

Strategies for helicase recruitment and loading in bacteria

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DNA replication initiation in prokaryotes and eukaryotes requires the recruitment and loading of a helicase at the replication origin. To subsequently unwind the double-stranded DNA, the helicase must be properly positioned on the separated DNA strands. Several studies have revealed similarities and differences in the mechanisms used by different autonomously replicating DNA elements (replicons) for recruitment and activation of the appropriate helicase. Of particular interest are plasmid replicons that are adapted for replication in diverse bacterial hosts and are therefore intriguingly able to exploit the helicases of distantly related bacterial species. The different molecular mechanisms by which replicons recruit and load helicases are only just beginning to be understood.

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Introduction

The replication of eukaryotic and prokaryotic chromosomes, viruses and bacterial plasmids involves several analogous events and similarities in replisome architecture. For many systems, it has been demonstrated that specific initiation proteins, including bacterial DnaA protein, phage λ O protein, SV40 T antigen, plasmid replication initiation proteins (generally termed Rep) and the eukaryotic origin recognition complex (ORC), form complexes at the origin that serve as platforms for subsequent DNA replication initiation events. Despite certain similarities, the specific mechanism for replication initiation of a given replicon is dependent on both the structure of the replication origin and the nature of the replication initiation protein. The replication of extra-chromosomal replicons, such as plasmids, phages and viruses, is generally limited to a single host or a few closely related hosts (narrow host range). However, promiscuous plasmids of bacteria are able to replicate and maintain themselves in many distantly related bacterial species (broad host range). Consequently, versatile interactions of plasmid-encoded proteins and replication origins with host-specific replication factors might determine the mode of broad-host-range replicon initiation.

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Origin structure and opening

The origins of prokaryotic and some eukaryotic replicons such as DNA viruses and *Saccharomyces cerevisiae* possess characteristic functional elements, including specific binding sites for the appropriate initiation protein and an AT-rich region where DNA duplex destabilization occurs. Plasmid origins usually contain multiple binding sites (iterons) for the plasmid-specific replication initiation protein as well as one or more binding sites for the host replication initiation protein, DnaA (DnaA boxes; Fig. 1).

Several lines of evidence suggest that these structural elements of the origin are employed for broad-host-range plasmid replication and maintenance in different host bacteria species. For example, the minimal origin of the broad-host-range plasmid RK2 (*oriV*; Fig. 1) possesses five iterons and is functional in *Escherichia coli*. However, the presence of three additional iterons stabilizes RK2 plasmid maintenance in *Pseudomonas putida* (Schmidhauser *et al.*, 1983). In addition, the region with four DnaA boxes is essential for RK2 replication in *E. coli*, but is dispensable for replication of the plasmid in *Pseudomonas aeruginosa* (Shah *et al.*, 1995; Doran *et al.*, 1999). In the *E. coli* chromosome, the replication origin (*oriC*) contains five DnaA box sequences (Fig. 1).

The binding of multiple DnaA molecules in the presence of the histone-like HU protein and the site-specific DNA-binding protein IHF (integration host factor) results in destabilization of the duplex DNA within the nearby AT-rich sequences of the *oriC* of *E. coli* (Messer *et al.*, 2001). Origin opening of the narrow-host-range plasmids P1, F, R6K and pSC101 requires, in addition to *E. coli* DnaA, HU and/or IHF proteins, the binding of plasmid-encoded replication initiation proteins (Mukhopadhyay *et al.*, 1993; Kawasaki *et al.*, 1996; Lu *et al.*, 1998; Park *et al.*, 1998; Kruger *et al.*, 2001; Sharma *et al.*, 2001). Similarly, the formation of an open complex at the replication origin of the broad-host-range plasmid RK2 by the plasmid-encoded TrfA initiation protein requires *E. coli* HU, and is stabilized by *E. coli* DnaA (Konieczny *et al.*, 1997).

In contrast to the chromosomal *oriC*, but similar to bacteriophage λ , plasmid origins do not require ATP for open complex formation (Schnos *et al.*, 1988; Mukhopadhyay *et al.*, 1993; Kawasaki *et al.*, 1996; Lu *et al.*, 1998; Park *et al.*, 1998). A basis for this lack of dependence on ATP might be the intrinsic DNA curvature of these origins as well as origin bending, induced in an ATP-independent mode, by the complex of the plasmid-encoded Rep protein and the host HU or IHF (Stenzel *et al.*, 1991; Doran *et al.*, 1998; Lu *et al.*, 1998; Komori *et al.*, 1999; Sharma *et al.*, 2001).

DnaB and other replicative helicases

The DnaB protein, the major replicative DNA helicase in *E. coli* (LeBowitz & McMacken, 1986), is a member of the hexameric DNA helicase family, which includes the T4 and T7 DNA helicases and plasmid RSF1010-encoded RepA, as well as the SV40 T antigen and the human MCM (minichromosome maintenance) protein (Patel & Picha, 2000). Although no sequence identity has yet been defined, these helicases form a ring structure with a central opening and are associated with DNA replication complexes. Mammalian MCM helicase is a complex of several different but related peptides. Interestingly, it was recently shown that the *Methanobacterium thermoautotrophicum* MCM protein can form heptameric rings (Yu *et al.*, 2002). Several lines of evidence suggest that DNA passes through the central opening of the helicase ring, although an alternative model of DNA wrapping around the outside of the helicase ring has also been proposed (Patel & Picha, 2000).

E. coli DnaB is a multifunctional enzyme with a number of distinct activities including DNA binding, ATP hydrolysis, DNA unwinding and the stimulation of the DnaG primase for primer synthesis, which is required to start the polymerization reaction by the DNA polymerase holoenzyme. DnaB interacts with a number of proteins, including *E. coli* DnaA (Marszalek & Kaguni, 1994), DnaC (Wickner & Hurwitz, 1975), DnaG primase (Lu *et al.*, 1996; Tougu & Marians, 1996) and the τ subunit of DNA polymerase (Kim *et al.*, 1996), as well as the plasmid-encoded replication initiation proteins RepA of pSC101 (Datta *et al.*, 1999), π of R6K (Ratnakar *et al.*, 1996) and TrfA of RK2 (Pacek *et al.*, 2001). The *E. coli* DnaB hexamer is present *in vivo* in a protein complex with six monomers of the DnaC protein and six ATP molecules (Wickner & Hurwitz, 1975; Lanka & Schuster, 1983; Fig. 2).

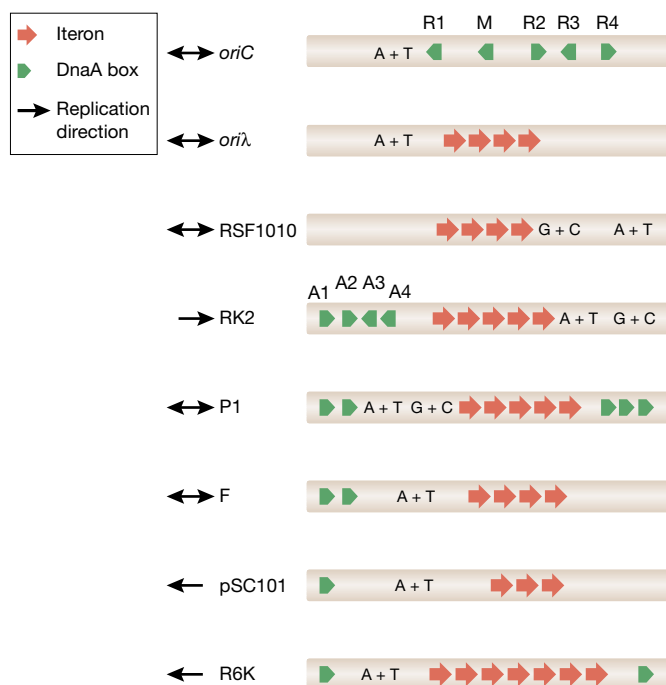


Fig. 1 | Structural organization of some prokaryotic origins. The multiple repeat sequences (iterons), AT-rich and GC-rich regions, and DnaA box sequences are indicated. (Maps are not drawn to scale.)

E. coli DnaB helicase recruitment and loading

The *E. coli* DnaB helicase binds to single-stranded DNA (ssDNA) in an ATP-dependent manner. However, this activity alone is not sufficient for helicase loading at a replication origin because the DnaB hexamer by itself has no affinity for ssDNA bound by SSB (single-stranded binding protein). Thus, entry of the DnaB helicase complex into the unwound *oriC* depends on additional protein factors, and the mechanism behind this event is not fully understood. Chemical crosslinking, enzyme-linked immunosorbent assays and monoclonal antibody interference studies have shown that a physical interaction between *E. coli* DnaA and DnaB is essential for delivering the helicase to *oriC* (Marszalek & Kaguni, 1994). The loading of DnaB probably depends not only on DnaA binding to the DnaA boxes present in the *E. coli* *oriC* sequence (Fig. 2A) but also on DnaA binding to the open region of the origin, which is then stabilized for subsequent helicase loading (Speck & Messer, 2001). A specific physical interaction between DnaA and DnaB was also shown to be crucial during plasmid RK2 replication initiation in *E. coli* (Konieczny & Helinski, 1997; Fig. 2D). This DnaA–DnaB complex was found at the DnaA box region of *oriV*, which is separated by more than 200 base pairs (bp) from the RK2 origin opening and has a strict DnaA box sequence requirement for stable formation (Pacek *et al.*, 2001).

In addition to the interaction between DnaB and DnaA, the helicase accessory ATPase protein, DnaC, is also required for helicase complex formation and helicase loading at *E. coli* *oriC*, as well as at several plasmid origins including RK2 (Konieczny & Helinski, 1997), R6K (Lu *et al.*, 1998) and pSC101 (Datta *et al.*, 1999). The T4 gp59 and *B. subtilis* DnaI proteins have a role similar to that of DnaC, serving as helicase loading factors during bacteriophage T4 and *B. subtilis* DNA replication initiation, respectively (Kreuzer & Morrical, 1994; Imai *et al.*, 2000). Two eukaryotic proteins, Cdc6 and Cdt1, the latter recently identified as a novel component of the pre-replication complex, have been proposed to recruit the MCM complex in *Xenopus* and *Saccharomyces* (Baker & Bell, 1998; Bell & Dutta, 2002). During the replication of bacteriophage λ, the role of DnaC is performed by the λP protein, which is also an ATPase but shares no sequence similarity with DnaC. λP protein binds the *E. coli* DnaB helicase and delivers it to the λ origin (*oriλ*) by means of an interaction with the λO protein (Dodson *et al.*, 1985; Fig. 2B). After the helicase complex has been formed at *oriλ*, it must be remodelled by the concerted actions of the *E. coli* chaperones DnaK, DnaJ and GrpE (Konieczny & Zyllicz, 1999). At this step, DnaB is released from a tight interaction with λP. The requirement for molecular chaperones is decreased by a mutation in the λP gene, which weakens the interaction between the λP protein and the DnaB helicase (Konieczny & Marszalek, 1995).

The detailed molecular mechanism of helicase loading onto ssDNA is not fully understood. It has been shown that ssDNA-binding activity of λP and DnaC is involved in DnaB loading (Learn *et al.*, 1997), and also that DnaC is released with concomitant hydrolysis of ATP during helicase loading onto *oriC* (Funnell *et al.*, 1987; Allen & Kornberg, 1991). Recently, it was proposed that DnaC is a dual ATP/ADP switch protein, with DnaB and ssDNA triggering ATP hydrolysis by DnaC (Davey *et al.*, 2002). Surprisingly, ATP is not required for the loading of DnaB by DnaC onto ssDNA, and the model proposes that DnaC–ATP loads the helicase onto *oriC*, but that conversion to DnaC–ADP is required before the helicase is active (Davey *et al.*, 2002).

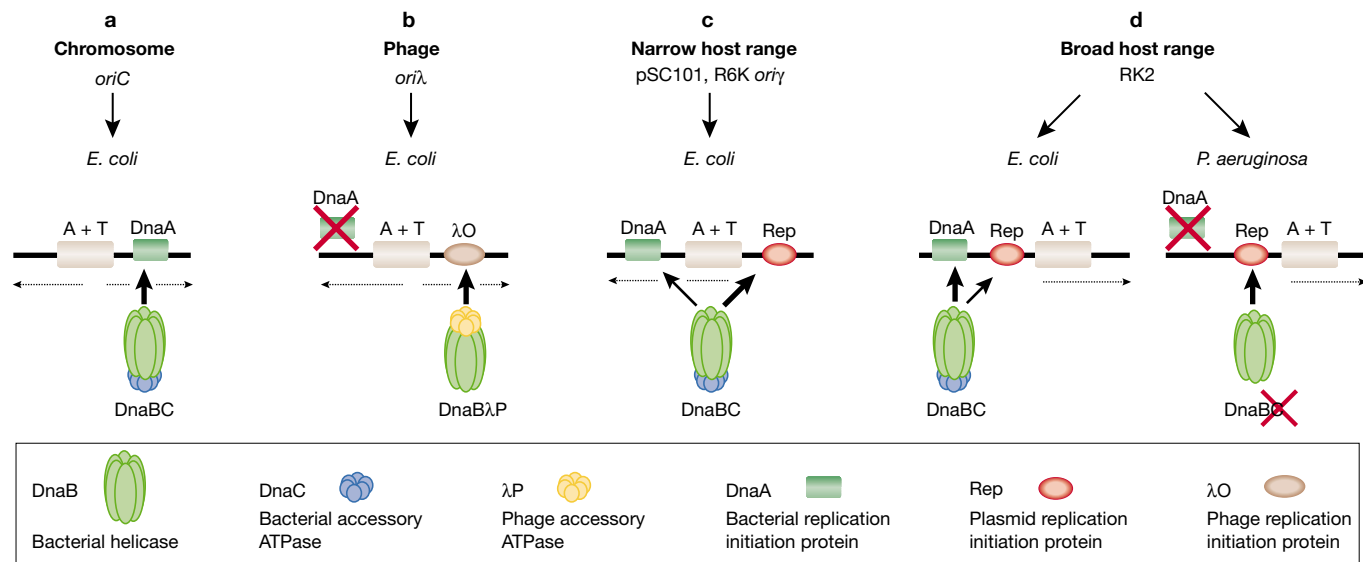


Fig. 2 | Models for helicase recruitment and loading at plasmid, phage and bacterial chromosomal origins. Protein requirements and interactions required for helicase recruitment and loading are depicted. Thick arrows indicate the crucial interactions; dotted arrows indicate the direction of replication. (A) A physical interaction between *E. coli* DnaA and DnaB helicase as well as the activity of an accessory DnaC ATPase are essential for delivering the helicase to *E. coli* *oriC*. (B) During bacteriophage λ replication, the role of DnaC is performed by the λ P protein, which binds the *E. coli* DnaB helicase and delivers it to *oriλ* by means of an interaction with the λ O protein. (C) In addition to *E. coli* DnaA and DnaC proteins, helicase recruitment at narrow-host-range plasmid origins requires plasmid-specific replication initiation proteins (Rep). (D) Alternative mechanisms for helicase recruitment and loading at the origin of the broad-host-range plasmid RK2. The host-specific DnaA–DnaB interaction used for helicase recruitment at the DnaA boxes of the RK2 plasmid origin is applicable to plasmid replication in *E. coli*. In *P. aeruginosa*, helicase is recruited and loaded onto the RK2 origin in a DnaA- and DnaC-independent mode, through a specific interaction with the plasmid TrfA-44 replication initiation protein.

During replication initiation of plasmid RK2 in *E. coli*, the DnaA protein directs DnaB, in complex with DnaC, to the DnaA boxes in *oriV* of RK2. However, the helicase complex fails to unwind the template unless the plasmid initiation protein TrfA is also present (Konieczny & Helinski, 1997). It has been proposed that the helicase is repositioned from the DnaA boxes onto the AT-rich region via a direct contact with TrfA (Pacek *et al.*, 2001). The loading of DnaB onto the ssDNA depends on the precise positioning of the DnaA boxes at *oriV*, as was shown by the disruption of helicase loading by the insertion of 6 bp between the DnaA boxes and the iterons at *oriV*, although opening was normal (Doran *et al.*, 1998). Interactions between *E. coli* DnaB and plasmid Rep proteins have also been reported for the plasmids R6K (Ratnakar *et al.*, 1996) and pSC101 (Datta *et al.*, 1999), and these have been shown to be crucial for initial helicase complex formation at these plasmid origins. A mutant form of the DnaB protein that does not interact with pSC101 RepA fails to activate replication initiation at this origin. However, the mutant maintains its ability to support replication initiation at *oriC* (Datta *et al.*, 1999). The plasmid R6K π protein and pSC101 RepA have also been shown to interact with the *E. coli* DnaA initiator (Lu *et al.*, 1998; Sharma *et al.*, 2001), which suggests a complex interaction involving the plasmid Rep protein in the formation of the prepriming complex, helicase loading, and activation.

Species-specific helicase recruitment and loading?

An intriguing question pertaining to DNA replication is whether or not the mechanism for helicase recruitment and loading described for *E. coli* *oriC* is also responsible for replication initiation of chromo-

somal and plasmid origins in other bacterial species. *E. coli* has traditionally been used as a model organism, but it is not clear whether these studies really do provide universal rules for all prokaryotes. This limitation can be overcome by studying promiscuous plasmids, which provide unique systems for exploring species-dependent replication mechanisms. Genetic and biochemical studies have suggested that these replicons have developed two major strategies to facilitate DNA replication in different genetic backgrounds: (1) initiation independent of host-DNA replication initiation factors, and (2) initiation dependent on versatile communication between plasmid and host-DNA replication initiation factors. Broad-host-range plasmids belonging to the IncQ incompatibility group (for example RSF1010) employ the first strategy by encoding three replication proteins that obviate the need for certain host proteins. The product of the *repA* gene of RSF1010 was found to have ssDNA-dependent ATPase and DNA helicase activity (Scherzinger *et al.*, 1997), the *repC* product binds to the iterons and opens the origin region, creating the entry site for the RepA helicase (Scherzinger *et al.*, 1991), and the *repB* product encodes a primase.

Broad-host-range plasmids belonging to the IncP group (e.g. RK2) rely on replication proteins from the host cell, and might therefore use a helicase loading mechanism adapted to the genetic background of the specific host bacterium. Recent results indicate that this is so (Caspi *et al.*, 2001). *In vitro* experiments with purified helicase from *E. coli*, *P. putida* and *P. aeruginosa* revealed that, unlike the *E. coli* DnaB helicase, both *Pseudomonas* helicases could be delivered and activated at the RK2 *oriV* in the absence of a DnaC-like ATPase accessory protein (Fig. 2D). Further versatility is provided by two forms of the

RK2 initiation protein (TrfA; 44 and 33 kDa), which are generated by alternative in-frame translational start sites (Kornacki *et al.*, 1984; Shingler & Thomas, 1984). The requirement for each of these forms is host-specific. Either form of the TrfA protein binds to the iterons located at *oriV* (Perri *et al.*, 1991) and opens the origin at the AT-rich region (Caspi *et al.*, 2001). Both are also functional in *E. coli* and *P. putida*, but only the 44-kDa protein is active in *P. aeruginosa* (Durland & Helinski, 1987; Fang & Helinski, 1991). Consistent with these observations are *in vitro* experiments showing that *E. coli* or *P. putida* DnaB is active with either TrfA-33 or TrfA-44, whereas *P. aeruginosa* DnaB specifically requires TrfA-44 for helicase complex formation and template unwinding (Caspi *et al.*, 2001). The molecular basis for this difference has recently been elucidated (Y. Jiang, M. Pacek, D.R. Helinski, I.K. and A. Toukdarian, unpublished observations). Size-exclusion chromatography and helicase-activity assays with altered *oriV* templates showed that neither DnaA nor the DnaA box sequences were required for the formation and activity of the *Pseudomonas* helicase complex at RK2 *oriV*. Furthermore, biospecific interaction analysis with BIAcore revealed that *Pseudomonas* helicases form complexes with TrfA-44 but not with TrfA-33 bound to the *oriV* iterons. The deletion of a putative helical region at the amino terminus of TrfA-44 completely abolished *Pseudomonas* helicase complex formation at the iterons (Z. Zhong, D. Helinski and A. Toukdarian, unpublished observations).

These results suggest that, depending on the bacterial host, RK2 uses either a DnaA-dependent or a DnaA-independent pathway for helicase recruitment and activation (Fig. 2D). The DnaA-dependent pathway is specific for RK2 replication initiation in *E. coli*. The second pathway, employed in *P. putida* and *P. aeruginosa*, involves helicase recruitment through its interaction with TrfA-44 bound to iterons. Moreover, for the *Pseudomonas sp.* helicases, the host DnaA protein is not essential for helicase complex formation and activity at *oriV*.

Conclusions

Although the analysis of helicase recruitment is still limited to a few bacterial species and a few plasmid replicons, some general conclusions can already be drawn. It is obvious that replicons use self-specific mechanisms for helicase recruitment and loading (Fig. 2), and it has been found that in some cases the helicase is recruited and loaded at the replication origin by an interaction with the DnaA protein bound to DnaA boxes. This mechanism requires an accessory DnaC ATPase and is essential during the initiation of chromosomal replication in *E. coli* (Fig. 2A). It might also be used by some plasmid replicons (Fig. 2C and D). In addition to *E. coli* DnaA and DnaC proteins, helicase recruitment at plasmid origins (namely P1, R6K, pSC101 and RK2) requires plasmid-specific replication initiation proteins. A different mechanism is used during bacteriophage λ DNA replication, in which a phage-encoded accessory ATPase (λ P protein) has a crucial role in recruitment of the host helicase (Fig. 2B). In a third mechanism, an accessory ATPase is not required and loading of the helicase is solely dependent on interaction with the replication initiation protein (namely plasmid RK2 replication in *Pseudomonas sp.*; Fig. 2D).

Interestingly, certain broad-host-range plasmids (such as RK2) can use host-specific pathways for helicase recruitment and loading. Plasmid RK2 shows versatility in terms of its requirement for certain origin structural elements (DnaA boxes and number of iterons) in various bacterial hosts. Another important factor is the nature of the protein interactions between the two forms of the RK2 replication

initiation protein and the host replication machinery. Recruitment of the helicase through the N-terminal domain uniquely present in the larger form of the plasmid-replication initiation protein is crucial in *P. aeruginosa* but has no role in helicase recruitment in *E. coli*. Similarly, the necessity of host-specific DnaA–DnaB interactions used for helicase recruitment at the plasmid origin depends on the bacterial species.

It is evident that the structure of the origin and the properties of the replication initiation protein contribute to the mechanism of helicase recruitment. Furthermore, helicase can be loaded onto a replication origin via different interactions. Genetic background and probably other conditional factors influence the way in which the helicase is recruited. Conceivably, alternative pathways might exist in the same bacterium under different growth conditions. It is of particular interest to determine the factors that affect replication initiation specificity.

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