaA-bound ATP hydrolysis, releasing ADP-DnaA back into the DnaA cycle. The interaction between the AAA^+ domains of Hda and DnaA is crucial, and is assisted by the interaction between Hda and DnaA domain IV (DNA-binding domain). N, Hda-amino terminus; I-II, DnaA domain I-II; III, DnaA domain III (AAA^+ domain); IV, DnaA domain IV (DNA-binding domain).

et al. 2008). The Hda AAA^+ domain bears a specific Arg residue (i.e., Arg finger) that is crucial for promoting DnaA-ATP hydrolysis (Su'etsugu et al. 2005). This residue may participate in forming the ATP hydrolysis catalytic center by direct DnaA-Hda interaction. This is a common characteristic of many AAA^+ proteins (Neuwald et al. 1999; Ogura et al. 2004; Indiani and O'Donnell 2006). In addition to the Arg finger, specific residues within the AAA^+ domains of DnaA and Hda are required for DnaA-Hda interaction and DnaA-ATP hydrolysis (Nakamura et al. 2010). DnaA domain IV (DNA binding domain) promotes this interaction by binding to Hda (Fig. 4) (Keyamura and Katayama 2011).

During DNA elongation, the clamps remain on the lagging strand after Okazaki fragments are synthesized and the DNA polymerase III core is released (Yuzhakov et al. 1996; Balakrishnan and Bambara 2013; Goodman and Woodgate 2013; Hedglin et al. 2013; MacAlpine and Almouzni 2013). DNA-loaded, DNA polymerase-free clamps bind ADP-Hda, resulting in the activation of RIDA (Su'etsugu et al. 2004, 2008). In addition, it is possible that because a clamp is a homodimer, Hda and DNA polymerase III subunit α bind to each protomer of the same clamp to allow Hda to hydrolyze DnaA-ATP as soon as replication forks are under way (Johnsen et al. 2011). DNA-free clamps are inactive in RIDA, although they can bind Hda, which ensures the timely and replication-coupled activation of RIDA. The dsDNA region flanking the clamp is required for RIDA, and may be recognized by DnaA (Fig. 4) (Su'etsugu et al. 2004).

The ADP-Hda-clamp-DNA complex is stable, whereas the affinity of this complex for DnaA is weak (Su'etsugu et al. 2008). This is

consistent with the fact that the complex is reused for cyclically interacting with multiple ATP-DnaA molecules, and that there are only about 100 Hda molecules per cell (Katayama et al. 2010).

The main principle of RIDA, which is the use of DNA-loaded clamps for replication-coupled negative feedback to the initiator protein, is evolutionarily conserved from bacteria to eukaryotes, including yeast, *Xenopus*, and human cells (Table 1) (Katayama et al. 2010; Zielke et al. 2012).

Coordination of Regulation for *oriC* and DnaA

SeqA binds to hemimethylated *oriC* immediately after initiation and replication of the *oriC* region. In a culture with a doubling time of 30 min, the binding of SeqA to *oriC* lasts for about 10 min. The ratio of ATP-DnaA to ADP-DnaA peaks at initiation, and then undergoes a gradual decrease because of RIDA activity. It takes approximately 15 min to decrease this ratio to the lowest levels, and probably less than 10 min to decrease the ratio enough to prevent initiation from occurring when the origins are released after 10 min of sequestration. Thus, SeqA inactivates *oriC* early during the inter-initiation period, whereas RIDA represses the DnaA initiation activity for the remainder of this period. Coordination of these two steps is crucial for repressing untimely initiation events (Fig. 3) (Lu et al. 1994; Kurokawa et al. 1999; Skarstad and Løbner-Olesen 2003; Katayama et al. 2010). During multifork replication, in which replication goes on continuously and only the number of replication forks under way changes through the cycle (Fig. 1), it could be that RIDA needs to be temporarily inhibited to achieve a high enough ratio of ATP-DnaA to ADP-DnaA at initiation. Whether such a temporary inhibition occurs remains to be elucidated.

The balance between Dam methylase and SeqA activities governs the duration of the sequestration period (Bach et al. 2003). If Dam methylase is overexpressed, extra rounds of replication occur (Boye and Løbner-Olesen 1990), indicating that high levels of Dam methylase shorten the sequestration period, resulting in origin availability before the initiation potential has dropped (Skarstad and Løbner-Olesen 2003). Likewise, if DnaA is overexpressed or DnaA inactivation by RIDA has not occurred, extra rounds of DNA replication occur because the initiation window has stretched beyond the sequestration window (Atlung et al. 1987; Skarstad et al. 1989; Nishida et al. 2002; Camara et al. 2003).

Regulation of replication initiation in *E. coli* does not involve control over the concentration of origins. All origins present in the cell are initiated at the same time once per generation irrespective of how many there are. This can be seen when minichromosomes (plasmids dependent on an *oriC* sequence for replication initiation) are present in the cell; they initiate at the same time as the chromosomal *oriC*s (Helmstetter and Leonard 1987; Løbner-Olesen 1999). Thus, the mechanism is the following: all origins are initiated during a short time interval and all new origins are sequestered until initiation is no longer possible. Although replication fork speed can vary significantly (Morigen et al. 2009; Odsbu et al. 2009; Stokke et al. 2012), it is not known whether the speed of each individual replication fork is regulated. In *Bacillus subtilis,* it has been found that replication elongation can be regulated by ppGpp (Wang et al. 2007) and metabolic enzymes (Janniere et al. 2007).

Regulation of *oriC* and DnaA in *B. subtilis* and *C. crescentus*

Regulation by the Clamp-Binding Protein

Bacillus subtilis (*Bs*) is a representative of Gram-positive bacteria and is a member of Firmicutes, whereas *E. coli* is representative of Gram-negative bacteria and is a member of γ-Proteobacteria. This means that these species are evolutionarily distant. In *B. subtilis,* the *dnaA* gene is located between the clusters of the cognate DnaA boxes on the genome (Messer 2002) that form the replication origin (Moriya et al. 1992, 1994; Krause et al. 1997; and see Leonard and Méchali 2013) and *dnaA* gene transcription is autoregulated like in *E. coli* (Table 1) (Ogura et al. 2001).

Subcellular localization analysis indicates that a considerable number of BsDnaA molecules are titrated to the replication forks in a clamp- and YabA-dependent manner (Noirot-Gros et al. 2006; Soufo et al. 2008; Goranov et al. 2009). Although YabA has no sequence similarity to *E. coli* Hda, it binds the clamp and BsDnaA, like Hda (Table 1) (Noirot-Gros et al. 2006; Cho et al. 2008). The YabA-clamp system is required to repress overinitiation of replication (Noirot-Gros et al. 2006; Soufo et al. 2008; Goranov et al. 2009), which may be sustained by the sequestration of many DnaA molecules from *oriC*. Whether DNA-clamp-YabA complexes induce BsDnaA-ATP hydrolysis is not yet known.

Caulobacter crescentus (*Cc*) is an α-Proteobacterial species that is evolutionarily distant from both *E. coli* and *B. subtilis*. *C. crescentus* cells undergo asymmetrical cell division, yielding a stalked cell and a swarmer cell; only a stalked cell can replicate the chromosome whereas a swarmer cell must first differentiate into a stalked cell before replication can occur (Marczynski and Shapiro 2002). In *C. crescentus*, HdaA, the *E. coli* Hda ortholog, colocalizes with replisomes and is required to repress overinitiation (Table 1) (Collier and Shapiro 2009). *Cc*DnaA Arg-357 corresponds to the *E. coli* DnaA AAA^+ sensor II motif Arg-334, and, like *E. coli* DnaA R334A, *Cc*DnaA R357A can cause overinitiation of the cognate chromosomal replication (Fernandez-Fernandez et al. 2011). Taken together, the data indicate that replication-dependent DnaA-ATP hydrolysis is important for repressing *Cc*DnaA activity and extra initiation events.

Regulation by *oriC*-Binding and Various Factors

In *B. subtilis*, the chromosome partition proteins Soj and Spo0J play regulatory roles in replication initiation at *oriC* (Ogura et al. 2003; Lee and Grossman 2006; Murray and Errington 2008; Scholefield et al. 2011). Soj and Spo0J are members of the same protein family as ParA and ParB that are plasmid-partitioning proteins. The ParS region is a centromere-like region located near *oriC* and partitions the sister *oriC* regions by binding to both Soj and Spo0J. DNA-free Soj binds to DnaA assembled on *oriC*, and represses untimely initiation events (Table 1). ATP-bound Soj binds to ParS and stimulates initiation. Spo0J negatively regulates this Soj action by stimulating Soj-ATP hydrolysis and dissociation from the DNA (Murray and Errington 2008; Scholefield et al. 2011). In addition, the binding of BsDnaA to DnaA box clusters near *oriC* is also important for preventing premature initiation (Okumura et al. 2012). Similar role for DnaA box clusters is also found in *Streptomyces* (Table 1) (Smulczyk-Krawczyszyn et al. 2006).

When *B. subtilis* cells sporulate, the initiation of chromosome replication is inhibited by SirA (Wagner et al. 2009; Rahn-Lee et al. 2011). SirA is expressed specifically on sporulation and binds BsDnaA, which inhibits BsDnaA-*oriC* binding. *E. coli* does not contain homologs of Soj, Spo0J, and SirA, whereas *B. subtilis* does not contain homologs of SeqA and Dam (Table 1).

In *C. crescentus*, CtrA is a major cell cycle-dependent transcriptional regulator present in swarmer cells. It binds to the cognate *oriC*, thereby inhibiting replication initiation (Table 1) (Quan et al. 1998). ClpP degrades CtrA when swarmer cells differentiate into stalked cells (Gorbatyok and Marczynski 2005; McGrath et al. 2006). Homologs of SeqA and Dam are absent in this bacterium.

The Stringent response is activated on carbon starvation and primarily inhibits transcription (Strivatsan and Wang 2008). Carbon starvation activates the synthesis of ppGpp from GDP by RelA or SpoT. ppGpp directly binds to RNA polymerase and alters transcription modes, specifically repressing stable RNA (rRNA and tRNA) synthesis. In *E. coli*, the initiation of chromosomal replication is inhibited on carbon starvation, which may be caused by changes in RNA polymerase function (Zyskind and Smith 1992) but the entire mechanism is not revealed. In *B. subtilis*, elongation, but not initiation, is inhibited on carbon starvation, which is indicated to be caused by ppGpp-dependent inactivation of primase (Wang et al. 2007). Similar mechanisms may also exist in *E. coli* (Maciag et al. 2010). In *C. crescentus*, SpoT, the ppGpp synthetase, degrades DnaA on carbon starvation (Lesyley and Shapiro 2008).

CONCLUDING REMARKS

Multiple systems regulate *oriC* and DnaA in a concerted manner to ensure that replication initiation occurs only once per origin per generation. Some of these regulatory systems are coupled with specific events that are important for cell cycle regulation or chromosomal replication. In *E. coli*, *oriC* becomes hemimethylated temporarily just after initiation, which allows the binding of SeqA, an inhibitor of initiation. The DNA-loaded clamps bind Hda protein, and the resultant complexes stimulate DnaA-ATP hydrolysis, thereby inactivating DnaA. This clamp-dependent feedback regulation is also conserved in eukaryotes. Also, *dnaA* gene transcription is repressed in a SeqA-dependent and temporal manner. These three negative-feedback mechanisms regulate initiation in a replication-dependent manner in *E. coli*. In addition, excessive DnaA molecules are titrated to the *datA* locus on the chromosome, which adds another level of negative regulation. *dnaA* transcription is also autoregulated in both *E. coli* and *B. subtilis*. DARS reactivates DnaA by stimulating ADP-to-ATP exchange, thereby supporting timely initiation as well as the DnaA-DiaA interaction.

The *B. subtilis* clamp-YabA complex and the *C. crescentus* clamp-HdaA complex regulate initiation in a replication-dependent, negative-feedback manner. YabA and HdaA are considered to be the functional homologs of *E. coli* Hda, but only HdaA displays structural similarity with Hda. In *B. subtilis*, the ordered interaction of Soj, Spo0J, and the *parS* locus regulates the initiation activity and assembly of BsDnaA at *oriC* in a cell cycle-coordinated manner. Like *E. coli datA*, DnaA box clusters near *oriC* are also important for repressing initiation potential. SirA, which is expressed specifically before sporulation, inhibits the binding of BsDnaA to *oriC*. In *C. crescentus,* the cell cycle-specific binding of CtrA to the origin may regulate initiation. The stringent response, which is induced on carbon starvation and increases the level of ppGpp, an RNA polymerase inhibitor, stimulates DnaA degradation in *C. crescentus,* and inhibits elongation in *B. subitilis* through the ppGpp-primase interaction and initiation in *E. coli*.

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